

DISSERTATION

**GENETIC FACTORS AFFECTING RADIATION
SENSITIVITY AND GENOMIC STABILITY**

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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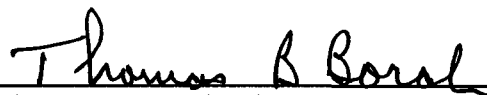
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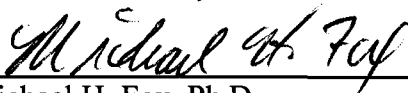
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED
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BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY.

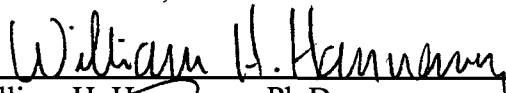
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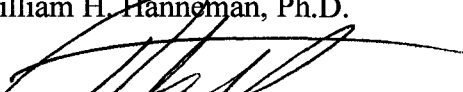
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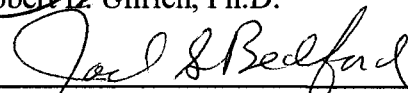
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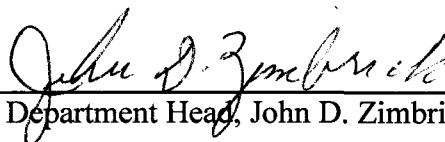
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ABSTRACT OF DISSERTATION

**GENETIC FACTORS AFFECTING RADIATION
SENSITIVITY AND GENOMIC STABILITY**

A report of the unexpected radiosensitivity of fibroblasts derived from unaffected parents of children with bilateral retinoblastoma (Fitzek, *et al.*, *Int. J. Cancer* **99**, 2002) prompted the studies discussed in this dissertation. The *Rb1*^{+/+} parents may harbor unidentified mutations in DNA repair, cell cycle control, or genomic stability genes, influencing their radiosensitivity and possibly the increased transmission of mutant *Rb1* alleles to their offspring. These studies sought to validate the moderate to hyper-radiosensitivity phenotype documented in the Fitzek report in this laboratory, and determine whether a continuous low dose-rate (LDR) irradiation protocol could amplify the survival differential between radiosensitive Rb family member and apparently normal control fibroblast strains sufficiently for genetic complementation and correction purposes.

Measurements of fibroblast survival following acute irradiation and 24-hour potentially lethal damage (PLD) repair and G2 chromosomal radiosensitivity were made. Rb family member strains were ~1.5 to 4-fold more radiosensitive than the CIMR control strains, with high degrees of tetraploidy and endoreduplication observed in some cases in the G2 assay. The ability of fibroblasts to form colonies during continuous LDR (0.5–8.4 cGy/h) irradiation was successful in identifying radiosensitive strains (both Rb family

members and some CIMR controls), achieving a >100-fold survival differential at ~2–3 cGy/h for some strains.

Continuous LDR (1–15 cGy/h) colony formation measurements were also made with *atm*^{-/-}, *atm*^{+/-}, and *tp53*^{-/-} mouse embryo fibroblasts (MEFs). The *atm*-heterozygote MEFs were intermediate in survival between the hyper-radiosensitive *atm*-homozygote MEFs and wild-type controls; the *tp53*^{-/-} MEFs were the most radioresistant. The frequency of giant cells in *tp53*^{-/-} MEF cultures was significantly increased by continuous LDR exposures but not by acute exposures. Cytogenetic and immunocytochemical analyses of *tp53*^{-/-} MEFs revealed micronuclei with γ -H2AX foci and aberrant centrosomes. Telomere analysis by fluorescence *in situ* hybridization (FISH) revealed a novel phenotype of telomere dysfunction, which I term “telomere fission,” characterized by large numbers of extra-chromosomal telomeric repeats and telomeric double-minutes. Massive chromosomal rearrangements were revealed by multiplex FISH (mFISH) analysis.

These studies indicate continuous LDR irradiation could provide a powerful genetic screening strategy for studying mechanisms involved in the mutation of crucial tumor suppressors underlying carcinogenesis.

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not ‘Eureka!’ but rather ‘Hmmm... that’s funny...’ ”

Isaac Asimov, 1920–1992

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CHAPTER 1

Introduction

1.1. Radiation Sensitivity and Cancer Predisposition

A phenotype of *in vitro* cellular radiosensitivity has been well-documented in a number of cancer predisposition and chromosomal instability syndromes, most notably ataxia-telangiectasia (A-T), spurring interest in the use of radiobiological techniques to identify other genes involved in these pathways and their contribution to the overall burden of cancer. However, aside from severely hyper-radiosensitive A-T patient and non-homologous end joining (NHEJ) pathway-deficient *scid* cells, the degree of hyper-radiosensitivity documented in other genetic conditions is relatively small (<2–3-fold). To confound matters further, this degree of moderate radiosensitivity often overlaps with the lower end of a broad range of responses measured in cells derived from “apparently normal” individuals available at the national cell banks for research purposes. Surveys of radiation sensitivity of large numbers of cell strains derived from apparently normal individuals (often unaffected, first-degree family members of patients with various genetic disorders) have indicated a significant proportion (~25–35%) are moderately radiosensitive, a testament to the presence of low-penetrance cancer gene polymorphisms in these individuals. This degree of differential radiosensitivity between cells derived from moderately radiosensitive patients and those derived from apparently normal individuals makes ionizing radiation (IR) a relatively inefficient selection tool for genetic

testing purposes to identify radiation-responsive genes differentially expressed between the two groups (of interest are those that may likewise influence a patient's disease etiology).

The early success of studies linking mutation of the retinoblastoma susceptibility gene *Rb1* (on chromosome 13q14) to the childhood cancer retinoblastoma (Rb) has attracted researchers to use Rb patient-derived cells as a model system to help identify and characterize more of the hundreds of cancer susceptibility and radiation sensitivity-modifying genes (Wood, *et al.*, 2005). Rb is associated with a high incidence of radiotherapy and chemotherapy treatment-related secondary cancers, the broadest spectrum of cellular radiosensitivity observed in any single human genetic condition, and a similarly broad spectrum of genomic instability observed in both somatic and tumor cells (discussed in Section 2.1 in Chapter 2). This indicates that mutations of other critical genes, other than just the *Rb1* gene alone, are likely to be involved in these phenotypes. Despite the terminology used, the large majority of Rb cases (both “hereditary” bilateral/unilateral cases and “sporadic” unilateral cases) are thought to represent *de novo* mutations of the primarily paternal allele of the *Rb1* gene in the genome of the developing zygote, and this may involve defects in one or more genomic maintenance genes of the parents. As with other radiosensitivity syndromes, studies of somatic cells derived from unaffected parents or siblings in Rb pedigrees that are considered radiosensitive could yield valuable evidence of the nature and frequency of such genetic defects, in a “wild-type” *Rb1*^{+/+} background.

Reports of cellular hyper-radiosensitivity in unaffected first-degree relatives of Rb patients have indeed been documented, including a recent report of Fitzek and co-

workers who observed hyper-radiosensitivity for cell killing and cell cycle delay responses in fibroblasts derived from fourteen members of five unrelated Rb families, both unaffected parents and affected children (Fitzek, *et al.*, 2002). These 14 Rb family member fibroblast strains and a group of 14 age- and sex-matched apparently normal fibroblast control strains obtained from the Coriell Cell Repositories (CCR) at the Coriell Institute for Medical Research (CIMR, Camden, NJ) are the subject of the research presented in this dissertation. A series of high dose-rate (HDR, acute delivery) and low dose-rate (LDR, chronic delivery) irradiation protocols were employed to confirm and better accentuate the degree of hyper-radiosensitivity of these Rb family member fibroblast strains, in the hope of achieving a large enough survival differential using continuous low dose-rate (LDR) radiation exposures to undertake a series of genetic complementation and correction experiments with some of these cells.

1.2. Radiation Sensitivity in Retinoblastoma Patients and Families

Studies of retinoblastoma (Rb), a rare childhood eye cancer, have provided key information in our current understanding of the genetic events underlying malignant transformation. A high occurrence of secondary cancers, notably following radiation treatment, has spurred much interest in the role of DNA damage and genomic stability pathways in the etiology of Rb. Insight into these pathways is limited by a broad spectrum of radiosensitivity in somatic and tumor cells of Rb patients and interestingly, their first-degree relatives. Likewise, a broad spectrum of functions ascribed to the pRb protein in the maintenance of key cellular regulatory mechanisms, including evidence of an ever-increasing *direct* role in genomic stability, is reflected in the range of genomic

instability phenotypes associated with Rb cells. A broad spectrum of radiation responses likewise seen in apparently normal individuals in the population, along with the large majority ($\geq 90\%$) of unaffected parents of affected children in Rb families, suggests the involvement of additional genetic modifying factors.

The majority of Rb radiosensitivity studies involve measurements of IR-induced cell killing of non-tumor somatic cells (primarily fibroblasts and lymphoid types) derived from Rb patients and unaffected first-degree relatives. These studies have reported a wide range of survival curve parameters (D_0 , D_{10} , SF_2 , *etc.*) calling into question any direct connection between pRb protein function and radiation sensitivity (see Chapter 2). This is best illustrated by Figure 1.1 (reproduced from Deschavanne, *et al.*, 1986), a probability-frequency (probit) plot of the mean inactivation dose, $\bar{D}$ (the mean of the survival curve when treated as a cumulative probability distribution), for 21 Rb fibroblast strains gathered from 6 of the reports discussed below. The radiation response of Rb cells (green line) extends from moderate to hyper-radiosensitivity at the lower limit to almost transformed cell-like radioresistance at the upper limit, extending above the range of response of the 70 apparently normal fibroblast strains reviewed (blue line). The range of radiation response of the A-T heterozygote fibroblast strains (grey line) likewise falls within the normal range, the lower range of which approaches the upper range of the hyper-radiosensitive A-T homozygote patient fibroblast strains (red line). Deschavanne and Fertil (1996) further extended this meta-analysis to include a total of 147 apparently normal fibroblast strains in 47 reports (but only one strain with Rb, from Wang, *et al.*, 1986a), which resulted only in a slight extension of the upper end of the normal range from that seen in Figure 1.1.

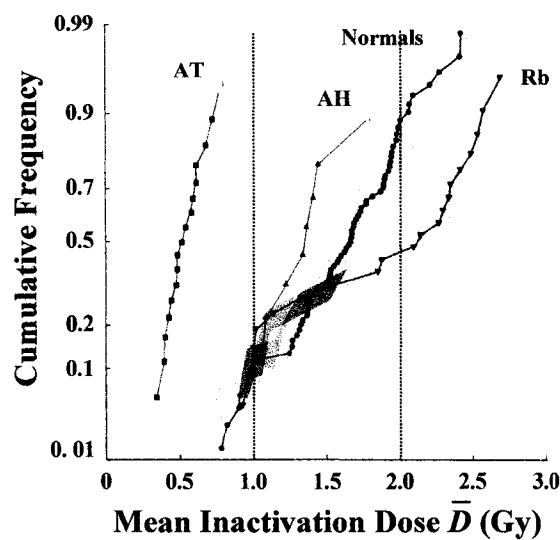


Figure 1.1. Probability probit plot of mean inactivation dose, $\bar{D}$, versus cumulative frequency of the four groups of fibroblast strains discussed in the text: ataxia-telangiectasia homozygotes (AT, red line) and heterozygotes (AH, gray line), retinoblastoma heterozygotes (Rb, green line), and apparently normal control fibroblasts (blue line). Plot adapted from Deschavanne, *et al.* (1986).

The early Rb radiosensitivity reports suggested that the presence and possible disruption of other DNA repair or cell cycle control genes besides *Rb1* (and *BRCA2*) in the 13q region may influence radiosensitivity, earlier onset or severity of the disease, and the generation of secondary radiogenic tumors. However, conflicting results regarding the extent of disruption of the 13q region and the degree of radiosensitivity were reported. Only *some* 13q-deletion and bilateral Rb patient fibroblast strains demonstrated a significant degree of radiosensitivity compared to unilateral patient fibroblast Rb strains and the admittedly broad range of response seen in the apparently normal control strains (Weichselbaum, *et al.*, 1977, 1978, 1980a, 1980b, 1983; Nove, *et al.*, 1979, 1981; Cox and Masson, 1974, 1980; Arlett and Harcourt, 1980; Fujiwara, *et al.*, 1981; Zampetti-Bosseler and Scott, 1981; Ejima, *et al.*, 1982; Kossakowska, *et al.*, 1982; Morten, 1986; Wang, *et al.*, 1986a; Little, *et al.*, 1988, 1989; ICRP, 1999).

Direct comparisons of radiosensitivity parameters (*e.g.*, D_0 values) can typically be made for results obtained with identical protocols shared between different research groups or individual researchers within the same laboratory. In the Weichselbaum, Nove and Little series of reports referenced above, the experimental protocol entails cells being plated at low density without the use of feeder cells, irradiated 18–20 hours later when cells are in asynchronous growth phase, and scored for colony formation two weeks later (per Nove, *et al.*, 1980; the same protocol employed for the Rb family member fibroblast strains reported in Fitzek, *et al.*, 2002, and Table 1.1 and Figures 1.2 and 1.3 discussed below). D_0 values for the 12 13q-deletion and 33 bilateral fibroblast strains examined in these studies ranged from 94–149 cGy and 84–145 cGy, respectively. The unilateral Rb and normal control fibroblast responses ranged from 90–175 cGy, with most normal

strains ranging from 125–150 cGy, making definitive (*i.e.*, statistically significant) assignment of moderate or hyper-radiosensitivity impossible in most cases.

The studies by Cox and Masson (1974, 1980), Arlett and Harcourt (1980), and Pledger, *et al.* (1987) using heavily-irradiated feeder cell layers, and exposures conducted with density-inhibited (confluent) G0/G1-phase fibroblast cultures (Nagasawa and Little, 1983 and 1988) produced similar results and conclusions to those above. Interestingly, Nagasawa and Little (1983) reported that 70% of Rb strain AG1880 fibroblasts suffered an irreversible G1 block (up to 120 hours) after 4 Gy irradiation and immediate subculture, compared to only 20% in normal fibroblast strains. Survival studies employing the alkylating agents ethyl-methane sulfonate (EMS), methyl-methane sulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and 4-nitroquinoline 1-oxide (4NQO; Barfknecht and Little, 1982), or the DNA binding agent actinomycin D and the DNA cross-linking agent mitomycin C (MMC; Woods and Byrne, 1986) only suggested a mild sensitivity of bilateral Rb cells to these DNA-damaging agents. Fewer studies have been included the unaffected first-degree relatives in Rb families. The parents of one family in Arlett and Harcourt (1980) were shown to have normal radiosensitivity but 4 of 14 (29%) of the unaffected members of a large family examined in Pledger, *et al.* (1987) were hyper-radiosensitive.

Cytogenetic reports examining the production of MNNG- and radiation-induced chromosomal aberrations (Gainer and Kinsella, 1983; Morten, *et al.*, 1980; Nagasawa and Little, 1983; Heras and Coco, 1987), radiation-induced micronuclei (Goddard, *et al.*, 1985), and MMC-, TPA-, or radiation-induced sister chromatid exchanges (SCEs; Der-Sarkissian, *et al.*, 1982; Gainer and Kinsella, 1983; Takabayashi, *et al.*, 1983;

Abramovsky-Kaplan and Jones, 1984) have likewise demonstrated normal to moderately sensitive responses in Rb patient cells. Cytogenetic responses to G2-phase irradiation or bleomycin treatment were measured in Chaum, *et al.* (1984) and Sanford, *et al.* (1989, 1996), and are discussed further with the G2 chromosomal radiosensitivity assay experiments performed with the Rb family member fibroblast strains in Chapter 4.

The possibility of two distinct sub-groups of bilateral Rb patients, one typified by hyper-radiosensitivity and genomic instability of their somatic cells and a higher incidence of radiotherapy-induced secondary cancers, has been suggested. The study by Heras and Coco (1987) identified two distinct radiation response groups, with the radiosensitive group displaying a significant, approximate 2-fold increase in chromosome exchanges over normal controls and the remaining bilateral cases. The tumors of Rb patients examined by van der Wal, *et al.* (2003) were likewise divided almost equally into two groups on the basis of low or high levels of chromosomal instability, with each group associated with differences in clinical presentation. Examination of the radiosensitivity of non-tumor fibroblast or epithelial cell strains from these Rb patients could perhaps yield some concordance to the finding of chromosomal instability seen in matched tumor cells (Heras and Larripa, 1988).

Similar frequencies of 6-thioguanine-resistant (6TG or S6G, a toxic purine analog) hypoxanthine phosphoribosyltransferase (*hprt*) mutants and transformation to anchorage-independent growth in soft agar were induced in bilateral Rb patient and normal control fibroblast strains following irradiation (Wang, *et al.*, 1986b). Despite the attractive notion that radiosensitivity observed in some Rb patients may be due to an inherent defect in DNA repair, the induction and rejoining kinetics of X and gamma-ray

induced DNA single and double-strand breaks has been shown to be normal by elution assays (Cleaver, *et al.*, 1982; Woods, *et al.*, 1982).

1.3. Introduction to the Retinoblastoma Families Studies of Fitzek and Coworkers: Radiosensitivity in the Unaffected Parents and some Apparently Normal Controls

An unexpected hyper-radiosensitivity in low-passage primary fibroblasts strains derived from the unaffected parents of five Rb families, as measured by poorer clonogenic survival and an enhanced radiation-induced G1 arrest (measured by ^3H -TdR cumulative labeling indices, CLI) following acute, HDR ^{60}Co irradiation, was recently reported by Fitzek and coworkers (Fitzek, *et al.*, 2002). In each of these families, the parents' fibroblast strains were equally or more radiosensitive than fibroblasts strains derived from their own affected children (the probands), two of which (MF-7R and MF-14R) fell within the normal range of response. The published survival curves, pedigrees, *Rb1* mutations, and reported D_0 values are shown in Figure 1.2 and Table 1.1 (reproduced with permission, from Fitzek, *et al.*, 2002).

Rb family member fibroblast strains with the suffixes "M" are mothers, "F" are fathers, and "R" are affected proband children; the prefix "MF" refers to the primary author of the report, Dr. Markus Fitzek. The CIMR (Coriell Institute for Medical Research) fibroblast strains (used in both the Fitzek report and this dissertation) with prefixes "AG" were obtained from the National Institutes on Aging (NIA) cell collections and those with prefixes "GM" were obtained from the National Institute of General Medical Sciences (NIGMS) cell collections, both housed at the Coriell Cell Repositories

Table 1.1. Characteristics of the 14 retinoblastoma family members and radiosensitivity parameters for asynchronous cultures of low-passage fibroblast strains initiated from these individuals, as reported in Fitzek, *et al.* (2002).

Fibroblast Strain	Sex	Age at Biopsy	<i>Rb1</i> Genotype	Reported Radiosensitivity Parameters			Fitzek D_0 Range ^b	Source ^c	Comment
				PE Range	SF ₂ ^a	Acute D_0 (cGy)			
MF-2M Rb Family I	F	33 yr	+/+	0.194–0.295	0.14 ± 0.03	81 ± 4 cGy	70–90 cGy	HSPH	Unaffected Parent
MF-3R Rb Family I	F	14 mo	+/-	0.301–0.389	0.12 ± 0.02	74 ± 5 cGy	70–90 cGy	HSPH	Affected Proband
MF-4F Rb Family I	M	33 yr	+/-	0.359–0.468	0.09 ± 0.02	68 ± 4 cGy	50–70 cGy	HSPH	Unaffected Parent
MF-6F Rb Family II	M	24 yr	+/+	0.334–0.404	0.08 ± 0.01	69 ± 3 cGy	50–70 cGy	HSPH	Unaffected Parent
MF-7R Rb Family II	M	14 mo	+/-	0.374–0.396	0.20 ± 0.01	97 ± 6 cGy	90–110 cGy	HSPH	Affected Proband
MF-10R Rb Family III	M	4 mo	+/-	0.251–0.313	0.10 ± 0.03	91 ± 2 cGy	90–110 cGy	HSPH	Affected Proband
MF-11F Rb Family III	M	41 yr	+/-	0.255–0.329	0.09 ± 0.02	78 ± 3 cGy	70–90 cGy	HSPH	Affected Parent
MF-12M Rb Family III	F	36 yr	+/+	0.194–0.203	0.04 ± 0.01	74 ± 6 cGy	70–90 cGy	HSPH	Unaffected Parent

Table 1.1. Continued.

Fibroblast Strain	Sex	Age at Biopsy	<i>Rb1</i> Genotype	Reported Radiosensitivity Parameters			Fitzek D_0 Range ^b	Source ^c	Comment
				PE Range	SF ₂ ^a	Acute D_0 (cGy)			
MF-13M Rb Family IV	F	23 yr	+/+	0.298–0.350	0.05 ± 0.01	64 ± 3 cGy	50–70 cGy	HSPH	Unaffected Parent
MF-14R Rb Family IV	F	4 mo	+/-	0.398–0.485	0.21 ± 0.03	98 ± 16 cGy	90–110 cGy	HSPH	Affected Proband
MF-15F Rb Family IV	M	24 yr	+/+	0.286–0.373	0.06 ± 0.01	66 ± 4 cGy	50–70 cGy	HSPH	Unaffected Parent
MF-16M Rb Family V	F	35 yr	+/+	0.061–0.169	0.03 ± 0.01	52 ± 5 cGy/ 91 ± 4 cGy (Biphasic)	50–70 cGy	HSPH	Unaffected Parent
MF-17R Rb Family V	F	24 mo	+/-	0.076–0.140	0.03 ± 0.003	53 ± 4 cGy/ 89 ± 6 cGy (Biphasic)	50–70 cGy	HSPH	Affected Proband
MF-18F Rb Family V	M	45 yr	+/+	0.364–0.413	0.04 ± 0.01	58 ± 1 cGy	50–70 cGy	HSPH	Unaffected Parent

^a SF₂ = relative surviving fraction at 2 Gy.

^b The reported acute D_0 values for the exponential survival data sets provide the basis of the assigned Fitzek D_0 ranges (90–110 cGy, 70–90 cGy, 50–70 cGy) used in Chapters 5 and 7 for group-based radiosensitivity comparisons.

^c HSPH = Harvard School of Public Health/Massachusetts Eye and Ear Infirmary.

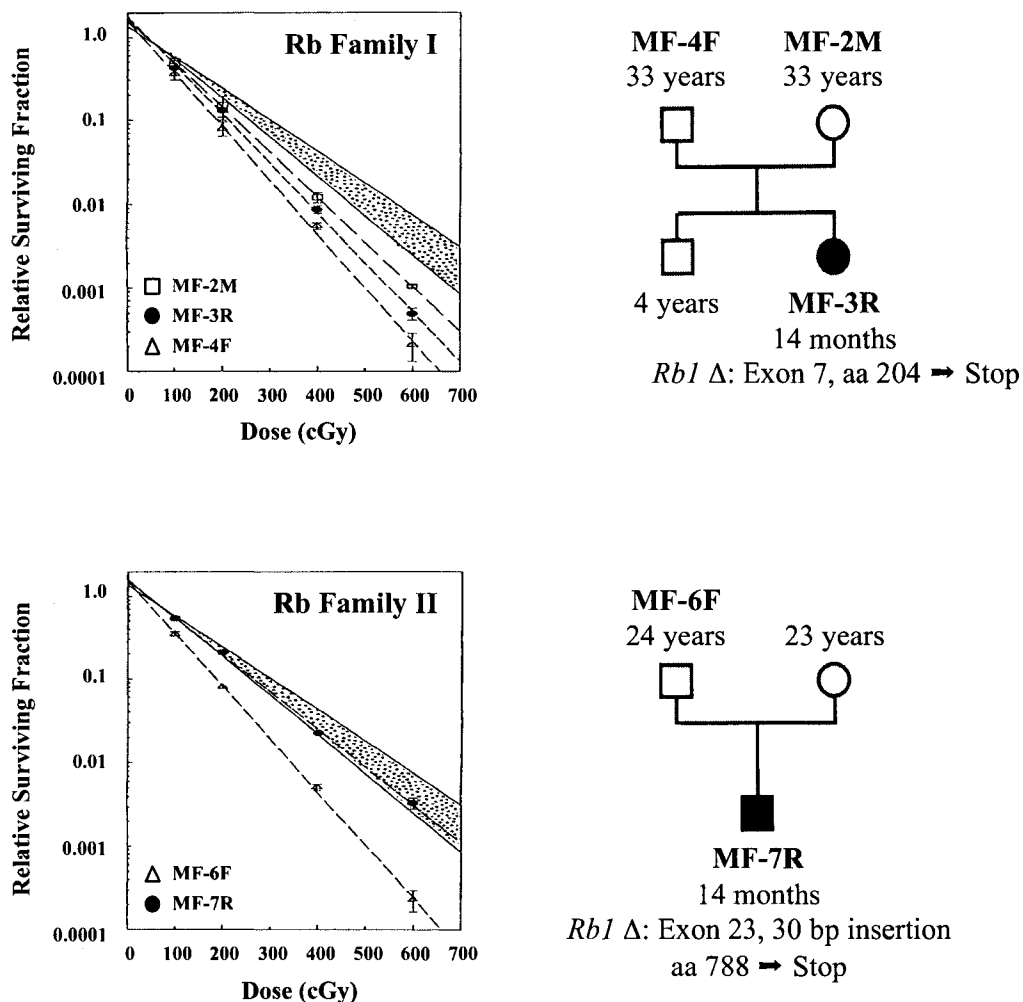


Figure 1.2. Acute dose irradiation survival curves, pedigrees, and identified *Rb1* mutations for fibroblast strains derived from the 14 individuals of the five Rb families [reproduced, with permission, from Fitzek, *et al.* (2002)]. The survival data was determined from acute ^{60}Co gamma irradiation of asynchronous cultures plated at low density 18 hours prior to irradiation. The cross-hatched area represents the range of response for the 6 apparently normal CIMR controls. The reported Rb family member strain characteristics and radiosensitivity parameters are listed in Table 1.1. Strains denoted “M” are mothers, “F” are fathers, and “R” are affected proband children; “MF” refers to the primary author of the report, Dr. Markus Fitzek.

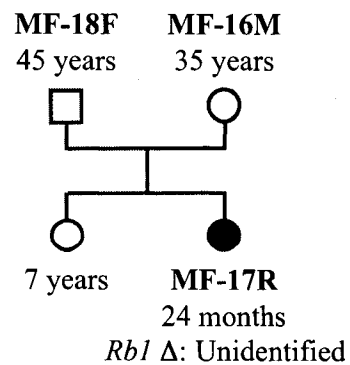
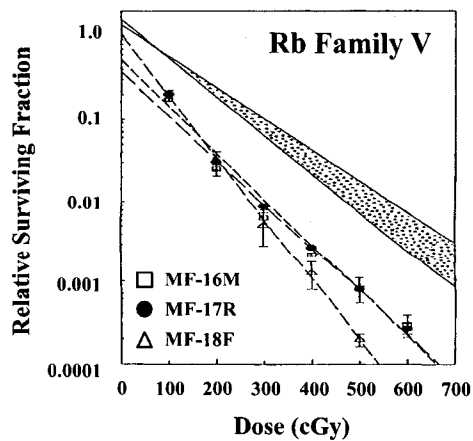
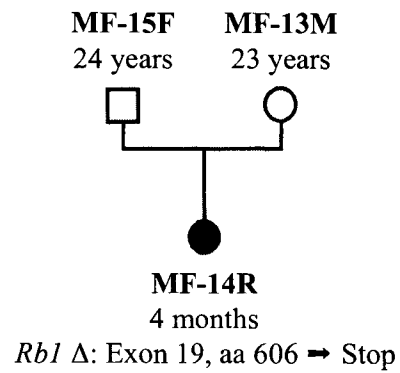
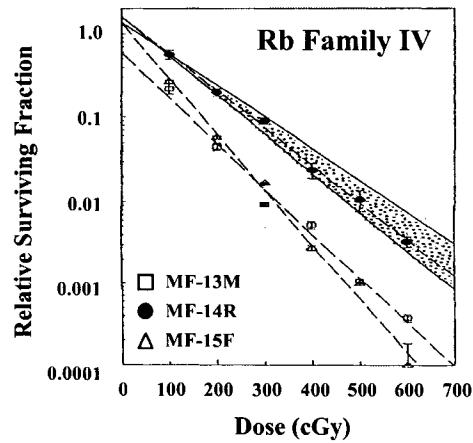
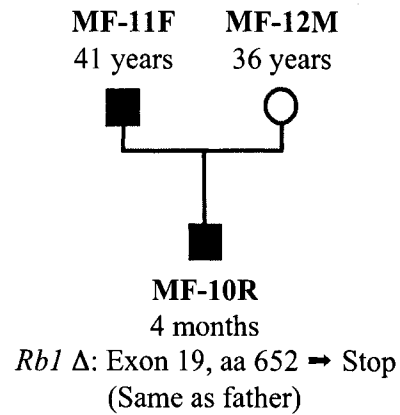
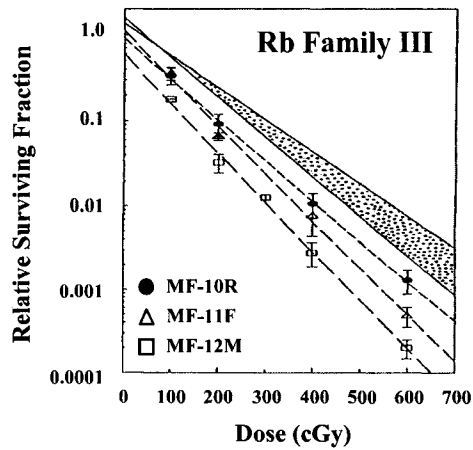


Figure 1.2. Continued.

(CCR) in Camden, New Jersey. This fibroblast strain nomenclature is likewise used throughout this dissertation.

All the probands were classified as hereditary Rb cases from the occurrence of multifocal, bilateral tumors (Reese-Ellsworth stage V, *i.e.*, tumors involving more than half than retina with vitreous seeding) and were treated with surgical enucleation and radiation therapy. The family histories were negative for Rb (except for an affected father MF-11F in Rb family III, whose affected eye was enucleated at age 2 and whose remaining eye displayed spontaneously regressed retinomas), and for any other known cancer susceptibility or radiosensitivity syndromes. Fibroblast strains were initiated from punch biopsies of the thigh (*i.e.*, distant from the radiation treatment field in the affected children) with informed consent. *Rb1* mutations were determined by exon-specific PCR analysis and direct DNA sequencing, and the absence of the proband *Rb1* mutations was verified in the peripheral blood cells of both parents (except for MF-11F, who transmitted his *Rb1* mutation to his affected son, MF-10R).

A group of six apparently normal CIMR male fibroblast strains (AG01522, GM05565, GM05658, GM05758, GM08333, and GM08429) were used in the Fitzek report as reference normal controls. Interestingly, two of these strains (GM05565 and GM05658) were equally or even more radiosensitive than many of the Rb family member fibroblast strains in terms of post-irradiation clonogenic survival (D_0 values of ~90–95 cGy) and radiation-induced G1 arrest ($^3\text{H-TdR}$ cumulative labeling indices of 33% and 51%, respectively, after a dose of 4 Gy). The 4 remaining apparently normal CIMR strains had an average D_0 value of ~125 cGy and maximum cumulative labeling indices of ~77% after 4 Gy, consistent with previous published survival measurements from that

laboratory. Three of these four CIMR strains (AG01522, GM08333, and GM08429) were included in the group of age- and sex-matched apparently normal controls for the experiments detailed in Chapters 3 and 5, and are likewise considered typical normal reference strains in this laboratory.

The overlap of response of MF-7R and MF-14R into the normal range of response for clonogenic survival in the Fitzek report is due to the inclusion of the two moderately radiosensitive apparently normal CIMR strains in the normal range (the cross-hatched areas of the survival curves in Figure 1.2), without which all the Rb family member fibroblast strains would be considered moderately radiosensitive to hyper-radiosensitive. These two proband strains did experience a more moderated post-irradiation G1 arrest, with maximum cumulative labeling indices of 64% and 68% after 4 Gy, respectively, that was more similar to the response of the four resistant CIMR strains (average CLI of ~77%) than the remaining Rb family members (average ~28%, range of 17%–44%).

A histogram of the D_0 value distribution for the 14 Rb family member and six apparently normal CIMR control fibroblast strains are shown in Figure 1.3 (the “MF,” “AG,” and “GM” prefixes have been omitted). The distribution appears trimodal, reflective of the moderate and hyper-radiosensitivity classifications of the Rb family member fibroblast strains made in the Fitzek report. The two apparently normal CIMR strains GM05565 and GM05658 obviously fall into the moderately radiosensitive group, well separated from the remaining four radioresistant apparently normal CIMR strains. Similar histograms have been generated for the other radiosensitivity parameters measured in the following chapters of this dissertation for comparison purposes.

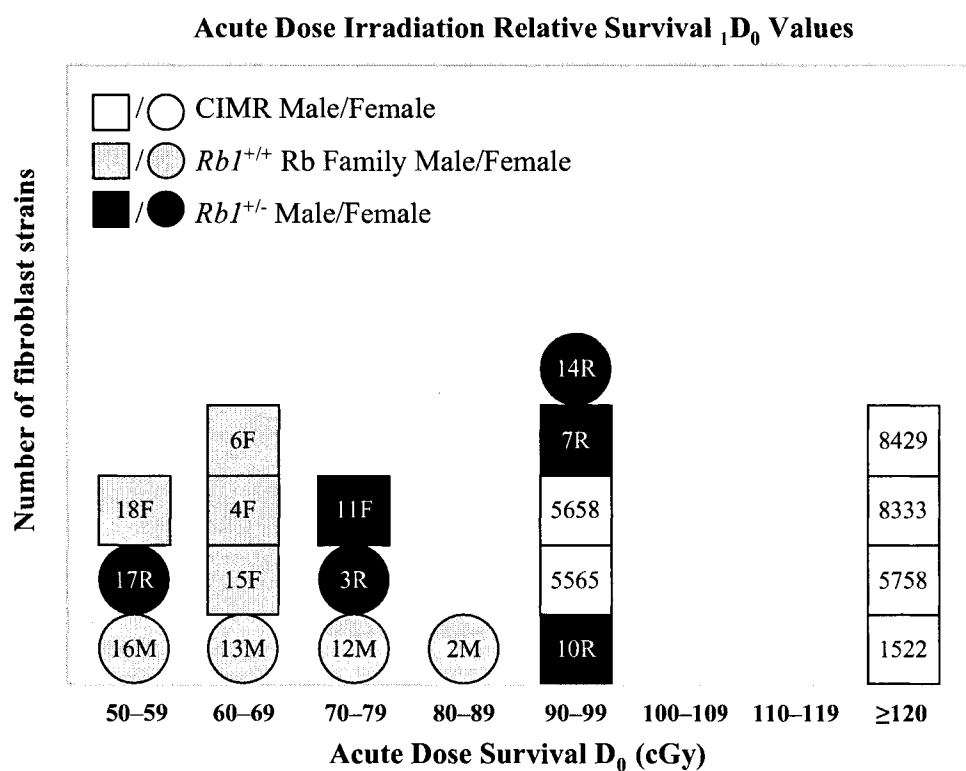


Figure 1.3. Distribution of acute dose irradiation survival D_0 values for the 14 Rb family member and 6 apparently normal CIMR control fibroblast strains reported in Fitzek, *et al.* (2002; see Table 1.1).

As mentioned earlier, previous radiosensitivity surveys of apparently normal fibroblast strains conducted by Little and coworkers and by other research groups suggest that ~25–35% of these strains are moderate or hyper-radiosensitive (Weichselbaum, Nove, and Little series discussed above; see also Nagasawa and Little, 1988; Deschavanne and Fertil, 1996). The probit plot of Deschavanne, *et al.* (1986) seen in Figure 1.1 likewise demonstrates a significant radiosensitive subpopulation (10–15%) in their large meta-analysis of “apparently normal” fibroblast survival data. Cumulative labeling index (CLI) measurements of radiation-induced G1 delay in Nagasawa and Little (1988) demonstrated that these moderately radiosensitive apparently normal cells suffer an irreversible G1 block (as they first observed with the bilateral Rb strain AG1880 in their 1983 report). Nagasawa and Little (1988) also stressed the importance of choosing validated reference normal cell strains for use as controls in the measurement of radiosensitivity in various other genetic conditions.

It must be noted that the parents of probands in the Rb families are also “apparently normal” individuals, without positive family history for any known genetic abnormality. The moderate radiosensitivity of these parents and the two reference normal strains in Fitzek, *et al.* (2002) suggests the probability that variable combinations of modifying genetic factors (*i.e.*, coding variants in radiation-responsive genes) may be influencing their radiation response, as described by Mohrenweiser, *et al.* (2003). If so, it would be expected that these could be inherited and translated to a variable degree of genomic instability and pathology of primary and secondary tumors in their affected children (Sigurdson and Jones, 2003).

Fitzek, *et al.* (2002) offered a hypothesis relating the parents' hyper-radiosensitivity phenotype to the possible enhanced transmission of mutant *Rb1* alleles to the offspring in Rb families and the occurrence of radiation-induced secondary cancers in Rb patients. I have modified this hypothesis to incorporate aspects of the transmission and sex-ratio distortion phenomena observed in Rb pedigrees discussed by Naumova and Sapienza (1994) and genomic imprinting defects recognized in female germ-line cells (Judson, *et al.*, 2002). Unexpectedly, the four most hyper-radiosensitive normal controls in the LDR studies discussed in Chapter 5 are all female. Other sex-specific differences in LDR radiation response (including cellular epigenetic modifications separate from potential hormone-related effects) in mice are only recently becoming better appreciated (Kovalchuk, *et al.*, 2004). These imprinting and radiosensitivity phenotypes may be different manifestations of a common underlying genetic deficiency in genomic maintenance and epigenetic control in these Rb family member cells.

The ~10% incidence of sporadic, unilateral Rb attests to the fact that the *Rb1* locus can be a target for aberrant methylation and epigenetic silencing. In female germ cells with defective imprinting, the paternally-inherited *Rb1* locus may become a target of aberrant methylation and silencing due to permissive spreading of methylation from the nearby and normally paternally-silenced 5-hydroxytryptamine (serotonin) receptor 2A gene (*Htr2A*, 13q14.2) during epigenetic imprint re-establishment of the new germ-line following fertilization. In this silenced, heterochromatic state, the paternal *Rb1* locus might then be relieved of selective pressure within the cell, with transcription of maternal *Rb1* gene alone supplying the cell with pRb protein, and suffer from a stochastic mutational event. Without proper DNA repair, this silenced paternally-derived *Rb1*-

mutation is then “fixed” and passed on to the next generation where *proper* imprint erasure early in embryogenesis unmasks the (now maternal/grand-paternal) mutant *Rb1* allele, serving as the first hit in Knudson’s hypothesis in this affected child. While this hypothesis is not directly tested in this work, future research in this area (e.g., 5-azacytidine-induced passive demethylation studies that may turn a potentially silenced gene back on in these fibroblast strains) may yield informative results.

1.4. Objectives of Dissertation

The main objectives of this dissertation are two-fold. First, we wanted to confirm the moderately radiosensitive and hyper-radiosensitive responses (and degrees thereof) for the 14 Rb family member fibroblast strains made in Fitzek, *et al.* (2002) using other radiobiological assays: survival following acute irradiation and 24 hours potentially lethal damage PLD repair in G0/G1-phase cultures and G2-phase chromosomal radiosensitivity. This provided laboratory baseline data for these fibroblast strains in these assays. Second, we wanted to try to enhance the degree of differential radiation survival between the radiosensitive Rb family members and a group of age- and sex-match apparently normal CIMR controls using a continuous low dose-rate (LDR) irradiation colony formation assay protocol previously employed in this laboratory to isolate the *prkdc*-deficient CHO 10B2 mutant *irs-20* [*prkdc* encodes the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs, the cardinal protein in the primary DNA double-strand break repair pathway in mammalian cells, non-homologous end-joining (NHEJ)]. If this degree of differential LDR survival was large enough, we could apply continuous LDR radiation exposure as a selection tool in a genetic complementation and correction

strategy to possibly identify mutant genes (other than, of course, *Rb1*) in the Rb family member strains (Grimm, 2004). The hyper-radiosensitivity of the unaffected parents in the Rb families attests to the potential presence of such genetic variants (in a unconfounded, wild-type *Rb1*^{+/+} genetic background). This genetic deficiency could be corrected by the transfection of a normal copy of a gene from a cDNA library pool of expressed human genetic sequences that restores the wild-type phenotype (in this case, the typical “normal” radioresistance of *validated* control fibroblast strains exposed to continuous LDR irradiation). Identification of such a gene could also provide critical evidence and impetus for further research into how mutations of this gene could contribute to bilateral Rb and possibly other genetic diseases.

To provide background for the research presented in this dissertation, Chapter 2 presents short reviews on a pertinent number of topics related to the cellular, biochemical, and genetic basis of cancer predisposition and radiation sensitivity. These discussions focus primarily on *in vitro* radiobiological experiments with human and rodent cells with mutations in the critical tumor suppressor proteins pRb, tp53, and ATM, which are responsible for the diseases retinoblastoma (Rb), Li-Fraumeni syndrome (LFS), and ataxia-telangiectasia (A-T), respectively. Collectively, mutations of these three genes have been clearly associated with tumorigenesis in a very large majority of human cancer types (over 200 specific cancers in every human tissue have been documented), besides the symptoms classically associated with these conditions. Despite the application of more modern molecular biology techniques, questions continue to the total number of roles these proteins play in governing the extensive regulatory networks associated with DNA damage response and repair, cell cycle control, and genomic

stability in mammalian cells. Also included are summaries of relevant research protocols and results of studies employing these protocols with various normal and tumor cell types derived from affected individuals. This provides the background rationale for the experimental protocols employed in Chapters 3 through 6.

The purpose of the studies described in Chapters 3 and 4 are to measure the acute, high dose-rate (HDR) radiation response of the Rb family member and some apparently normal CIMR fibroblast strains using the endpoints of: 1) clonogenic survival following acute, HDR ^{137}Cs irradiation and 24 hours of potentially lethal damage (PLD) repair in confluent, G0/G1-phase cultures, and 2) G2-phase chromosomal radiosensitivity (induction of chromatid-type aberrations in the first post-irradiation mitosis) in asynchronously growing (log-phase) cultures. Hypersensitive responses in both these particular assays have been reported for bilateral Rb patients (Weichselbaum, *et al.*, 1985; Sanford, *et al.*, 1989, 1996) and were considered potentially informative for use with the Rb family member strains. As discussed previously, ~25–35% of apparently normal fibroblast strains examined in these studies have also demonstrated moderately radiosensitive to hyper-radiosensitive responses as well.

The purpose of the studies described in Chapter 5 is to measure the continuous low dose-rate (LDR) irradiation colony formation ability of asynchronous cultures of the 14 Rb family member and 14 apparently normal CIMR control fibroblast strains after a two-week exposure period. We hypothesized that we could achieve a larger degree of differential survival between the hyper-radiosensitive Rb family member and radioresistant apparently normal CIMR strains than is possible with an acute, HDR

protocol. This would also provide the selection basis for attempts to complement and correct the responsible genetic defects in these parental cells.

Earlier work by Stackhouse and Bedford (1993) involved in the initial isolation of the *prkdc*-deficient CHO 10B2 mutant *irs-20* in this laboratory, provides the basis for the application of the continuous LDR colony formation to the radiosensitive Rb family member fibroblast strains in Chapter 5. Priestly, *et al.* (1998) later applied the continuous LDR colony formation assay to wild-type CHO 10B2 cells and C57BL/6 wild-type fibroblasts, and their radiosensitive counterparts, the CHO 10B2-derived *prkdc*-deficient *irs-20* and *scid prkdc^{-/-}/tp53^{-/-}* SGCER11 CB17 mouse fibroblasts, demonstrating a very large survival differential between the radiosensitive and wild-type cells at dose rates <15 cGy/h. This report provides the basis for the study reported in Chapter 6.

In Chapter 6, the continuous LDR irradiation colony formation assay is applied to mouse embryo fibroblasts (MEFs) deficient for the two other critical tumor suppressor proteins, Tp53 (mutated in LFS patients) and ATM (mutated in A-T patients). MEFs deficient for these genes were chosen to demonstrate the probable limits of response in the continuous LDR colony formation assay for future researchers, and for comparison to data presented in Priestly, *et al.* (1998). The *tp53^{-/-}* MEF clone 6 cell line (a high-passage, clonal isolate of MEF cells generated in Donehower, *et al.*, 1992) was tested to determine if these cells, previously shown to be radioresistant to wild-type control cells in acute, HDR radiation survival studies (along with the majority of other human and rodent cell types deficient for *tp53*), would similarly be radioresistant during continuous LDR colony formation. The *atm^{-/-}* and *atm^{+/-}* MEF strains were likewise chosen to determine the probable lower limit of response (in the former case), and to determine if the LDR

survival response of the *atm*^{+/-} heterozygote strains would be significantly different from that of the wild-type control strains (an issue further discussed in Section 2.4.6 in Chapter 2). Also included in Chapter 6 are a series of cytogenetic and immunocytochemical experiments that further characterize the high degree of spontaneous and radiation-induced genomic instability observed in a sub-population of the *tp53*^{-/-} MEF clone 6 cell line, whose phenotypes include giant, aneuploid cells; aberrant mitotic figures; and massive chromosome aberrations in metaphase preparations.

In Chapter 7, I begin by briefly summarizing the experimental results for the 14 Rb family member fibroblast strains recorded in Chapters 3 through 5, including a comparison of the relative responses of the family members cells in each of the three assays. I continue by discussing the benefits of the continuous LDR irradiation colony formation assay, as compared to the more traditional HDR and LDR exposure strategies that have been employed in the past, in terms of the ability to generate a large differential degree of cell killing for radiosensitive human cell strains at dose rates where a significant fraction of cells with normal radioresistance may be recovered. The basis and preliminary results of genetic complementation and correction experiments employing viral-based cDNA library transfections and continuous LDR radiation exposure (2–3 cGy/h) as the selection agent are discussed as well. Finally, I conclude by discussing reasons why such chronic, LDR radiation exposures can be so effective for cell killing, chromosome aberration induction, and tumorigenesis, when one considers the potential damage that can be caused by a *single* electron track traversal in a mammalian cell nucleus.

CHAPTER 2

Review

2.1. Retinoblastoma

2.1.1. Introduction to Retinoblastoma

Retinoblastoma (Rb) is a malignant neoplasm of the retina resulting from inactivating mutational events involving both alleles of the *Rb1* retinoblastoma susceptibility gene, located on human chromosome 13q14.1–14.2 (Knudson, 1971, 1983; Abramson, *et al.*, 2003). Rb occurs in one in 18,000 to 30,000 live births worldwide equally among males and females, appearing predominantly in children under four years of age, and is responsible for 2.9% of childhood malignancies and ~11% of cancers developing in the first year of life (Young, *et al.*, 1999; American Cancer Society, 2005). Although fairly rare, Rb is considered a classic genetic disease because of the well-documented genetic patterns and underlying molecular mechanisms that characterize these tumors (Knudson, 1971, 1983, 2001). Prior to the advent of eye enucleation in 1865 and more modern cancer treatment modalities, retinoblastoma was often invariably fatal due to local spread into the brain and more distant metastases (Kleinerman, *et al.*, 2005). Survival of Rb patients is now greater than 93%, and the increased transmission of mutant *Rb1* alleles in the population due to relaxed selection may lead to an increased incidence of Rb in the future (American Cancer Society, 2005).

Children may be unilaterally (one eye) or bilaterally (both eyes) affected with Rb with one or multiple tumors per eye, depending upon the type of retinal progenitor cell and timing of the mutational events of the *Rb1* gene. Formation of the retina takes weeks in mice and months in humans, during which retinal progenitor cells divide and differentiate into 55–60 functionally distinct cell types, divided into seven main classes: amacrine, bipolar, cone, ganglion, horizontal, Müller glia, and rod cells (Dyer and Bremner, 2005). *De novo Rb1* mutations occurring in an established pool of one of the classes of cells give rise to usually one tumor in one eye, as the low frequency of loss of both wild-type *Rb1* alleles and other requisite mutations will serve to limit the initiation of additional neoplastic precursors. However, inheritance of a mutated *Rb1* allele through either parental germ line or a somatic mutation occurring very early in embryonic development prior to the generation of the retinal stem cell pool will result in multifocal tumors in both eyes, as most (in the case of mosaicism) or all somatic cells throughout the body (including all retinal precursor cells) will carry one mutated *Rb1* allele. Concomitant loss of the other functional *Rb1* allele and additional mutations could thus occur in a higher percentage of the retinal precursor cells, resulting in multiple initiated cells (derived from either the retinal stem cells or a more differentiated class of cells) capable of forming multiple tumors in both eyes. The developmental timing of retina formation and retinal precursor cell division and differentiation pathways in embryonic/neonatal development limits the occurrence of Rb to children.

Sporadic Rb (resulting from *de novo* somatic mutations in the *Rb1* gene in the pool of retinal precursor cells) accounts for approximately 67% of Rb cases and presents at an average age of 23–26 months with a single tumor in one eye. The remaining 33%

of Rb cases result from germinal mutations of the *Rb1* gene and present earlier with multifocal tumors (mean of 5.3 foci) by 10–16 months of age (Vogel, 1979; Petty, *et al.*, 1999; Young, *et al.*, 1999). Eighty-five percent of germinal Rb patients are bilaterally affected, while the remaining 15% are unilaterally affected; however the large majority of these patients have multifocal tumors in the same eye (Young, *et al.*, 1999; American Cancer Society, 2005). Only a minority (approximately 8–10%) of patients with germinal Rb are truly “hereditary” and involve previous family history of the disease, the remaining “non-hereditary” cases represent novel parental germ-line mutations or mosaicism of a mutation generated during early embryonic development (Vogel, 1979; Lohmann, *et al.*, 1996; Lohmann, 1999).

Knudson’s now classic “two-hit” model was officially formulated in 1971 to explain the observed frequencies and modes of transmission in Rb pedigrees (Knudson, 1971, 1983, 2001). Statistical analyses of the germ-line carrier tumor incidence frequencies mentioned above (roughly 70–80% bilaterally affected, 15–20% unilaterally affected and 10–15% unaffected) was viewed as evidence of a Poisson distribution-based tumor distribution, with a mean of 2–3 genetic “hits,” or mutations (see Figure 7.3 for an example of a 2-hit Poisson distribution). The relationship between age of onset and the frequencies of diagnosed bilateral and sporadic cases was likewise best fit on semi-logarithmic plots with one-hit (linear) and two-hit (curvilinear) curves, respectively, providing more support for Knudson’s model. The earlier presentation in bilateral Rb cases is consistent with this notion, as one of the tumor hits is present in all retinal precursor cells. Sporadic Rb cases present later in childhood as both tumor hits must

occur in a retinal precursor cell, along with other requisite mutations (see below), resulting in delayed presentation of the disease.

The penetrance of *Rb1* mutations has been estimated as 85–90% (Knudson, 1971; Vogel, 1979), *i.e.*, 10–15% of *Rb1* mutation carriers will not develop retinoblastoma requiring medical intervention, although ophthalmologic investigations of such individuals often reveals spontaneously regressed tumors or benign masses (retinomas) (Fitzek, *et al.*, 2002; Abramson, *et al.*, 2003). Incomplete penetrance and variable expressivity of *Rb1* mutations is likewise evident by generational skipping in some Rb pedigrees, and the presence of both bilateral and unilaterally affected siblings in a single generation (Vogel, 1979). Thus, the chance of a bilateral Rb patient having an affected child with Rb is ~45% (50% chance by random meiotic segregation of the mutated chromosome 13 times ~90% penetrance).

Approximately 7.5–10% of hereditary Rb cases and 1–5% of sporadic Rb cases involve cytogenetically visible disruptions of the 13q14 region (Ejima, *et al.*, 1988). Megabase-scale deletions of the 13q region are most commonly observed, but stable translocations with breakpoints in the 13q14 region have been documented in a small minority of cases. These so-called “D-deletion” (so-called because cytogeneticists first noted deletions involving the “D” group of human chromosomes, *i.e.*, the medium-sized acrocentric chromosomes 13, 14, and 15) or “13q-deletion” Rb patients possess characteristic physical traits including high foreheads, broad nasal bridges, thin lips, and are often mentally retarded (Young, *et al.*, 1999).

A third form of Rb, known as trilateral Rb, involves bilateral Rb and pinealoma. The pineal gland is attached to the brain’s third ventricle and has been referred to as the

“primitive third eye” (Abramson, *et al.*, 2003). It functions to secrete melatonin and plays an essential role in the proper regulation of diurnal/nocturnal circadian cycles, which, if chronically disturbed, can contribute to increased cancer incidence rates in individual cohorts (*e.g.*, airline workers, “jet-lagged” mice; Filipski, *et al.*, 2004). Trilateral Rb accounts for approximately 3% of bilateral cases (Amoaku *et al.*, 1996) and usually affects individuals in the second or third generation of Rb pedigrees (Kivella *et al.*, 1999), possibly a reflection of the phenomenon of “genetic anticipation.”

Retinoblastoma patients can be treated with surgery (enucleation), laser photocoagulation, cryotherapy, thermotherapy, external beam radiotherapy, intraocular brachytherapy, chemotherapy regimens, or combinations thereof (Petty, *et al.*, 1999). Patients with bilateral Rb have a fairly high incidence of secondary cancers (primarily osteosarcomas, soft tissue sarcomas, and malignant melanoma), often occurring after chemotherapy or in the radiation treatment field in a dose-responsive manner (Tucker, *et al.*, 1987; Eng, *et al.*, 1993; Wong, *et al.*, 1997; Kleinerman, *et al.*, 2005). External beam radiotherapy treatment options include the use of orthovoltage X-ray sources, megavoltage photon sources, and ^{60}Co sources delivering doses of 15–115 Gray (Gy; mean of ~45–50 Gy). Brachytherapy treatments include ^{222}Rn seeds and ^{60}Co plaques, ranging in strength from 150 to 800 milligram hour radium (mgh Ra) equivalents (Kleinerman, *et al.*, 2005). Chemotherapeutic agents employed include carboplatin, cisplatin, vincristine, etoposide, teniposide, cyclophosphamide, doxorubicin, and combinations thereof (American Cancer Society, 2004).

In the most recent report by Kleinerman, *et al.* (2005), the standardized incidence ratio (SIR), the ratio of secondary cancers in Rb patients to the overall expected

population frequencies, was 19 (95% CI, 16–21) for hereditary cases and 1.2 (95% CI, 0.7–2.0) for non-hereditary cases. Radiation treatment further increased the risk of secondary cancers in hereditary cases by 3.1-fold (95% CI, 2.0–5.3). The cumulative incidence of secondary cancers at 50 years after initial diagnosis was 36% (95% CI, 31–41%) for hereditary Rb cases and only 5.7% (95% CI, 2.4–11%) for non-hereditary Rb cases (Kleinerman, *et al.*, 2005). This observation has spurred much interest in a possible connection between the etiology of Rb, the use of cancer therapy treatment regimens, and DNA damage-induced genomic instability and transformation. This connection will be discussed in more detail in later sections, following a brief introduction to the retinoblastoma gene, *Rb1*, and protein, pRb, whose multitude of cellular functions provide numerous routes to tumorigenesis.

2.1.2. The Retinoblastoma Susceptibility Gene *Rb1*

The retinoblastoma susceptibility gene, *Rb1*, was the first tumor suppressor gene to be isolated by positional cloning approaches (Friend, *et al.*, 1986). Guided by the karyotypic abnormalities observed in 13q-deletion Rb patients and Rb tumors, the gene was localized to the long arm of chromosome 13, band 14.1–14.2, using restriction fragment length polymorphism (RFLP) analysis (Cavanee, *et al.*, 1983, 1985). Further molecular characterization of the *Rb1* gene revealed that it spans over 183 kilobases (kb) and is composed of 27 exons (Toguchida, *et al.*, 1993; Richter, *et al.*, 2003). The *Rb1* gene encodes a 4.7-kb mRNA transcript ubiquitously expressed in all adult tissues, but demonstrates developmental expression patterns correlated with the timing of terminal differentiation pathways (Jiang, *et al.*, 1997). Why germinal mutation of the *Rb1* gene

leads predominantly to retinoblastomas and osteosarcomas and not other tumor types still remains unknown, but is most probably related to a tissue-specific function of the pRb protein in the terminal differentiation pathways of retinoblasts and osteoblasts (Dyer and Bremner, 2005).

The mutation spectrum of the *Rb1* gene is somewhat reflective of the mode of inheritance (sporadic/unilateral, hereditary/bilateral, or 13q-deletion type), and all classes of mutations have been observed distributed across 26 of the 27 exons of the *Rb1* gene (Lohmann, *et al.*, 1996; Lohmann, 1999; Richter, *et al.*, 2003). The heterogeneity of *Rb1* mutations and large size of the *Rb1* gene requires more complicated genetic techniques such as direct DNA sequencing, quantitative multiplex PCR, and methylation-specific (bisulfite method) PCR for mutational analyses of individual patients (Richter, *et al.*, 2003). The germline *Rb1* mutations observed in unilateral Rb cases reported in Richter, *et al.*, were 57% null, 40% in-frame, and 3% in the promoter region (primarily methylation-based silencing of CpG-rich tracts). The remaining sporadic unilateral Rb cases consisted of 89% null, 3% in-frame, and 8% promoter methylation. Mutations detected in the bilateral Rb cases were 93% null, 6% in-frame, and 1% promoter methylation. The characterized mutations in the affected members of the Rb families that are the subject of this report are all null mutations (Fitzek, *et al.*, 2002; see Figure 1.2).

Null *Rb1* mutations include whole gene deletions, deletions involving one or more exons, small frame-shift intra-exon insertions and deletions, and nonsense mutations resulting in complete loss or premature truncation of the mRNA transcript (Richter, *et al.*, 2003). Following transcription, unstable *Rb1* mRNA is predicted to be degraded via nonsense-mediated decay (NMD, a form of RNA surveillance), resulting in the absence

of measurable pRb protein levels in the cell (Dunn, *et al.*, 1989; Frischmeyer and Dietz, 1999). Measurable levels of mutant pRb protein in tumor cells are thought to occur from down-regulation of this NMD pathway or higher levels of transcription of the mutant *Rb1* alleles (Dunn, *et al.*, 1989). Most of the missense mutations that result in expressed mutant forms of the pRb protein cluster in the A and B regions of the “pocket” domain (Lohmann, 1999, see below); such mutations are infrequent (7%) in bilateral cases, but account for 43% of germline mutations in unilateral Rb cases (Richter, *et al.*, 2003).

In about 50–65% of Rb tumors, mutation of the second, functional allele of *Rb1* occurs by a chromosomal loss-of-heterozygosity (LOH) event, accompanied by the loss of nearby polymorphic microsatellite markers (Squire, *et al.*, 1985; Zhu, *et al.*, 1992; Hagstrom and Dryja, 1999; Richter, *et al.*, 2003). Such LOH events can occur from whole or partial chromosome deletion, somatic recombination/gene conversion, or mitotic nondisjunction events (Cavanee, *et al.*, 1983; Zhu, *et al.*, 1992). Interestingly, the likelihood of such a LOH event depends on the form of the initial *Rb1* mutation. In the study by Richter, *et al.* (2003), only 23% of tumors with whole-gene deletions and 27% with exon deletions of the first *Rb1* allele demonstrated LOH of the second *Rb1* allele. However, in 88% of the tumors due to methylation-induced silencing of the promoter of the first *Rb1* allele, the second allele was lost in a LOH event.

Cytogenetic analysis of Rb tumors indicates other chromosomal regions besides 13q are also likely to be involved in the full progression of retinoblastoma, *i.e.*, biallelic inactivation of the *Rb1* gene is necessary but not sufficient for the full onset of Rb. It was noted in the 1970s that metaphase spreads prepared from Rb tumor samples often contained additional marker chromosomes (Yunis and Ramsay, 1978), these were

identified by chromosome banding techniques and more recently with the molecular cytogenetic techniques of fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH). Rb tumor karyotypes include additional copies of chromosome regions 1q21–1qter, 2p23–25, 5p15, 6p11–12, 6p22, and 9q; translocations involving 10q25, 14q32, and 17p12; and losses involving 9p, 13q14, 16q22, 19q13, and Xq among others (Gardner, *et al.*, 1981; Squire, *et al.*, 1984; Squire, *et al.*, 1985; Mairal, *et al.*, 2000; Chen, *et al.*, 2001; van der Wal, *et al.*, 2003; Huang, *et al.*, 2003; Lillington, *et al.*, 2003; Amare, *et al.*, 2004; Zielinski, *et al.*, 2005). A candidate oncogene *RBKIN/KIF13A*, that encodes a kinesin-like protein, has been identified in a 0.6 Mb-sized region of 6p22 that was amplified in 59% of Rb tumors examined (Chen, *et al.*, 2002).

Less than 20% of Rb tumors possess cytologically visible double minute (DM) chromosomes or chromosomes with homogenously staining regions (HSRs); in such cases, the *MYCN* (N-myc) and *INT1* proto-oncogenes and genes encoding Id1–4 helix-loop-helix transcription factors are frequently amplified (Lee, *et al.*, 1984; Squire, *et al.*, 1985; Sakai, *et al.*, 1985; Arheden, *et al.*, 1988; Mairal, *et al.*, 2000; Chen, *et al.*, 2001). Analysis of constitutional fragile site expression in Rb patients revealed a higher prevalence of fragile site loci at 13q14 and 13q22, 3p14, 6p23, 10q25, 16q22–q23, and 17p12 (Squire, *et al.*, 1995; Amare, *et al.*, 2004). Consistent with this more likely “multi-hit” nature of Rb tumor formation, introduction of a wild-type copy of the *Rb1* gene into transformed tumor cells is often insufficient to reverse the malignant phenotype (Muncaster, *et al.*, 1992).

2.1.3. Inheritance of Retinoblastoma

Although Rb is technically an autosomal recessive condition, in that both functional *Rb1* alleles must be inactivated to initiate tumor progression, susceptibility to Rb is inherited as an autosomal dominant trait; inheritance of a single mutated *Rb1* allele will almost always give rise to a tumor (Vogel, 1979). Given the retinal stem cell compartment contains an estimated four million cells and the large number of cell divisions occurring in the retinal epithelium during development, the chance that the second, functional *Rb1* allele in heterozygote children could be mutated due to genetic or epigenetic mechanisms is quite high (Knudson, 2001; Dyer and Bremner, 2005). This large number of cell divisions also prevents the need to invoke a “mutator phenotype”-like phenomenon of accelerated mutagenesis to explain the multiple hits required for carcinogenesis (Knudson, 2001).

Indeed, genomic instability is not a common feature of all retinoblastomas and Rb-associated secondary tumors, as will be discussed further in the following sections. This heterogeneity provides evidence that multiple routes of genetic deregulation in a precursor cell are capable of generating the same tumor type. By identifying the critical mutational events along these routes, more effective genetic therapies could be specifically targeted to such changes. As discussed previously, the cell lineages (and their many class-specific differentiation variants) primarily responsible for generating these initial neoplastic precursor cells has yet to be identified. This is complicated by the fact that Rb tumors contain multiple cell types and individual tumor cells may display cell surface markers from several different cell types found in the retina (DiCiommo, *et al.*, 2000; Dyer and Bremner, 2005).

Sex-ratio distortion in retinoblastoma has been documented for a number of phenomena in Rb. Analysis of tumor tissue obtained from bilateral retinoblastoma and osteosarcoma patients demonstrates the preferential retention of mutated paternal alleles, *i.e.*, the maternal, wild-type alleles are more frequently the target of LOH events, the “second-hit” in Knudson’s two-stage model (Ejima, *et al.*, 1988; Dryja, *et al.*, 1989; Zhu, *et al.*, 1989). Reports by Munier, *et al.* (1992, 1994) revealed an unanticipated transmission-ratio distortion of affected Rb children of bilaterally affected males versus females. This bias was further supported by a report by Naumova and Sapienza (1994), who also identified a sex-ratio distortion as an excess of male children overall *and* affected male children in the offspring of affected fathers (perhaps, they suggest, at the *expense* of female children overall). To explain these findings, they attribute this effect to either a defectively imprinted X-linked locus or perhaps to the more frequent transmission of sperm bearing a mutant *Rb1*. To address this latter issue, a study by Girardet *et al.* (2000) examining six Rb families found no evidence of an unbalanced transmission of *Rb1* mutant sperm using single sperm PCR genotyping. They likewise postulate a defective imprint maintenance gene exists on the X chromosome, perhaps involved in meiotic chromosome segregation, and a defectively imprinted gene on 13q14 (other than *Rb1*) may represent targets of a deregulated imprinting protein (perhaps a DNA methyltransferase). Finally, deaths from secondary tumors in Rb patients are significantly more frequent among females (relative risk = 39) than males (relative risk = 22; Eng, *et al.*, 1993).

2.1.4. The Retinoblastoma Protein pRb

The 4.7-kb mRNA transcript of the *Rb1* gene encodes a 110-kDa nuclear phosphoprotein (105-kDa on SDS-PAGE) composed of 928 amino acids (921 in mouse), denoted pRb (Friend, *et al.*, 1986), as seen in Figure 2.1. Retinoblastoma protein-mediated pathways play a critical role in regulating properly timed cell cycle transitions (most notably the G1 to S phase transition), differentiation programs (by inducing tissue-specific gene expression and permanent withdrawal from the cell cycle), and Tp53-mediated apoptotic responses after DNA damage. As such, *Rb1* presents an attractive target for deregulation, as evidenced by the gene's mutation in ~80% of sporadic human cancers (Scherr, 1996). Oncoproteins encoded by various DNA tumor viruses [*e.g.*, human papilloma virus (HPV) E7, adenovirus E1A, and polyoma virus SV40 large T antigen] target pRb and disrupt its function, and have proven to be invaluable tools in the study of the mechanisms of oncogenic transformation (Morris and Dyson, 2001). Amplification of the cyclin-dependent kinase genes *CDK4* and *CDK6* or their cyclin partners *CCND1–3* (cyclin D1–D3), or loss of *CDKN2A* (p16^{Ink4a}), a specific CDK4/6 inhibitor, through direct deletion or by promoter silencing provide yet other routes of pRb pathway disruption (Weinberg, 1995; Mittnacht, 1998; Scherr, 2001).

The pRb protein is a member of the so-called “pocket protein” family, named for the A and B “pocket” domains (see Figure 2.1), that also includes the proteins p107 and p130. The primary function of these pocket proteins is to bind these E2F/DP1 transcription factor family members in transcriptional repressor complexes through their conserved LxCxE motif (reviewed in Weinberg, 1995). The original six E2F proteins (E2F1–6) and two DP proteins (DP1–2) form heterodimers while the newest E2F family

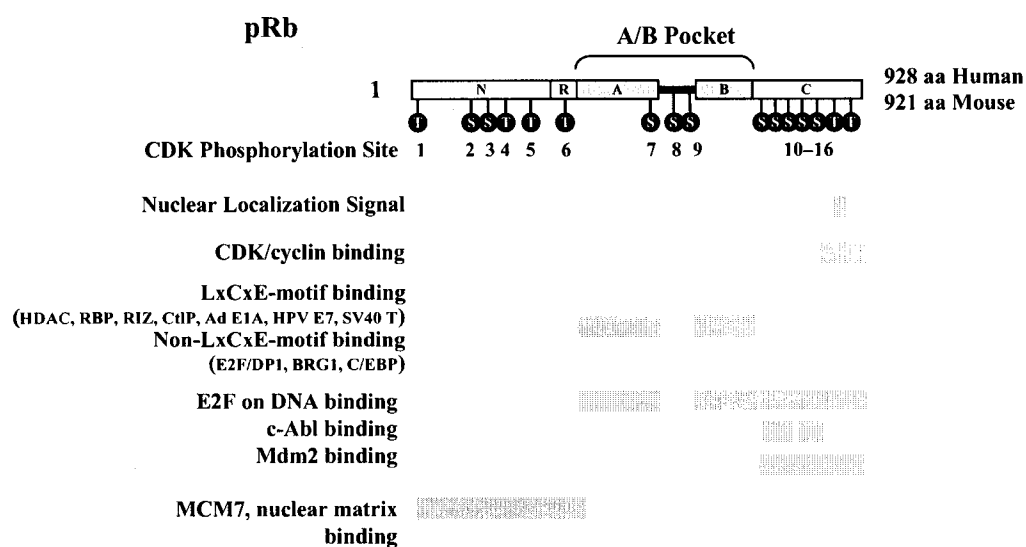


Figure 2.1. Structural domains of the pRb protein illustrating sites of CDK4/6–cyclin D and CDK2–cyclin E-mediated serine (S) and threonine (T) phosphorylation and other important protein–protein interactions. Adapted from DiCiommo, *et al.* (2000).

members, E2F-7 and E2F-8, do not require a DP partner and dimerize with themselves (De Bruin, *et al.*, 2003; Stevens and La Thangue, 2004; Maiti, *et al.*, 2005). Unlike *Rb1*, very few reports of cancers due to *p107* or *p130* mutations exist in the literature. pRb-mediated transcriptional repression is also modulated by pRb complex binding to RNA polymerases I and III, and recruitment of histone deacetylases, histone and DNA methyltransferases, BRG1 and hBrm1 (homologues of yeast SWI/SNF-family chromatin remodeling complexes), polycomb group proteins, and other heterochromatin maintenance proteins that function to tighten or relax chromatin compaction in the promoter regions of target genes (reviewed in Morris and Dyson, 2001; Zheng and Lee, 2002).

The activity of the pRb protein (and its associated complexes) is controlled by its phosphorylation status, which provides a sensitive regulatory mechanism for the G1 to S-phase transition (Scherr and McCormick, 2002). The active form of pRb is hypophosphorylated and functions in the early G1-phase to bind members of the E2F/DP1 transcription factor family and a multitude of other interacting proteins. This prevents the premature transcription of E2F target genes involved in the onset of S phase and the initiation of DNA synthesis, including PCNA, DNA polymerase α , and nucleotide biosynthesis genes (Dyson, 1998; Lavia and Jansen-Durr, 1999; Stevaux and Dyson, 2002; Polager, *et al.*, 2002; Stevens and La Thangue, 2004). The DP1 and DP2 proteins function as transcriptional repressors, providing the basis of an auto-regulatory loop that eventually halts transcription of the E2F-responsive genes as cells complete DNA synthesis and prepare to enter G2 phase.

As cyclin D1–D3 levels rise in mid-G1, complexes of CDK4/6-cyclin D bind C-terminal (R/K)xL sequences of pRb and partially phosphorylate the protein, releasing some of the E2F transcription factors that induce the transcription of cyclins A and E. Complexes of CDK2-cyclin E and CDK2-cyclin A complete the phosphorylation of the 16 serine and threonine sites of pRb, disrupting the assembled transcriptional repressor complexes, and DNA synthesis is initiated as cells progress into S phase (DiCiommo, *et al.*, 2000). Essentially all pRb-associated interacting proteins fail to bind hyper-phosphorylated pRb, and phosphorylation site mutants of pRb are potent inhibitors of cell cycle progression and transcription in virtually all cell types studied (Morris and Dyson, 2001).

Apart from its critical role in regulating E2F transcriptional networks, the pRb protein has been shown to interact with at least 110 different protein partners in addition to those already mentioned, forming complexes of ≥ 10 MDa in molecular weight (Morris and Dyson, 2001; Zheng and Lee, 2002). A recent review of the GeneCards[®] database (Weizmann Institute of Science, <http://bioinfo1.weizmann.ac.il/genecards/index.shtml>) identified a total of 140 pRb-interacting proteins. These proteins are involved in a number of cellular pathways including RNA and protein metabolism, growth factor signaling, chromatin remodeling, nuclear matrix attachment, and DNA repair (see Figure 2.1). Chromatin immunoprecipitation (ChIP)-coupled microarray and GFP-pRb fusion protein studies have revealed hypo-phosphorylated pRb repressor complexes are enriched in nucleolar regions and E2F-responsive promoter regions, and are subsequently excluded from these regions upon hyper-phosphorylation (Angus, *et al.*, 2003; Wells, *et al.*, 2003). These studies suggest that hyper-phosphorylated pRb becomes highly mobile

in the nucleus and is no longer subject to chromatin-associated sequestration by the other members of the transcriptional repressor complexes.

Critical for the assembly and perinucleolar localization of pRb-associated complexes are the nuclear lamins A and C (alternate splice forms of the *Lmna* gene), knockouts of which disrupt this localization and induces proteasomal degradation of pRb (Johnson, *et al.*, 2004). *Lmna*^{-/-} cells have ~5-fold lower pRb protein levels (mRNA levels were not affected) and resemble *Rb1*^{-/-} cells by having altered cell cycle kinetics and a reduced capacity to undergo DNA damage-induced cell cycle arrest. As an interesting side note, use of lamin A/C siRNA controls in siRNA “knockdown” experiments may lead to previously unrecognized off-target (pRb-related) effects that are not generally appreciated in such studies.

The pRb protein also plays a critical role in the S-phase checkpoint responses following DNA damage. Cells deficient in pRb fail to slow DNA synthesis following DNA damage, a major feature of the intra-S-phase checkpoint that is activated by DNA double-strand breaks occurring outside active replicons (Harrington, *et al.*, 1998; Knudsen, *et al.*, 2000; Falck, *et al.*, 2001, 2002). This checkpoint is distinct from the S-phase replication checkpoint, which becomes activated by dNTP pool depletion, chemical inhibition of the DNA polymerases, or replication fork stalling and/or collapse, in an ATR-dependent manner. This second checkpoint inhibits the firing of new replication origins and stabilizes broken replication forks for repair by mismatch and excision repair, HRR, and Fanconi complex members, in a Tp53-independent manner (Bartek, *et al.*, 2004).

Rb1-deficient cells exposed to ionizing radiation or other DNA-damaging agents may undergo a cycle of chromosome endoreduplication (or “hyper-replication”) as a result of S-phase DNA damage and mitotic inhibition (Di Leonardo, *et al.*, 1997; Niculescu, *et al.*, 1998; Flatt, *et al.*, 2000). This cellular phenotype is evident as paired chromosome homologs in metaphase cell cytogenetic preparations (see Figure 4.1, final panel for an example), with the original sister chromatids still held together by cohesin-based binding complexes and flanked with a *new* pair of sister chromatids after a second round of DNA synthesis in the cell without the execution of a proper mitosis. Following DNA damage in *normal* late-G1 and S-phase cells, levels of hypophosphorylated, active pRb rise (by protein phosphatase 2A-mediated dephosphorylation); and pRb-mediated repressor complexes are thought to participate in the maintenance of the S-phase arrest and the subsequent proper re-initiation of DNA replication after repair is complete, or, alternatively, signal for Tp53-mediated apoptosis if the damage level is too great (Avni, *et al.*, 2003; Bartek, *et al.*, 2004).

2.1.5. Mouse Models of Retinoblastoma

Studies of pRb function have been greatly extended by the generation of knockout mice carrying heterozygous and homozygous deletions of *rb1* (Lee, *et al.*, 1992; Jacks, *et al.*, 1992; Clarke, *et al.*, 1992), located on mouse chromosome 14, band D3 (per Jackson Laboratory Mouse Genome Informatics, <http://www.informatics.jax.org>). Inactivation of *rb1* in mice results in unscheduled cell proliferation, excessive tp53-dependent apoptosis, and widespread developmental defects (Chau and Wang, 2003). Most interestingly, *rb1*-mutant mice do not develop retinoblastoma; the additional loss of *p107* or *p130* is

required for the initiation of this tumor type (Robanus-Maandag, *et al.*, 1998; MacPherson, *et al.*, 2004). Germline inactivation of either *p107* or *p130* alone does not induce formation of other tumor types either (Vooijs and Berns, 1999), except in the BALB/c strain where *p107* deficiency results in growth retardation and myeloid hyperplasia and *p130* deficiency results in embryonic lethality (LeCouter, *et al.*, 1998). This illustrates the important influence of strain-specific modifier genes (*e.g.*, *prkdc* and *cdkn2a*) in the phenotypes of BALB/c mice (see Ullrich and Ponnaiya, 1998).

Heterozygous *rb1*^{+/-} mice appear normal at birth but develop thyroid medullary carcinomas and pituitary adenocarcinomas at an early age (Jacks, *et al.*, 1992). Mice homozygously lacking *rb1* (nullizygous) die mid-gestation by embryonic day 14.5 (E14.5) due to hematopoietic and nervous system abnormalities linked to ectopic cellular proliferation and differentiation and massive apoptosis (Lee, *et al.*, 1992; Jacks, *et al.*, 1992; Clarke, *et al.*, 1992). Interestingly, such phenotypes are partly due to pRb deficiency in extra-embryonic tissues (*i.e.*, the placenta); when *rb1*^{-/-} mutant mice are supported by a wild-type placenta, they survive to birth but die soon after from lens and severe skeletal muscle defects (Wu, *et al.*, 2003). A larger variety of cancers are induced in *rb1* heterozygote mice backcrossed into a *tp53*-deficient background, implicating the essential role of *tp53*-dependent apoptosis in limiting potential *rb1/p107*-deficient or *rb1/p130*-deficient neoplastic precursors (Howes, *et al.*, 1994; Vooijs and Berns, 1999; Chau and Wang, 2003).

Targeted inactivation of the pRb protein family in B6D2F1 mouse mammary epithelium tissues by expression of a truncated SV40 large T antigen fragment (T₁₂₁) that specifically disables the three Rb family member pocket proteins (but not *tp53*) leads to

primarily malignant adenocarcinomas within 10–16 months (Simin, *et al.*, 2004). This incidence was accelerated by heterozygosity for *tp53*, and tumors were repeatedly shown to lose the wild-type *tp53* allele in a LOH event, but were not considered chromosomally unstable by chromosome and microarray CGH. When BALB/cJ strain backgrounds were used, the latency time was reduced to 8.5 months, another demonstration of the importance of modifier genes in this strain. Interestingly, MMTV-promoter driven over-expression of *CCND1* (cyclin D1), a commonly observed route of pRb pathway deregulation in human sporadic cancers, in the mouse mammary epithelium likewise leads to mammary adenocarcinomas (Wang, *et al.*, 1994). Centrosome amplification, a potent source of genomic instability (see Chapter 6), in mouse embryo fibroblasts (MEFs) deficient for *Rb1* was not observed (Fukasawa, *et al.*, 1996).

Microarray profiling experiments of quiescent wild type, *rb1*-null, or *p107/p130*-null MEFs obtained from E13.5 C57BL/6 embryos were reported in Black, *et al.* (2003). Similar to previous reports (*e.g.*, Herrera, *et al.*, 1996), *rb1* deficiency resulted in the many-fold up-regulation of E2F-responsive DNA replication and cell cycle control genes. Such trends were not observed in the *p107* or *p130*-null MEFs, rather genes encoding proteins with roles in growth factor signaling (*e.g.*, PDGF, TGF- β , Stat1) and extracellular matrix (ECM) maintenance (*e.g.*, procollagen, fibulin, dynactin, and catenin) were deregulated.

2.1.6. Genetic Instability in Retinoblastoma Somatic and Tumor Cells

A strong link between cancer susceptibility and genomic instability is exemplified by a number of the so-called chromosome breakage syndromes including ataxia-

telangiectasia (A-T), Nijmegen breakage syndrome (NBS), Fanconi anemia (FA), and Bloom syndrome (BS), among others (reviewed in Khanna, *et al.*, 1998; Lengauer, *et al.*, 1998; van Gent, *et al.*, 2001; Thompson and Schild, 2002). High frequencies of spontaneous chromosomal aberrations and extreme chromosomal and cellular sensitivity to many classes of genotoxic and clastogenic agents are cardinal features of these syndromes. As mentioned earlier, a wide range of chromosomal instability (CIN) has been noted in somatic and tumor cells derived from retinoblastoma patients.

However, only limited reports of microsatellite instability (MIN) have been reported for this disease. Microsatellite instability, another and usually mutually exclusive form of genomic instability exemplified by the condition of hereditary non-polyposis colon cancer (HNPCC), is related to the expansion and contraction of repetitive microsatellite DNA sequences distributed throughout the genome generated by deregulated DNA mismatch and nucleotide excision repair systems (MMR and NER; Lengauer, *et al.*, 1998). MIN serves as a potent form of genetic deregulation if microsatellite inflation or deflation occurs near critical coding gene sequences (*e.g.*, *TGFBR2* in HNPCC), and is a classic example of the “mutator phenotype” of cellular hyper-mutability (Knudson, 2001).

In a report by Huang, *et al.* (2003), MIN affecting one or more loci on chromosomes 19–22 and X was identified in 3 of 15 (20%) Rb tumor specimens. A later report by the same group (Choy, *et al.*, 2004) observed MIN due to methylation of the 5' promoter region of the *MLH1* gene (encoding one of the core proteins of the mismatch repair system) in 5 of 26 (19%) Rb tumors examined. Other reports have failed to observe MIN in Rb cases (van der Wal, *et al.*, 2003). Only two reports of MIN status in

radiation therapy-induced secondary tumors in bilateral Rb patients are available. The first report by Gafanovich, *et al.* (1999) identified MIN in dinucleotide repeat tracts within the *tp53* and *rb1* genes, along with five other loci, in both radiogenic osteosarcomas examined. The second report by Lefèvre, *et al.* (2001) found no evidence of MIN in five osteosarcomas, one peripheral sheath nerve tumor, and one leiomyosarcoma all induced by radiation therapy treatment. In primary *BRCA1*-related ovarian and fallopian tube carcinomas demonstrating high degrees of LOH at 13q, microsatellite instability was demonstrated in only 6 of 36 (17%) of tumors examined (Jongsma, *et al.*, 2002).

The diminished or absent activity of the pRb protein in the retinal epithelium may alone be sufficient to generate instability at other chromosomal loci associated with full onset of the primary disease. Besides the well-established role pRb plays in the regulation of the G1–S transition, it has increasingly been shown to play a role in the G2–M transition and in mitosis itself (reviewed in Morris and Dyson, 2001; Zheng and Lee, 2002). This occurs via interactions with protein phosphatase 1 α (PP1 α), cyclin B1, and topoisomerase-II α , and by mediating Tp53/p21/Mdm2-dependent G2-phase and mitotic arrest and apoptosis following DNA damage (Flatt, *et al.*, 2000). The pRb protein may also participate directly in chromosome segregation, through association with the anaphase-promoting complex (APC), Hec1, and structural maintenance of chromosomes (SMC) protein family members (Zheng, *et al.*, 2000; Zheng and Lee, 2002).

The pRb protein also functions to monitor the integrity of the actin cytoskeleton in a tp53-independent manner, with pocket protein triple-knockout mouse embryo fibroblasts (MEFs) and SV40 large T-antigen-transformed rat embryo fibroblasts (REFs)

failing to arrest at the G1–S transition point or the G1 tetraploidy checkpoint after treatment with the actin inhibitory drug, dihydrocytochalasin B (DCB; Lohez, *et al.*, 2003). Likewise, treatment of Rb-deficient cells with microtubule-depolymerizing agents (nocodazole and colcemid) induces premature mitotic exit and subsequent chromosome endoreduplication (Di Leonardo, *et al.*, 1997). Recently, *Rb1* mutation was shown to promote aneuploidy by the deregulation and enhanced expression of the E2F-responsive gene *MAD2*, whose protein product is involved in proper mitotic spindle checkpoint control (Hernando, *et al.*, 2004).

In one study examining the fate of an integrated chromosomal marker in wild-type, *rb1*^{+/-}, and *rb1*^{-/-} mouse embryonic stem (ES) cells, partial or complete abrogation of pRb protein function led to a one to three orders of magnitude increase (from a baseline of 10⁻⁸) in the loss of this marker per cell per generation (Zheng, *et al.*, 2002). Loss of this marker was generated through chromosomal mechanisms, not via point mutations, and further implies a function of the Rb protein in the maintenance of chromosome stability. This relationship may be directly reflected in the subset of Rb primary (van der Wal, *et al.*, 2003; Amare, *et al.*, 2004) and secondary, radiogenic tumors that display chromosomal instability (Lefèvre, *et al.*, 2001). Interestingly, this is also seen in other tumor types (*e.g.*, B-CLL, ovarian and breast cancer) that involve loss-of-heterozygosity (LOH) at the *Rb1* locus (Liu, *et al.*, 1993; Jongsma, *et al.*, 2002; Przybytkowski, *et al.*, 2003). Przybytkowski, *et al.* (2003) suggested the existence of a second locus at 13q13 specifically associated with *intrachromosomal* instability, detectable by inter-simple sequence repeat (SSR) PCR techniques, in 43% of sporadic

breast cancers examined. They noted this phenotype was distinct from the gross chromosomal instability (primarily aneuploidy) observed in the remaining tumors.

Other circumstantial evidence of additional essential genes located nearby to the *Rb1* locus comes from the observation that relatively few (<5–10%) cases of bilateral Rb involve larger chromosomal-scale microdeletions or cytogenetically visible deletions, *i.e.*, such cases are limited by a concomitant deletion of a gene adjacent to *Rb1* essential for survival (DiCiommo, *et al.*, 2000; Richter, *et al.*, 2003). The existence of a second cancer gene nearby *Rb1* on 13q has also been suggested in studies of B-cell chronic lymphocytic leukemia (B-CLL; Liu, *et al.*, 1993), HPV-associated cutaneous squamous cell carcinomas (O'Connor, *et al.*, 2001), prostate carcinomas (Fiedler, *et al.*, 2001), *BRCA1*-related ovarian and fallopian tube carcinomas (Jongsma, *et al.*, 2002), and sporadic breast cancer (Przybytkowski, *et al.*, 2003), among many others. Candidate genes in this region include those encoding protocadherin-9, the protein kinase EMK, ubiquitin C-terminal esterase, and XPG/ERCC5 (defective in xeroderma pigmentosum complementation group G) (Jongsma, *et al.*, 2002).

2.2. Effects of G0/G1-Phase Irradiation on Mammalian Cells

2.2.1. Ionizing Radiation-induced DNA Damage and Repair

Ionizing radiation (IR) exposure produces the most complex damage patterns in DNA of any single genotoxic and clastogenic agent found in nature. The stochastic and highly penetrating nature of IR produces immediate damage through direct ionization of cellular material and indirect production of highly reactive oxygen and nitrogen species

(ROS/RNS or “free radicals”) within microseconds (reviewed in Ward, 1998). In the DNA molecule and the associated water hydration shell (120 water molecules per nucleotide) of human cells, this damage includes a large variety of nucleotide and deoxyribose sugar/phosphate backbone damage types (~3000 per cell per Gray; 1 Gray (Gy) = 1 J/kg = 100 rads), single strand breaks (SSBs, ~1000 per cell per Gy), double strand breaks (DSBs, ~30–80 per cell per Gy), and complex damage sites (“multiply-damaged sites”) with compound types of this damage, that result from densely localized energy depositions along the particle’s track as it traverses the chromatin territories within a cell nucleus (Ward, 1998; Sutherland, *et al.*, 2000; Rothkamm and Löbrich, 2003). Inherent difficulty in correctly repairing such clustered, complex damage involving potential critical sequence losses on both strands of the DNA double helix sets IR apart from other chemical mutagens/carcinogens in terms its potential for genetic mutation and oncogenic transformation (Little, 1998).

The differences between the “high” and “low” linear energy transfer (LET) ionizing radiations stem from the amounts of energy deposited per unit track length and the density of energy deposition on the microscopic level. While sufficient for current illustration purposes, LET is considered a *macroscopic* measure of energy deposition (with typical units of keV/ μ m in water) averaged over the entire track. More detailed microdosimetric studies employ more appropriate parameters such as lineal energy (y , same units) for sub-microscopic *intratrack* analyses (see Semenenko, 2002). Low-LET ionizing radiations (β particles, secondary electron recoils from X- and γ -rays) deposit energy stochastically in matter by liberating secondary electrons with extremely tortuous track structures with LET values on the order of 0.3–1 keV/ μ m (β particles are primary

electrons released from radioactive decay processes directly from an unstable nucleus). High-LET ionizing radiations [protons, α particles, nuclear fragment recoils from high-energy neutron interactions, and other heavy charged particles (HCP) such as accelerated heavy ion nuclei, available at the Brookhaven National Laboratory accelerator facilities in Upton, New York] deposit their energy along more direct tracks producing both nuclear (*i.e.*, atomic) fragments and scattering secondary high-energy δ -ray electrons with *combined* LET values that may approach 1000 keV/ μ m or more (Hall, 2000).

For most radiobiological endpoints measured, high-LET radiations are more effective per unit dose [*i.e.*, have a higher relative biological effectiveness (RBE)] than low-LET radiations due to both the higher energy depositions per unit volume and the continuous nature of track energy depositions (Hall, 2000). The relative biological effectiveness (RBE) of a radiation quality of interest is defined as the ratio of the dose of the test radiation to the dose of ^{137}Cs or ^{60}Co reference γ -radiations producing the same radiobiological endpoint (ICRP, 2003). The ICRP recommends that *only* these two radioisotopes higher-energy γ -radiations (662 keV for ^{137}Cs and 1173 and 1332 keV for ^{60}Co), and not X-ray sources (operated at 30–250 kV_p), be used for such RBE calculations due to significant microdosimetric energy deposition differences between photons of these energy ranges (ICRP, 2003; Chen, 2004).

Depending on the biological endpoint measured and other factors such as total dose and dose-rate, RBE values from ~ 1 for low-LET radiations to >100 for high-LET radiations have been calculated (Hall, 2000). In measurements of radiation-induced cell killing, mutation, and oncogenic transformation, RBE values increase from ~ 1 with increasing LET, reaching a maximum of ~ 8 – 10 at ~ 100 keV/ μ m, and subsequently

falling and approaching unity again at LET values of 500 keV/ μ m or more (NRC BEIR, 1990; ICRP, 2003). Quality factors (Q) and radiation weighting factors (w_R) are conservatively related to experimentally-based RBE values and are used by regulatory agencies to determine effective dose equivalents (dose times Q or w_R , measured in Sieverts (Sv); 1 Sv = 100 rem) for individuals (nuclear power workers, astronauts, members of the general public, *etc.*) that may be exposed to mixed radiation fields (NRC BEIR, 1990; ICRP, 2003).

Nuclear DNA, and the genetic information it encodes, is generally considered the principal target for radiation effect in mammalian cells, a concept supported by a very large assemblage of radiobiology research conducted over the last 100 years (well reviewed in Elkind and Whitmore, 1967; Hall, 2000). This is best illustrated by the effects of halogenated pyrimidine analog incorporation in DNA, resulting in enhanced radiosensitization for subsequent X- or γ -ray exposures [*e.g.*, 5-bromo-2'-deoxyuridine (BUdR) incorporation; Dewey and Humphrey, 1965; Mitchell, *et al.*, 1989; Cecchini, *et al.*, 2004, 2005] or in direct cell killing when conjugated to various radioisotopes [*e.g.*, ^{125}I -iododeoxyuridine ($^{125}\text{IUdR}$) incorporation; Sedelnikova, *et al.*, 2002]. The use of nuclear/cytoplasmic-targeted charged particle microbeams with micrometer or sub-micrometer accuracies (available *e.g.*, at the Columbia University, Gray Cancer Institute, and Pacific Northwest National Laboratory microbeam facilities), and the hyper-radiosensitivity observed in DNA DSB repair-deficient cells and animals (including humans, see below) also provide conclusive evidence of the central role of DNA damage induction and repair in determining cellular, tissue, and organism response following IR exposure (Hall, 2000).

However, the high-LET-“like” decay of plasma membrane-bound ^{125}I (conjugated to succinyl-concanavalin-A; Kaplan and Morgan, 1998) or microbeam-targeted *cytoplasmic* irradiation (Wu, *et al.*, 1999) has demonstrated a small, but significant, toxicity and mutagenicity induced from this non-nuclear damage localization. Interestingly, the majority of the mutation types recovered from these cytoplasmic-targeted cells or from their non-irradiated bystander companions are primarily point mutations, rather than larger chromosome-scale deletions and re-arrangements characteristic of radiation exposure (Wu, *et al.*, 1999; Huo, *et al.*, 2001; Nagasawa, *et al.*, 2003). The demonstration of the protective effects of ROS/RNS radical scavengers (DMSO) and interference with gap junction-mediated cell-to-cell communication [by membrane permeability disruptors (*e.g.*, lindane, fillipin) or direct mutation of the connexin-43 gene *GJA1*], and the significantly higher yields of mutations and chromosome aberrations in DNA DSB-deficient repair mutants implies that radiation damage detected at the cell membrane is translated through molecular signaling pathways and diffusion of damage species (ROS/RNS), to register a response in the cell nucleus (Nagasawa and Little, 1992; Nagasawa and Little, 1999; Wu, *et al.*, 1999; Azzam, *et al.*, 2001; Huo, *et al.*, 2001; Little, *et al.*, 2003; Nagasawa, *et al.*, 2003; Shao, *et al.*, 2004; Nagasawa, *et al.*, 2005).

Most forms of single-stranded DNA damage generated by radiation exposure and oxidative cellular metabolism may be accurately repaired using the opposite strand as a repair template through a number of evolutionarily conserved repair pathways [base excision and nucleotide excision repair (BER/NER), mismatch repair (MMR); Lindahl, 1993]. Agents that produce primarily DNA single-strand breaks (SSBs), such as

hydrogen peroxide (H₂O₂), are not effective in cell killing unless cells are treated with high concentrations (Ward, 1998). This cell killing effect is thought to be due to the high density of SSBs in the DNA, which if closely localized (within 10–15 bp apart), will generate a secondary DSB, either spontaneously or during cellular excision and repair activities (Ward, 1998).

Double-stranded DNA damage presents a more critical issue of potential sequence loss, including that damage formed immediately from the radiation exposure and that generated from subsequent cellular repair processes. Indeed, the intermediate steps of DNA repair often create aberrant DNA structures that could be considered damage moieties by the cell if left unattended by the DNA repair machinery. As such, the DNA double-strand break (DSB) is considered *the* determinant lesion for cell survival and, depending on genomic location and local chromatin organization, the underlying lesion responsible for chromosome aberration formation, mutagenesis, and carcinogenesis following IR exposure (Dewey, *et al.*, 1971; Carrano, 1973; Bender, *et al.*, 1974; Bedford, *et al.*, 1978; Thacker, 1982; Thacker and Cox, 1983; Cornforth and Bedford, 1983, 1987; Bedford, 1991; Cornforth, 1998; Olive, 1998; Hoeijmakers, 2001; van Gent, *et al.*, 2001). Radiation-induced cell death mechanisms include apoptosis, necrosis and premature senescence/mitotic cell death (also known as mitotic “catastrophe”), and are cell-type specific. Cells of lymphoid origin are prone to radiation-induced apoptosis and cells of mesenchymal and epithelial origins tend undergo radiation-induced premature senescence/mitotic cell death with permanent cell cycle arrest but continued (and potentially altered) biochemical/metabolic activity (Darzynkiewicz, *et al.*, 1997; Hall, 2000; Campisi, 2003; Castedo, *et al.*, 2004).

Of the estimated 30–80 DSBs induced per Gy (Ward, 1998), only a fraction (10–15%) will result in a chromosome break, as measured by immediate post-irradiation premature chromosome condensation (PCC; Hittelman and Rao, 1974; Waldren and Johnson, 1974; Cornforth and Bedford, 1983, 1987; Iliakis and Pantelias, 1990; Iliakis and Okayasu, 1990; Iliakis, *et al.*, 1993), a technique that induces immediate chromatin condensation in interphase cell nuclei by fusion with a mitotic cell (Johnson and Rao, 1970). The large majority of these radiation-induced DSBs and chromosome breaks (~5–6/Gy for low-LET and ~10–12 for high-LET radiations) are faithfully rejoined (>99%), as evidenced by the low frequency of terminal deletions seen in the first post-irradiation mitosis of irradiated G0/G1 cells (Cornforth and Bedford, 1983; Bedford and Goodhead, 1989; Cornforth, 1998).

With higher radiation doses, the occurrence of two DSBs proximal in both space and time increases, as does the chance they may illegitimately interact and form a chromosome exchange. This theory is known as the “Breakage-first” or “Breakage-and-Reunion” model of chromosome aberration formation (Sax, 1940, 1941; Lea and Catchside, 1942; Catchside, *et al.*, 1946; Lea, 1947; Bender, *et al.*, 1974; Savage, 1975; reviewed in Cornforth, 1998; Savage, 1998). With modeled by a single-hit, two-target linear-quadratic function ($Y = \alpha D + \beta D^2$ where Y is the yield of radiation-induced aberrations and D is the dose), the α component would reflect single chromosome breakpoints induced by independent particle tracks, and the β (dose-squared) component would reflect dual breakpoints induced by either the passage of a single particle through two different chromosome segments or by multiple, independent particle tracks (Lea, 1947; Neary, 1965; Savage, 1998). With the advent of combinatorial chromosome

painting techniques [*e.g.*, multiplex FISH (mFISH) and spectral karyotyping (SKY)], single-hit, *multi-target* effects are becoming increasingly appreciated with the demonstration of (sometimes *very*) complex chromosome aberration types, *i.e.*, those formed from ≥ 3 breakpoints in a single or multiple chromosomes (reviewed in Cornforth, 2001; Boei, *et al.*, 2002), even at very low doses and dose rates (Loucas, *et al.*, 2004). Indeed, frequencies of such complex chromosome aberrations may directly underlie the curvature of radiation dose response curves, superimposed on a fairly linear dose-*dependent*, but dose-rate-*independent*, response for “simple” exchanges (dicentrics and translocations; Simpson and Savage, 1995; Loucas and Cornforth, 2001; Loucas, *et al.*, 2004), as suggested in Bedford and Cornforth (1987).

An alternate to the Sax/Lea “breakage-and-reunion” model is Revell’s “exchange hypothesis” (1959), in which the radiation-induced “lesions” that are destined to interact and form a chromosome aberration are not direct DSBs, but lesions that, during the process of DNA repair, become involved in a failed chromosome exchange event involving entire chromatin domains (Bryant, 2004). Experiments performed by Cornforth and Bedford (1990) in which the genomes of BUdR-labeled, non-irradiated mitotic HeLa cells and irradiated, non-labeled AG01522 human fibroblasts were fused after irradiation demonstrated exchanges occurred only among the irradiated non-labeled chromosomes, and not between the mixed genomes of the fused heterokaryon. This is taken as a direct contradiction of the basis of Revell’s exchange hypothesis.

Bryant (1998, 2004) has proposed a “signal” model of chromatid-type aberration formation based on Revell’s exchange hypothesis (1959) to explain how a bp-scale DNA DSB could form a microscopically visible Mb-scale chromatid gap or break in mitosis

(Savage, 2004). Rather than requiring two “lesions” to interact to form the DSB/chromosome break as in Revell’s model, Bryant suggests that a single DSB “signals” to the DNA repair machinery, and during the repair process, suffers from a misrepair event resulting in a chromatin loop-sized exchange event and deletion. Support for Bryant’s signal model come from two sources: chromatid break induction in I-SceI-treated, genetically-engineered Chinese hamster fibroblasts containing a single 18-bp I-SceI consensus sequence (5’-TAGGGATAACAGGGTAAT-3’, not found otherwise in the mammalian genome; Rogers-Bald, *et al.*, 2000) and low-dose carbon K-shell ultrasoft X-ray induction of chromatid breaks (Bryant, *et al.*, 2003). The rapid exponential attenuation of dose delivery by such low-energy (278 eV) ultrasoft X-rays would likely produce only a few DSBs at low doses, randomly distributed along the bottom of the exposed cell nuclei (Raju, *et al.*, 1987; Cornforth, *et al.*, 1989).

2.2.2. DNA Double-Strand Break Repair Pathways

Prior to the engagement of the two major DNA DSB repair pathways in mammalian cells, non-homologous end-joining (NHEJ) or homologous recombinational repair (HRR), an “un-programmed” DNA double-strand break must first be detected by a member of a (growing) family of DSB sensor and mediator proteins. To date, these sensor proteins include the Ku heterodimer, the Mre11-Rad50-Nbs1 (MRN) complex, Tp53-binding protein 1 (53BP1), mediator of the DNA damage checkpoint-1 (MDC1/NFBD1), the PCNA-like Rad9-Rad1-Hus1 or “9-1-1” complex, the breast cancer susceptibility protein BRCA1, and DNA and RNA polymerases that become stalled on sites of DNA damage (Khanna, *et al.*, 1998; Schultz, *et al.*, 2000; Zhou and Elledge,

2000; Khanna and Jackson, 2001; Venkitaraman, 2002; Thompson and Limoli, 2003; Wang, *et al.*, 2000, 2005, and many other references).

Many of these sensor proteins share common protein-protein interaction motifs, such as the forkhead-associated (FHA) and BRCA1 carboxy-terminal (BRCT) domains found in 53BP1, BRCA1, and Nbs1 (nibrin), that when mutated, result in attenuated repair foci formation in the cell nucleus (D'Amours and Jackson, 2002; Cerosaletti and Concannon, 2003; Thompson and Limoli, 2003). The MRN complex member Rad50, a structural maintenance of chromosomes (SMC) cohesin-like protein, acts as a flexible, hinged clamp tethering the broken DNA ends, limiting further diffusion and unscheduled end-processing. With endonuclease, exonuclease, and homology-searching activities provided by Mre11, the MRN complex may direct un-programmed DSBs for further repair by NHEJ and HRR complexes and also safeguard programmed DSBs generated during DNA replication (D'Amours and Jackson, 2002; van den Bosch, *et al.*, 2003). Mre11 serine 649 and Nbs1 serines 278 and 343 are subject to Chk2 and CK2-mediated and ATM and ATR-mediated phosphorylation events, respectively, following treatment with DNA damaging agents, with partially overlapping agent specificity (Gatei, *et al.*, 2000; Lim, *et al.*, 2000; Kim, 2005). Mutations of two of the three members of the MRN complex are associated with clinical conditions: Nbs1 with Nijmegen breakage syndrome (NBS) and Mre11 with A-T-like disorder (ATLD), whose patients cells share similar, but not identical, phenotypes (*e.g.*, radioresistant/unscheduled DNA synthesis, chromosomal and telomere instability, checkpoint defects, and hypersensitivity to DNA-damaging agents) with cells derived from A-T patients (Khanna, *et al.*, 1998; D'Amours and Jackson, 2002; Thompson and Schild, 2002).

These sensor proteins transmit damage signals to the PIKK kinases ATM, ATR, and DNA-PK (Thompson and Schild, 2002; Bakkenist and Kastan, 2003; Thompson and Limoli, 2003). These proteins function to phosphorylate histone H2AX on serine 139 forming a γ -H2AX focus, recruiting additional DNA repair proteins to the site of damage whose activities are likewise modulated by subsequent phosphorylations by ATM, ATR and DNA-PK (Rogakou, *et al.*, 1998, 1999). These proteins likewise phosphorylate the effector kinases CHK1 and CHK2, whose downstream phosphorylations signal to halt the cell cycle machinery before attempting DNA replication or mitosis with damaged DNA. Extensive and partially overlapping auto-regulatory feedback mechanisms exist for every step of these pathways, providing redundant and damage level-responsive cellular response pathways (Paull, *et al.*, 2000; Zhou and Elledge, 2000; Khanna and Jackson, 2001; Thompson and Schild, 2002; Thompson and Limoli, 2003; Bakkenist and Kastan, 2003; Goldberg, *et al.*, 2003; Lou, *et al.*, 2003; Mochan, *et al.*, 2003; Stewart, *et al.*, 2003; Wang, *et al.*, 2000, 2005, and many other references).

The two major pathways for rejoining DSBs in mammalian cells are non-homologous end joining (NHEJ) and homologous recombinational repair (HRR), and are thought to participate in DSB repair in a cell cycle phase-dependent manner (Khanna and Jackson, 2001; Thompson and Schild, 2002; Rothkamm, *et al.*, 2003; Valerie and Povirk, 2003). NHEJ is the more prevalent DSB repair pathway in G1 and S-phase chromosomes prior to DNA replication, and HRR is more prevalent in duplicated S and G2-phase chromosomes which may use the duplicated sister chromatid as the template for recombinational repair. NHEJ does participate in G2-phase DSB repair as well, and possibly with increased fidelity (Krüger, *et al.*, 2004), implying these two predominant

repair pathways are not mutually exclusive during the cell cycle. Interchromosomal HRR between homologous or heterologous chromosomes in G1-phase chromosomes can be detected but occurs at such a low frequency ($\sim 10^{-6}$) to be of biological importance (Richardson, *et al.*, 1998; Richardson and Jasin, 2000; Sonada, *et al.*, 2001). As the majority of cells in the body are quiescent or slowly cycling, a large fraction of them are in the G0 or an extended G1 stage of the cell cycle (Campisi, 2003).

Local chromatin topography (compaction status, epigenetic and histone modifications, accessory proteins, *etc.*) also plays a role in the accessibility of various repair proteins to DSBs, as evidenced by the observation that both IR and restriction endonuclease-induced DSBs occur preferentially in G-light chromosome bands, *i.e.*, euchromatic, more “open” regions of active gene transcription (Bauchinger and Gotz, 1979; Winegar and Preston, 1988; Cornforth and Bedford, 1993; Folle and Obe, 1996; Folle, *et al.*, 1997; Martinez-Lopez, *et al.*, 1998; Forrester and Radford, 2004). NHEJ-mediated repair of radiation-induced DSBs may result in small deletions or nucleotide changes at the repair junction at low frequencies due to exonucleolytic processing (Taghian and Nickoloff, 1997; Elliott, *et al.*, 1998; Forrester and Radford, 2004). Such “error-prone” DSB repair through NHEJ-mediated pathways is thought to constitute the predominant source of potentially mutagenic lesions associated with radiation exposure and carcinogenesis (Little, 1998; Hoeijmakers, 2001; van Gent, *et al.*, 2001), and is thought only to be “tolerated” in mammalian cells because of the high percentage of non-protein coding DNA sequences in mammalian genomes (Sengupta and Harris, 2005).

After initial sensing of the DSB by the MRN complex (Mre11–Rad50–Nbs1), 53BP1, MDC1, or Ku itself, heterodimers of Ku70/Ku80 are recruited to form a ring-like

clamp around the free DSB end which translocates inward 16–18 bp, possibly with the direct assistance of the MRN complex (Paillard and Strauss, 1991; Gatei, *et al.*, 2000; Kysela, *et al.*, 2003). Inappropriate end structures (*e.g.*, 3'-phosphate groups or 3'-phosphoglycolate esters) are cleaved and the proper ends restored by APE1, TDP1, and PNK (Chappell, *et al.*, 2002). With the assistance of the XRCC4/LigIV complex, two molecules of the DNA-PK catalytic subunit (DNA-PKcs) bind both free Ku-laden DSB ends (Valerie and Povirk, 2003). As DNA-PKcs couples to the Ku protein dimers, the now functional DNA-PK holoenzyme aligns and anneals the broken DNA ends in synapsis, driven by base pairing of short stretches (1-10 bp) of complementarity known as microhomology patches at or near the DSB (Roth and Wilson, 1986).

As a member of the PI3-kinase-like protein kinase (PIKK) family that includes ATM and ATR, DNA-PK catalyzes a number of phosphorylation events, signaling to other downstream cell cycle and DNA repair effector proteins like Tp53, the WRN helicase, and Artemis, and auto-phosphorylates itself and the Ku dimers to signal for release off the DNA (Anderson and Lees-Miller, 1992; Chan and Lees-Miller, 1996; Ding, *et al.*, 2003). The reaction is completed by DNA polymerase μ/λ gap filling and final ligation by the XRCC4/LigIV complex (reviewed in Thompson and Limoli, 2003; Valerie and Povirk, 2003); the fidelity and precision of this latter step is facilitated by Tp53 (Akyüz, *et al.*, 2002; Lin, *et al.*, 2003). Work from this laboratory has shown that siRNA knockdowns of *prkdc* (the gene that encodes DNA-PKcs) in normal human fibroblasts results in increased radiation-induced chromosome aberration frequencies and cell killing, and interestingly, a down-regulation of *ATM* mRNA expression and protein levels in transfected cells, implying a previously unrecognized regulatory feedback loop

between the ATM- and NHEJ-mediated DNA DSB response and repair pathways (Peng, *et al.*, 2002, 2005).

DNA DSB repair through the “error-free” recombination mechanisms of HRR requires extensive regions of sequence homology (of >200 base pairs) usually only found in sufficiently close proximity in the sister chromatids after DNA replication (Rubnitz and Subramani, 1984; Liskay, *et al.*, 1987). While homology exists (by definition) in homologous chromosomes, these are not used due to physical proximity limitations in the nucleus (Thompson and Schild, 2002, Rothkamm, *et al.*, 2003). Homologous sequences are also available in the G1 nucleus on neighboring heterologous chromosomes in the form of the approximately 40% of moderately or highly repetitive sequence elements (*SINEs*, *LINES*, *etc.*) that are distributed across the genome. However, use of these sequences is discouraged due to the possibility of illegitimate exchanges, a very potent source of genomic instability (Rothkamm, *et al.*, 2003; Sengupta and Harris, 2005).

The Tp53 protein is thought to play a critical role in preventing inappropriate HRR, as normal cells expressing mutant *tp53* alleles or infected with SV40 or HPV have significantly higher levels (5–100-fold) of homologous recombination in transfected plasmid substrates and genomic loci (Mekeel, *et al.*, 1997; Dudenhoffer, *et al.*, 1999; Saintigny, *et al.*, 1999; Willers, *et al.*, 2001; Akyüz, *et al.*, 2002; Janz and Weismüller, 2002; Saintigny and Lopez, 2002; Dahm-Daphi, *et al.*, 2005). The Tp53 tetramer interacts directly with the Rad51 and Rad54 recombinases, the BLM and WRN helicases, and the single-stranded binding protein RPA (Süsse, *et al.*, 2000; Janz, *et al.*, 2002; Linke, *et al.*, 2003; Sengupta, *et al.*, 2003; Kumari, *et al.*, 2004; Romanova, *et al.*, 2004; Sengupta and Harris, 2005), implicating a critical function of the Tp53 protein in HRR of

radiation-induced DSBs and those generated from stalled replication forks in S-phase. Tp53 has also been shown to bind non-specifically (*i.e.*, not as a transcription factor) to sites of single- and double-stranded DNA damage (Liu and Kulesz-Martin, 2001; Zhou and Prives, 2003) and binds with greater affinity than the hMSH2–hMSH6 mismatch repair complex to mismatched DNA sequences caused by insertions and deletions, under the same binding conditions (Lee, *et al.*, 1995; Degtyareva, *et al.*, 2001).

Upon sensing a DSB in a duplicated S or G2-phase chromosome, repair may be directed to the HRR system by the MRN complex and BRCA1. An as-yet unidentified 5'–3' exonuclease (possibly ExoI or Tp53 itself; the MRN complex has primarily 3'–5' exonuclease function, Süsse, *et al.*, 2000; Llorente and Symington, 2003) resects the DNA to form a long 3' single-stranded overhang that becomes coated by molecules of the single-stranded binding protein known as replication protein A (RPA; Thompson and Schild, 2001). The BRCA2 protein, with the assistance of BRCA1 and the five Rad51 paralogs (XRCC2, XRCC3, Rad51B, Rad51C, Rad51D), loads molecules of Rad51 onto the DNA strand forming a nucleoprotein “filament.” This signals for the recruitment of Rad52 and Rad54 (a member of the SWI/SNF chromatin remodeling family), which direct Tp53-dependent homology searches of potential repair templates (found usually in the closely-associated, cohesin-bound sister chromatid; Linke, *et al.*, 2003). Once found, this repair template is opened to form a D-loop structure as the resected 3'-end of the broken DNA strand invades and base pairs appropriately (Mazin, *et al.*, 2003). Growth of the heteroduplex D-loop by DNA polymerase-mediated extension forms a pair of Holliday junctions, which are recognized and further processed by Tp53 (Süsse, *et al.*, 2000; Janz, *et al.*, 2002). Most often Holliday junctions are disengaged without a

crossover event and the remaining gaps filled and ligated by DNA LigIII (Johnson and Jasin, 2000; Thompson and Schild, 2001, 2002; Valerie and Povirk, 2003). Alternatively, isomerization and resolution of the Holliday junctions by resolvases may lead to a crossover event between the sister chromatids (Constantinou, *et al.*, 2001). Such an event may lead to gene conversion events, loss of DNA sequence, and cytologically visible sister chromatid exchanges (SCEs) when visualized by BUdR labeling and fluorescence-plus-Giemsa (FPG) or “Harlequin” chromosome staining techniques (Perry and Wolff, 1974; Nagasawa, *et al.*, 1992).

Deficiencies in DNA damage repair systems often lead to the increased sensitivity of mammalian cells to mutagenic or clastogenic agents, often in a cell cycle phase-specific manner, and an enhanced predisposition to various cancer types in affected patients, indicating the essential nature of these DNA repair systems in the maintenance of genomic integrity (reviewed in Hoeijmakers, 2001; van Gent, *et al.*, 2001; Thompson and Schild, 2001, 2002). Likewise, mutations in genes encoding various DNA damage sensors (*e.g.*, the MRN complex, 53BP1, the Rad9-Hus1-Rad1 “911” complex) and signal transducers (*e.g.*, ATM and ATR) that function to both stimulate repair functions and halt cell cycle progression until DNA damage is repaired can lead to deleterious consequences for a damaged genome and an enhanced predisposition to cancer. No other genetic condition epitomizes this connection more than ataxia-telangiectasia (A-T), a progressive neurodegenerative disease associated with hyper-radiosensitivity and predisposition to lymphomas (Taylor, *et al.*, 1975; Cox, 1982; see Figure 1.1). Consistent with the ATM protein’s role as the primary transducer of radiation-induced DNA damage signals, A-T cells are exquisitely sensitive to a large number of other DNA

damaging agents as well. Likewise, the UV hyper-sensitivity of xeroderma pigmentosum (XP) patient cells and MMC hyper-sensitivity of Fanconi anemia (FA) patient cells are due to deficiencies in nucleotide excision and interstrand cross-link/replication fork collapse repair, and both conditions are associated with increased cancer incidence (primarily melanoma and acute myeloblastic leukemia, respectively; Cleaver, 1968, Thompson, 1998; Khanna, *et al.*, 1998, Wang and D'Andrea, 2004).

In addition to the essential contribution of proper DNA repair function for the maintenance of genomic stability, complex protein regulatory mechanisms known as cell cycle checkpoints have evolved to inhibit the progression of cells into subsequent phases with damaged DNA (reviewed by Kastan and Bartek, 2004). First identified in yeast, each major transition of the eukaryotic cell cycle is overseen by extensive and redundant networks of sensor, signaling, and effector proteins to provide adequate time for the repair of DNA damage (Hartwell and Weinert, 1989; Hartwell and Kastan, 1994; Scherr, 1996, 2000; Zhou and Elledge, 2000). Such damage, if not correctly repaired, may become “fixed” as a permanent mutation through successive rounds of DNA replication. Cell cycle checkpoints are intimately connected with the apoptotic and cellular senescence-associated cell death programs that function to remove damaged, and possibly neoplastic, cells from the population (Zhou and Elledge, 2000; Campisi, 2003).

2.2.3. Definition of the G0 and G1-phases of the Somatic Cell Cycle

The G1 (gap 1) stage of the somatic cell cycle is intermediate between the stages of mitosis, in which chromosomes of the parental cell are evenly distributed to the daughter cells, and DNA replication in the following S phase. The duration of G1 (0–

100+ hours) is the most variable in the mammalian cell cycle, depending on species, tissue and cell type (Cleaver, 1967). Cells that are temporarily not cycling may exist in a quiescent G0 phase of the cell cycle, with much reduced transcriptional activity, until environmental factors (nutrients, growth factors, *etc.*) are sufficient to re-engage the cell cycle machinery. For example, work from this laboratory has shown G0 cells have very low levels of DNA polymerase α while cycling cells have much higher levels of this protein. Once the decision to re-enter the cell cycle is made, cells monitor their nutritional status and genomic integrity (presence of DNA damage or unfinished previous mitosis) in G1 at the so-called “restriction point” prior to initiating another round of DNA replication or returning to a quiescent state (Pardee, 1974; Cooper, 2003). Transition throughout the cell cycle is regulated through the coordinated oversight of cyclins (Cln), cyclin-dependent protein kinases (CDKs), and CDK inhibitors (CKIs). These CKIs are assigned to two groups, depending on their target kinases: the INK4 family (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}) specifically binds the G1-phase cyclin-D-associated CDK4 and CDK6 kinases, while the Cip/Kip family (p21^{Cip1/Waf1}, p27^{Kip1}, and p57^{Kip2}) can bind all cyclin/CDKs (reviewed in Scherr and Roberts, 1999; Scherr, 2000).

The G0 phase of the cell cycle represents a withdrawal from the active cell cycle associated with nutritional/serum deprivation, terminal differentiation or cellular senescence (reviewed in Cooper, 2003). Maintenance of the G0 state is maintained primarily through two cyclin-dependent kinase inhibitors, p27^{Kip1} and p57^{Kip2}, that function to inhibit CDK/cyclin-mediated ATP-dependent phosphorylation and inactivation of pRb, and the transcription of downstream proteins required for initiation of DNA synthesis. As part of the early response to mitogen stimulation, transcription of

the D-type cyclins (D1–D3) increases, under the influence of the Ras/MAPK/ERK, c-Myc, and Akt (and possibly hypophosphorylated pRb) growth factor signaling pathways (Gille and Downward, 1999; Mateyak, *et al.*, 1999). These D-type cyclins assemble into complexes with pre-existing CDK4 or CDK6 kinases (depending on the particular cell lineage), with the assistance of CDKN1A (p21^{Waf1/Cip1}) and PCNA (Zhang, *et al.*, 1993; LaBaer, *et al.*, 1997). Levels of the CKI p27^{Kip1} fall concomitantly with the rise of cyclin D/CDK4/6 complexes through binding and titration of p27^{Kip1}, and direct transcriptional repression (Mateyak, *et al.*, 1999; Scherr and Roberts, 1999; Scherr, 2000). Once the p27^{Kip1} “threshold” is overcome, sufficient CDK4/6/ClnD complexes are available for activation by phosphorylation on a single cyclin D threonine residue (Thr286) by CDK7/ClnH/p36MAT1 (CAK1) or glycogen synthase kinase-3 β (GSK-3 β ; Scherr and Roberts, 1999; Watcharasit, *et al.*, 2002).

The primary target of the activated CDK4/6/ClnD complex is the retinoblastoma protein, pRb, which is initially maintained in a hypophosphorylated, active form bound to inactive E2F/DP heterodimeric transcription factor complexes (Weinberg, 1995; Morris and Dyson, 2001). There are six E2F (E2F1-5 are transcriptional activators, E2F-6 is a transcription repressor) and two DP family members (DP-1 and DP-2) that form heterodimeric transcription factor complexes that play multifaceted roles in cellular proliferation, differentiation, and apoptosis (Stevens and La Thangue, 2004). Recently, two new E2F family members, E2F-7 and E2F-8, were identified. These E2F family members form transcriptional repressor complexes independent of a DP subunit and may play a role in maintaining G1 and G2-phase cell cycle arrest (de Bruin, *et al.*, 2003; Stevens and La Thangue, 2004; Maiti, *et al.*, 2005). It has been shown that pRb contains

at least sixteen consensus sequences for CDK phosphorylation, requiring successive phosphorylations by CDK4/6 and CDK2 for full inhibition of E2F/DP (or E2F-7/8) complex binding (Morris and Dyson, 2001), as discussed previously.

Upon initial phosphorylation of pRb by CDK4/6/ClnD, conformation of domain B of the pRb pocket is modified to prevent histone deacetylase (HDAC) from binding (Stiegler, *et al.*, 1998; Takaki, *et al.*, 2004). HDAC contains a LXCXE consensus sequence for binding pRb (also found in the D cyclins), and, by removing acetyl groups on histone tails, acts to tighten chromatin packing and repress transcription (Chan, *et al.*, 2001). This inactivation of HDAC promotes the transcription of the cyclin E gene, whose product complexes with another kinase, CDK2, and provides an auto-regulatory loop, promoting further cyclin E expression. These CDK2/ClnE complexes complete the phosphorylations of pRb, resulting in full inactivation of the binding pocket domain and release of the E2F/DP transcription factors. Two pocket proteins related to pRb, p107 and p130, serve partially overlapping functions in a cell type-specific manner (Morris and Dyson, 2001).

The E2F transcription factors subsequently induce expression of a number of genes necessary for DNA replication and regulation of entry into S phase (Stevens and La Thangue, 2004). These targets include: cyclins A and E, pRb, p107, E2F family members, DNA polymerase α , nucleotide synthesis genes, PCNA, and *c-myc*, among others (reviewed in Morris and Dyson, 2001; Stevaux and Dyson, 2002). The tandem genes for the CKIs p16^{Ink4a} and p15^{Ink4b} may be other targets. By competing with cyclin D for CDK4/6 binding, increasing levels of these proteins will sequester and inactivate CDK4/6 and promote cyclin D turnover (due to unstable PEST sequences) in an auto-

regulatory feedback loop. Another interesting E2F target is RanBP1 (Ran GTPase-binding protein 1), which is involved in nuclear/cytoplasmic protein transport and proper mitotic spindle assembly (Lavia and Jansen-Durr, 1999; Guarguaglini, *et al.*, 2000).

Association of CDK2/ClnE with E2F-p107 complexes in the cyclin A promoter region triggers transcription of the cyclin A gene. Cyclin A then complexes with CDK2, in competition with cyclin E, which is also rapidly degraded due to unstable PEST sequences. As cells enter S phase, complexes of CDK2/ClnA phosphorylate the E2F transcription factors, releasing them from promoters; maintains pRb phosphorylation; and continues to activate other proteins required for the initiation of DNA synthesis, *e.g.*, CDC45 (Kitagawa, *et al.*, 1995; Scherr, 1996). CDK2/ClnA also forms complexes with E2F-p107 that have been shown to have histone H1 kinase activity, indicating a role in mitotic progression (Yang, *et al.*, 1999).

2.2.4. Radiation Exposure in the G1-phase of the Mammalian Cell Cycle

Ionizing radiation-induced DNA damage in the G1 stage of the cell cycle results in activation of the cardinal transducer PIKK kinases ATM and DNA-PK (see section 2.2.2) and the subsequent stabilization of Tp53, via the rapid phosphorylation of serines 15/18 (human/rodent), 20 and 37 (Siliciano, *et al.*, 1997; Chao, *et al.*, 2000; Turenne, *et al.*, 2001). The Tp53 protein is constitutively expressed in most cell types and, without stabilization, the protein has a half-life of only ~6–15 minutes and is found in low levels in cells due to ubiquitin-targeted proteasome degradation mediated by its negative regulator, Mdm2 (Hainaut and Hollstein, 2000). Upon phosphorylation, inhibition of Mdm2 binding, and stabilization, Tp53 functions as a tetrameric transcription factor,

inducing the expression of a number of cell cycle control and repair genes to halt further cell cycle progression and initiate DNA repair (Kastan, *et al.*, 1991; Lu and Lane, 1993; Siliciano, *et al.*, 1997; DeSimone, *et al.*, 2003; Fei and El-Deiry, 2003). A number of additional post-translational modifications, including phosphorylation of an additional 14 serine and threonine residues by CHK1, CHK2, p38, CK1, and PKC (and other protein kinases), acetylation of lysine residues by p300/CBP acetyltransferases, and sumoylation by SUMO-1 (reviewed in Hainaut and Hollstein, 2000; Saito, *et al.*, 2003; Fei and El-Deiry, 2003) also contribute to the Tp53-mediated DNA damage response regulatory network.

The *tp53* gene is located on human chromosome 17p13.1 and mouse chromosome 11B2–C and consists of 11 exons that encode a 393 amino acid, 53-kDa protein (Hainaut and Hollstein, 2000; <http://www.informatics.jax.org>). The Tp53 protein includes a number of functional domains involved in transactivation, DNA binding (both Tp53-consensus sequences and damaged DNA sequences), oligomerization (tetramerization), and regulation of protein function and cellular distribution. Over 20,000 mutations have been identified in the *Tp53* gene in the majority of human cancer types, 80% of which occur in the DNA binding domain and result in loss of the Tp53-DNA major and minor groove contacts [per the International Agency for Research on Cancer who maintains an extensive *Tp53* mutation database at <http://www-p53.iarc.fr> (Release 10, June 2005; Olivier, *et al.*, 2002)].

The primary mediator of the Tp53-dependent G1-phase delay is CDKN1A (p21^{Waf1/Cip1}), a potent inhibitor of cyclin/CDK complexes, cyclin A expression, and the replication, but not repair, activities of PCNA (Hainaut and Hollstein, 2000; Fei and El-

Deiry, 2003). PCNA, the ring-like clamp thought to function as a scaffold protein for DNA damage signaling and repair (along with its traditional role as a DNA polymerase loader), and Gadd45, a stimulator of PCNA-dependent repair activities, are also Tp53 transactivation targets (Smith, *et al.*, 1994). To limit its own response, Tp53 also induces the expression of its own negative regulator, Mdm2, which binds the N-terminal of Tp53 and promotes rapid ubiquitin-mediated degradation in the 26S proteasome (Honda, *et al.*, 1997). Mdm2 is an E3-like ubiquitin ligase that has been shown to be phosphorylated by DNA-PK following IR exposure, preventing the binding and inactivation of Tp53 (Shieh, *et al.*, 1997; Mayo, *et al.*, 1997).

Tp53 is also subject to phosphorylation of at least four residues by CDK7/ClnH/p36MAT1 (CAK1), which serves an additional role as a subunit of the TFIIH damage recognition complex in nucleotide excision repair (NER; Ko, *et al.*, 1997; Wang, *et al.*, 2003). After pausing at the site of DNA damage on an actively expressing gene (a form of DNA repair referred to as transcription-coupled repair or TCR), RNA POL II signals TFIIH, XPC and Tp53 to investigate the nature of the lesion. This leads to signal transfer between TFIIH and Tp53, possibly by Tp53 binding of two other TFIIH subunits, the XPB and XPD helicases (Wang, *et al.*, 1996). Conversely, the activation and binding of Tp53 to CAK1 results in significant down-regulation of CAK1-mediated phosphorylation of CDK2, preventing its activation, and the C-terminal domain of RNA polymerase II, preventing further elongation of mRNA transcripts (Schneider, *et al.*, 1998).

The non-receptor tyrosine kinase c-Abl is also activated by ATM and DNA-PK and induces higher CDKN1A expression by stabilizing Tp53 (Kharbanda, *et al.*, 1997,

1998; Shangary, *et al.*, 2000). Other targets of ATM activation are Mre11 and Nbs1 (nibrin), the protein mutated in Nijmegen breakage syndrome (Lim, *et al.*, 2000; Wu, *et al.*, 2000; Girard, *et al.*, 2002; Kobayashi, *et al.*, 2004). Both are core components of the MRN complex (Mre11-Rad50-Nbs1) that functions as a DSB repair organizer early in the complex signaling pathways that activate downstream NHEJ and HRR proteins and cell cycle checkpoints (D'Amours and Jackson, 2002). Overall, stabilization of Tp53 followed by higher CDKN1A expression results in accumulation of hypophosphorylated pRb, preventing the E2F/DP-driven transition into S phase. The other pRb family members, p107 and p130, have also been implicated with an active (although minor) role in delaying this phase transition, possibly by silencing some of the activated E2F transcription factor complexes.

2.2.5. Mammalian Cell Survival Following G0/G1-Phase Irradiation

The G0/G1-phase survival of a large variety of mammalian cell types, including their various mutant and transformed counterparts, has been extensively investigated. A number of *in vitro* culture conditions, such as density-inhibited contact inhibition, serum deprivation, cold treatment, and various chemical agents that directly interfere with cell cycle progression, have been employed to generate nearly synchronous populations of G0/G1 cells. The use of such synchronous populations prevents the confounding effect of differential radiosensitivity of the various phases of the cell cycle (Terasima and Tolmach, 1954; Elkind and Whitmore, 1967). In general, G1 is considered a relatively radiosensitive cell cycle phase, transit into the S phase is generally accompanied by a gradual increase in radioresistance peaking in late S/early G2, late G2 and M are

considered more radiosensitive (Terasima and Tolmach, 1961; Hinz, *et al.*, 2005). Cells in late G2 and mitosis may not be blocked in the cell cycle to correct any deficiencies in the chromosome complement before completing mitosis and cell division (Terasima and Tolmach, 1961; Sinclair, 1968; Schneiderman, *et al.*, 1996; Cornforth, 1998; Hall, 2000).

Numerous mathematical models have been developed to describe the shape of the mammalian cell survival curve including various single/multi-hit single/multi-target, linear-quadratic, and various repair- and saturation-based models (Lea, 1946, 1955; Neary, 1966; Elkind and Whitmore, 1967; Kellerer and Rossi, 1972; Fertil, *et al.*, 1994; Sachs, *et al.*, 1997; Stewart, 2001; and many other references). These functions well illustrate models of DNA damage and chromosome aberration induction and repair, and their correlation to radiation-induced cell killing. Much focus has been placed on the way the parameters of these survival curve expressions change depending on biological, chemical, and physical conditions during radiation exposure (Deschavanne, *et al.*, 1986; Fertil, *et al.*, 1994; Deschavanne and Fertil, 1996; Fertil and Deschavanne, 1999; Hall, 2000).

For relative survival levels of $\geq 10\%$, both the linear and linear-quadratic models adequately fit most survival data (Elkind, 1977; Cox and Masson, 1980; Deschavanne, *et al.*, 1986). However at lower relative survival levels, a shoulder and pronounced curvature in most normal cell survival curves is observed and the two-parameter (α , β) LQ model becomes the model of choice until a survival level of 10^{-3} as repair activities become saturated and cell killing becomes almost exponential (Fertil, *et al.*, 1994). At relative survival levels $<10^{-3}$, the LQ model continues to bend and no longer fits the survival responses adequately, and the single-hit multi-target model best approximates

measured survival responses. It is interesting to note that all these models were developed with little initial understanding of the nature of the underlying mechanisms, and the recent rapid accumulation of molecular information in radiobiology has only served to strengthen the validity of these theories.

Deviations from these survival models can be quite informative of the radiation source or cell type employed in the survival measurements. High-LET irradiation produces a much reduced or absent shoulder and steeper slopes, possibly accounted for by the higher degree of complex DNA damage species induced along the particle's track and the difficulties involved in their resolution (Ward, 1998; Rydberg, *et al.*, 1998; Sutherland, *et al.*, 2000, 2001, 2002). Likewise, radiosensitive cells, in general, display much more shallow or absent shoulders and steeper slopes (and lower D_0 , D_{10} , SF_2 values, *etc.*), as compared to normal cells, presumably due to the reduced cellular capacity to *repair* this complex DNA damage, rather than the higher *induction* of such damage in these cells by high-LET radiation. The passage number of the same cell strain may also have pronounced effects on a radiation survival curve; Cox and Masson (1974) observed a shift from a straight exponential survival curve in low-passage fibroblasts to a more pronounced shouldered response at higher passages. They noted the appearance of more abnormal karyotypes and giant cells, reflective of changes seen in the process of cellular senescence (Hayflick, 1965), thought to be due mainly to telomere shortening (Shay and Wright, 2000).

Various curve-fitting parameters have been used to describe the radiosensitivity phenotype. For the purposes of comparing human cell radiosensitivity, these include the mean lethal dose D_0 (required to reduce relative survival on the exponential portion of the

survival curve to $1/e$ or 37%; Hall, 2000); D_{10} , the dose required to reduce relative survival to 10% (which reflects both the magnitude of the shoulder region and the exponential region; Little, *et al.*, 1988); the mean inactivation dose D (the mean of the survival curve if it is treated as a cumulative probability distribution; Fertil and Malaise, 1984, 1985); the relative surviving fraction at 2 Gy (SF_2 , a parameter commonly employed in relation to the effects of fractionated regimens in the radiotherapy clinic; Tucker, 1986); and α , the initial slope of the linear-quadratic survival curve (Elkind, 1977; Hall and Zaider, 1985; Malaise, *et al.* 1987; Fertil and Deschavanne, 1999). Fertil, Deschavanne, and Malaise have reported that survival responses in the low dose region (≤ 3 Gy), and the parameters describing survival in this region, are consistently the most informative for inter-comparison purposes of cell lines of different genetic and histological origins.

Unlike those generated by oxidative cellular metabolism, the more complex radiation-induced DNA lesions, such as crosslinks, DSBs, and complex, multiply damaged sites, are not routinely encountered by the cellular DNA repair machinery. Extensive DNA repair systems (base and nucleotide excision repair pathways) have evolved to repair the many thousands of these oxidative damage products generated per hour (Lindahl, 1993). These systems become saturated only after higher radiation doses, when high-density lesion clustering and excision repair activities creates additional sites of double-stranded damage, comprising $\sim 70\%$ of the complex lesions produced by IR (Rydberg, *et al.*, 1998; Sutherland, *et al.*, 2000, 2001, 2002). The induction of greater numbers of IR-induced lesions at higher doses may overcome the signaling and/or repair capacities of the cell, and the residual level of such unrepaired or misrepaired damage is

reflected in the shoulder of the survival curve (Löbrich, *et al.*, 1995, 2000). A correlation between DSB induction and repair, chromosome aberrations, and cell killing has been reported by a number of investigators (Dewey, *et al.*, 1971; Bedford, *et al.*, 1978; Radford, 1985; Iliakis and Okayasu, 1988; Cornforth and Bedford, 1987; Bedford, 1991; Rothkamm and Löbrich, 2002). This is best illustrated by the work of Revell and colleagues (1983) who observed an almost one-to-one correspondence between the absence or presence of radiation-induced acentric fragments and cell survival or cell death, respectively (also discussed in Bedford, 1991).

Much evidence has accumulated in recent years that the response of cells exposed to low doses (<1 Gy) of radiation contradicts those predicted from the classical linear-quadratic paradigms (reviewed in Joiner, *et al.*, 1996; Morgan, 2003; Bonner, 2004). Such phenomena include low dose hyper-radiosensitivity (HRS) and increased radioresistance (IRR), adaptive response, and bystander effects; each will be briefly reviewed. Radiation-induced genomic instability (RIGI) observed in descendants of irradiated cells generations following exposure can likewise alter the effects of radiation exposure in a population of cells. Rather than recapitulating the large and ever-growing body of research on RIGI, the reader is referred to an excellent series of extensive reviews (Ullrich and Ponnaiya, 1998; Morgan, 2003a,b; Little, 2003b).

High-accuracy cell sorting and automated microscopic observations of irradiated cells during colony formation has revealed a substructure in the low dose region of the acute dose survival response that deviates from predictions of the LQ survival model in more than 40 normal, mutant, and transformed human cell lines (Marples and Joiner, 1993; Skarsgard, *et al.*, 1996; recently reviewed in Marples, *et al.*, 2004). Experimental

uncertainties in cell counting/dilution, plating, and culture methods associated with traditional survival measurements [first described by Puck and Marcus (1956)] are too large to permit accurate survival level measurements at <1 Gy, which are not much different from unirradiated control levels (Boag, 1975). Using the more precise techniques, Marples and Joiner (1993) first identified an initial hypersensitive response in hamster V79 cells at doses ≤ 25 cGy, since termed low dose hyper-radiosensitivity (HRS). The low-dose HRS region is followed by a region of significantly increased radioresistance (IRR) from 40 to 100 cGy, after which cell survival follows that predicted by the LQ model. It must be noted that not all cell types examined by these groups have demonstrated evidence of HRS/IRR.

HRS/IRR phenomena were initially observed in asynchronously growing populations, which are comprised of several cell cycle phase subpopulations with radiosensitivities 2–3-fold different in magnitude (Terasima and Tolmach, 1961, 1963; Dewey, *et al.*, 1972), and it is possible that such HRS/IRR effects might be attributed to this differential cell cycle phase sensitivity. However, further work with cell populations synchronized in each cell cycle phase demonstrated HRS/IRR in G1, S, and G2/M, although the effect was most pronounced in this latter phase. Such effects are most probably related to inherent repair capacities associated with specific states of DNA metabolism and chromatin structure (Marples, *et al.*, 2004) and radiation quality (Schettino, *et al.*, 2001). More radioresistant cell strains generally show higher degrees of HRS and IRR than do radiosensitive ones, another reflection of the key requirement for functional DNA response and repair systems for survival at lower radiation doses (this phenomenon is reflected in the “shoulder” of radiation survival curves, as well).

The phenomena of increased radioresistance may be related to the concept of adaptive response, in which exposure of cells to small initial “priming” doses (as low as 10 mGy) may result in increased survival following exposures to subsequent higher doses (Raaphorst and Boyden, 1999; Skov, 1999; Marples, *et al.*, 2004). The stimulation of DNA repair activities by small priming doses (≥ 10 mGy) is thought to underlie both these responses (differing only in the timing of the initial doses). Alternatively, priming/HRS doses may be sufficient to kill selectively cells in the more sensitive phases of the cell cycle, partially synchronizing them in the more resistant phases, for which higher doses would be required to achieve the same survival level.

The concept that a certain radiation-induced DNA damage threshold must be reached to induce repair activities is supported by the study of Rothkamm and Löbrich (2002). By examining γ -H2AX focus kinetics in quiescent human fibroblasts (which they confirmed by higher-dose PFGE measurements to be directly correlated to the number of DSBs), the authors demonstrated that foci induced after ~ 1 mGy of X-irradiation (approximately 1 electron track per cell from Goodhead, 1992) remain unrepaired for several days. Higher doses induced rapid repair and disappearance of most foci by 24 hours after irradiation. Much research has recently focused on the validation of γ -H2AX foci as a sensitive marker for DSB induction and repair in other experimental systems (*e.g.*, Burma, *et al.*, 2001; Banath and Olive, 2003; Olive and Banath, 2004; T. Kato, personal communication).

The mammalian cell bystander effect was first identified by Nagasawa and Little (1992) in measurements of sister chromatid exchange (SCE) induction following low-dose alpha particle irradiation of CHO cells. Unexpectedly, low dose exposures in which

<1% of the cell nuclei were traversed by an alpha particle resulted in the induction of SCEs in 30% of the cell population. Since then, bystander effects for other radiobiological endpoints have been confirmed to varying extents for other cell systems and radiation qualities (both low and high LET radiations, many references), and are notably influenced by DNA repair status of the cells examined (Little, *et al.*, 2003a; Nagasawa, *et al.*, 2003, 2005; this latter report also includes my updated dosimetry of the original ^{238}Pu α -particle source used in the Nagasawa bystander experiments, completed with Dr. James Durham and Kinga Kröbl at CSU following the source's transfer from Harvard University). Both cell-to-cell signaling via gap junction-mediated communication and signaling by the release of extra-cellular signaling molecules into the cell culture medium have been implicated, but the true identity of the molecule or molecules responsible for the bystander effect has yet to be revealed (Nagasawa, *et al.*, 2002; Morgan, 2003a,b; Mothersill and Seymour, 2004). Interestingly, mutations measured in bystander cells are mainly small point mutations, not the typical chromosomal-scale rearrangements reflective of direct radiation exposure, suggesting radical-mediated (ROS/RNS) DNA DSB induction in the bystander cells (Nagasawa and Little, 1999; Zhou, *et al.*, 2000; Huo, *et al.*, 2001; Nagasawa, *et al.*, 2003; Little, 2003b; Sokolov, *et al.*, 2005).

2.2.6. Potentially Lethal Damage Repair

Pertinent to the discussion of radiation survival curves are the concepts of sublethal damage (SLD) and potentially lethal damage (PLD) repair; related phenomena that are operationally defined by changes in radiobiological response by manipulation of

experimental irradiation and cell culture protocols. Bedford and colleagues have argued that both phenomena are reflective of differences of DSB and chromosome break induction and processing caused by (usually deliberate) variation in these experimental protocols. SLD and its repair are related to effects of dose fractionation and low dose rate exposures and will be further discussed in Section 2.4.

PLD repair is an operational term that describes the influence of varying the cell culture conditions on the outcome of radiobiological measurements, either before or after irradiation (Phillips and Tolmach, 1966; Little, 1969, 1973; Cox, *et al.*, 1981; Arlett and Priestly, 1983; Iliakis, 1988; Bedford, 1991). For example, the survival of confluent, irradiated cultures may be significantly increased if the cultures are allowed to remain in density-inhibited contact inhibition (Little, 1969, 1973; Cox, *et al.*, 1981) or in serum-deprived culture medium (Mayne and Lehmann, 1982) for hours after irradiation. Likewise, the yield of induced DSBs, PCC fragments, and chromosome aberrations will diminish in confluent cultures of irradiated cells allowed to remain in the stationary phase prior to analysis (Iliakis, 1988; Cornforth and Bedford, 1987; Bedford, 1991; Stackhouse and Bedford, 1993).

This increase in post-irradiation survival is related to the post-irradiation repair of “potentially lethal” lesions, *i.e.*, DSBs and chromosome breaks. A number of reports have examined the kinetics of this response with various DSB repair assays [*e.g.*, elution techniques (Fornace, *et al.*, 1980; Iliakis and Okayasu, 1990; Schwartz, *et al.*, 2000) and constant/pulsed field gel electrophoresis (CFGE/PFGE; Metzger and Iliakis, 1991; Stackhouse and Bedford, 1994; Alsbeih, *et al.*, 2004)], demonstrating either first-order or biphasic “fast” and “slow” kinetics of repair. Cytologically, such experiments can be

performed by examining γ -H2AX and DNA repair protein foci kinetics and dynamics in interphase cells (Thompson and Limoli, 2003; Kuhne, *et al.*, 2004; T. Kato, personal communication) or by inducing PCC of such cells at different times after irradiation (Pantelias and Maillie, 1985; Cornforth and Bedford, 1987; Iliakis, *et al.*, 1990, 1993).

Iliakis, *et al.* (1990, 1993) measured an initial fast-repair component (half-times of 2–15 minutes) of PCC fragments, followed by a slow-repair component (half-times of 2–3 hours), in irradiated CHO cells. Pantelias and Maillie (1985) and Cornforth and Bedford (1987) identified only a first-order reduction in PCC breaks in irradiated unstimulated human lymphocytes and confluent AG01522 human fibroblasts, respectively, the majority (>90%) of which was complete within 6 hours. Interestingly, the generation and repair of DSBs in A-T patient fibroblasts were similar in kinetics to normal cells, but A-T cells maintained higher (~50%) levels of excess chromosome fragments (Cornforth and Bedford, 1987) and incorrectly rejoined DSBs (as measured by the *NotI* restriction fragment Southern hybridization FAR assay employed by Löbrich, *et al.*, 2000). However, whether this higher level of unrepaired and misrepaired DSBs and chromosome breaks, reflected in the high frequencies of γ -H2AX foci and simple and complex chromosome and *chromatid*-type aberrations in the first post-irradiation mitosis after G0/G1-phase irradiation that are characteristic of A-T cells, is the critical determinant of their poorer survival still remains to be resolved (Taylor, *et al.*, 1975; Taylor, 1978; Natarajan and Meyers, 1979; Cox, *et al.*, 1981; Cox, 1982; Nagasawa and Little, 1983; Nagasawa, *et al.*, 1985; Cornforth and Bedford, 1985, 1987; Astor and Morse, 1989; Mozdarani and Bryant, 1989; Löbrich, *et al.*, 2000; Little, *et al.*, 2002;

Mamon, *et al.*, 2003; Martin, *et al.*, 2003; Kuhne, *et al.*, 2004; Loucas and Cornforth, 2004; Chun and Gatti, 2004).

At the cellular level, numerous reports of post-irradiation colony formation ability have likewise measured similar kinetics of PLD repair (Little, 1969; Winans, *et al.*, 1972; Malcolm and Little, 1979; Cox, *et al.*, 1981; Thacker and Stretch, 1983; Cornforth and Bedford, 1987; Astor and Morse, 1989; Nagasawa, *et al.*, 1989; Stackhouse and Bedford, 1993). Repair of PLD has been noted in most cell lines examined (except *ATM*^{-/-} and *BRCA1*^{-/-} cells), including both normal and malignant in origin, making clinical use of chemical inhibitors of PLD processes ineffective for cancer treatment purposes (Hall, *et al.*, 1986; Hall, *et al.*, 1988; Mamon, *et al.*, 2003). Weichselbaum, *et al.* (1969) proposed a link between intrinsic PLD repair capability and the radiocurability of tumors, but further work (Hall, *et al.*, 1988) could not substantiate such a correlation. While the degree of cellular PLD response may be of little prognostic value in the radiotherapy clinic, such variations may be of great importance for the study of the responsible molecular repair mechanisms. For example, Schwartz, *et al.* (2000), Franken, *et al.* (2002), and Alsbeih, *et al.* (2004) have recently shown the PLD repair response to be a Tp53-dependent process. Functional inactivation by HPV-16 infection or direct mutation of Tp53 in human and isogenic mouse cell lines significantly moderated the increases in survival observed in normal cells following 24 hours of confluent holding at 37°C. As Tp53 inactivation is a classic feature of transformed cells, the observation that tumor cells demonstrate PLD repair is indicative that other (Tp53-independent) pathways in these cells must regulate this response.

Post-irradiation incubation temperatures and treatment with various inhibitors of DNA and protein synthesis or hypotonic salt treatments can also affect the survival and PLD response of irradiated cells. This phenomenon was first noted by Phillips and Tolmach (1966) who demonstrated that post-irradiation incubation with DNA synthesis inhibitors reduced survival while incubation with the protein synthesis inhibitor, cycloheximide, increased survival. Whitmore and Gulyas (1967), Winans, *et al.* (1972), and Raaphorst and Dewey (1979) later demonstrated that changes in post-irradiation incubation temperatures also modified the survival of mouse and Chinese hamster cells. Non-isotonic salt solution treatments also affect the degree of radiosensitivity (measured by survival or chromosome aberration induction) during or after irradiation, presumably by altering the state of chromatin compaction affecting both the initial induction and accessibility for repair of IR-induced DNA damage (Elkind and Whitmore, 1967; Winans, *et al.*, 1972; Belli and Shelton, 1969; Raaphorst and Dewey, 1979; Utsumi and Elkind, 1979; Evans and Horng, 1988; Bedford, 1991; Iliakis, *et al.*, 1993; Roos, *et al.*, 2002).

2.3. Effects of G2-phase Irradiation on Mammalian Cells

2.3.1. Definition of the G2 and M-phases of the Somatic Cell Cycle

The G2 (gap 2) stage of the somatic cell cycle is intermediate between the stages of DNA replication in S phase and segregation of the duplicated chromosomes in mitosis, and lasts for 2–5 hours in mammalian cells, depending on species and cell type (Cleaver, 1967). Efficient regulation of the G2–M phase transition ensures that a cell has

completely and accurately duplicated its genome and other essential cellular components (organelles, required proteins, *etc.*) for partitioning into daughter cells, and has repaired any DNA damage that may interfere with proper chromosome segregation to the daughter cells. This transition is tightly regulated by a combination of transcriptional, post-translational, and protein–protein interaction mechanisms, and multiple pathways of DNA damage sensors, transducers, and effectors exist to prevent the onset of mitosis and minimize the transfer of potentially mutagenic lesions to the progeny (Zhou and Elledge, 2000; Iliakis, *et al.*, 2003; Kastan and Bartek, 2004). Certain forms of DNA damage, such as base/nucleotide damage moieties and inter/intra-strand crosslinks, may fail to elicit a G2-phase checkpoint delay and may instead induce an S-phase checkpoint delay in the following cell cycle (Akkari *et al.*, 2000; Andreassen, *et al.*, 2003) when encountered by the DNA replication machinery.

The primary regulator of the G2 phase transition is the maturation-promoting factor (MPF), a complex composed of the CDK1 kinase (p34/Cdc2 from *S. pombe*, CDC28 in *S. cerevisiae*) and cyclin B1. The activity of this kinase complex is initially controlled by levels of cyclins B1 and A, another competing CDK1 partner, and subsequently, by the phosphorylation status of CDK1. As sufficient cyclin B1 levels accumulate during late S and G2, three critical phosphorylation sites of CDK1 (Thr14, Tyr15, and Thr161/167 depending on the particular species) become available upon cyclin B1 binding. Sequential phosphorylation and dephosphorylation events must be completed for full MPF activation, mediated by a number of upstream kinases and phosphatases. The kinases Myt1, Wee1, and CDK7/ClnH/p36MAT1 (CAK1) phosphorylate Thr14, Tyr15 and Thr161/167 respectively, keeping the complex inactive

but opening an ATP-binding domain (the T-loop) (Parker, *et al.*, 1992, Liu *et al.*, 1997). To initiate passage into mitosis, the Cdc25C phosphatase must dephosphorylate Thr14/Tyr15 for full activation of the complex.

Substrates of this activated MPF kinase include histones, nuclear lamins, cytoskeletal intermediate filaments and microtubule-associated proteins, and other structural proteins necessary for nuclear envelope breakdown and chromosome alignment. As the cell progresses through mitosis, MPF also activates the anaphase promoting complex (APC/C^{Cdc20}) or cyclosome, whose ubiquitin ligase E3-like function is necessary for chromosome separation at anaphase. This is accomplished by ubiquitin-mediated destruction of securin by APC/C^{Cdc20} that, in turn, releases separin to cleave the cohesin complexes binding sister chromatids. Upon successful completion of mitosis, the APC complex then recognizes the destruction box motif of the cyclin B1 subunit of MPF, targeting the cyclin's degradation in the 26S proteasome. The phosphatase INH completes the cycle by dephosphorylating CDK1 on Thr161/167, closing the ATP-binding domain and keeping the kinase inactive until cyclin B1 levels peak in the following G2.

2.3.2. Radiation Exposure in the G2-phase of the Mammalian Cell Cycle

Radiation exposure in the G2-phase of the cell cycle engages a checkpoint function to delay mitosis and induce repair processes to administer to defects in the DNA, chromosomes and spindle apparatus. Radiation-induced G2-phase delay has been widely studied in both asynchronous and synchronously cycling populations of mammalian cells (Terasima and Tolmach, 1963; Mitchell, *et al.*, 1979a, b; Nagasawa, 1980; reviewed in

Elkind and Whitmore, 1967; Okada, 1970; Kaufmann and Kies, 1998). These studies indicate the magnitude of this delay ranges from 0.3 to 5.5 hours/Gy and is mostly linear in the dose range of 2–8 Gy, depending on cell type and experimental conditions (Elkind and Whitmore, 1967; Okada, 1970; Mitchell, *et al.*, 1979; Kaufmann and Kies, 1998). Chemical treatments that do not induce prompt DNA DSBs, *e.g.*, camptothecin (a topoisomerase-I inhibitor) and sublethal fluences of 254-nm UV-C, are not effective at inducing a G2-phase delay, unlike treatment with bleomycin, etoposide, and topoisomerase-II inhibitors, which were quite effective (Kaufmann and Kies, 1998).

The cardinal DNA damage transducer kinases ATR (ATM/Rad3-related) and ATM (possibly with the assistance of BRCA1) activate the protein kinases Chk1 (via ATR-mediated phosphorylation of Ser317/345) and Chk2 (via ATM-mediated phosphorylation of Thr68), and both these downstream kinases have been shown to phosphorylate Ser216 of Cdc25C (Liu, *et al.*, 2000; Zhou, *et al.*, 2000). This inhibitory phosphorylation promotes the sequestration of Cdc25C by the 14-3-3 proteins and export out of the nucleus, displacing Cdc25C from the site of its activity (Sanchez, *et al.*, 1997; Peng, *et al.*, 1997). Chk1 has also been shown to phosphorylate Wee1 on Ser549 after irradiation, promoting inhibitory phosphorylation of the Tyr15 site of CDK1, keeping it inactive (Lee, *et al.*, 2001). Increased cyclin B1 mRNA instability, decreased cyclin B1 protein levels, and exclusion of cyclin B1 from the nucleus also contributes to the G2-phase delay (Bulavin, *et al.*, 2002; DeSimone, *et al.*, 2003).

ATM and ATR are both phosphatidylinositol-3 kinase-like kinases (PIKKs) that target protein serine and threonine residues for phosphorylation rather than lipids, and provide alternative, and partially overlapping, pathways for G2-phase delay. The timing

of these two complementary pathways is illustrated in Xu, *et al.* (2002) in which cells irradiated in G2 were first delayed in an ATM-dependent, but dose-independent, manner from entering mitosis. Cells in late S/early G2-phase at the time of irradiation were later delayed at the G2–M transition point in an ATM-independent, but now dose-dependent, manner by the parallel ATR-mediated pathway (see also DeSimone, *et al.*, 2003).

Originally, ATM-deficient cells were thought to experience a much shortened G2-phase delay (Painter and Young, 1980; Zampetti-Bosseler and Scott, 1981; Scott, *et al.*, 1994), but more careful examinations of mitotic indices using BUdR-labeled synchronous cell populations and flow cytometry techniques revealed that A-T cells, following the initial failure to block *late* G2-phase cells from entering mitosis, later experience a second and considerably longer G2-phase delay in the late-S/early-G2 proportion of cells (Ford, *et al.*, 1984; Bender, *et al.*, 1985; Beamish, *et al.*, 1994; Nagasawa, *et al.*, 1994), presumably mediated through this redundant ATR-mediated mechanism. The timing of these two events may underlie the argument of an abrogated or extended G2-phase delay in A-T cells, depending on the exact experimental conditions used to measure the delay. Cre-mediated deletion of a “floxed” (flanked by *lox* sites) ATR gene in human cells or siRNA knockdown of the ATR-interacting protein (ATRIP) abrogates the radiation-induced G2-phase delay and causes cell death (Cortez, *et al.*, 2001). This implies an essential role for the ATR–Chk1–Cdc25C pathway in maintenance of the G2-phase delay, perhaps in temporal continuation of its role as the cardinal mediator of the replication and intra-S phase checkpoints; disruption of these latter pathways lead to the phenotype of radioresistant (or unscheduled) DNA synthesis following DNA damage (Falck, *et al.*, 2001; Falck, *et al.*, 2002; Bartek, *et al.*, 2004).

In addition to the ATM–Chk2–Cdc25C damage response pathway, ATM also functions to stabilize Tp53 by phosphorylating serines 15/18 (human/rodent) and 37, providing another route to G2-phase delay (Bunz, *et al.*, 1998; Kastan, *et al.*, 1991; Powell, *et al.*, 1995; Falck, *et al.*, 2001; Taylor and Stark, 2001; DeSimone, *et al.*, 2003; Iliakis, *et al.*, 2003). Tp53 activation increases expression of CDKN1A (p21^{Waf1/Cip1}), a universal cyclin-dependent kinase inhibitor; the 14-3-3 proteins, which binds inhibited Cdc25C and activated Tp53 (increasing its affinity for DNA sequence-specific binding); PCNA (proliferating cell nuclear antigen) which is thought to function as a scaffold protein; and Gadd45, a stimulator of PCNA-dependent DNA repair (Smith, *et al.*, 1994; Taylor and Stark, 2001; Jin, *et al.*, 2002).

Stabilization of Tp53 after G2-phase damage also results in the down-regulation of cyclin B1 and CDK1 gene expression (Taylor and Stark, 2001; DeSimone, *et al.*, 2003). In addition, both the 14-3-3 proteins and Gadd45 have been shown to directly inhibit CDK1/Cyclin B1 complexes (Taylor and Stark, 2001; Jin, *et al.*, 2002; DeSimone, *et al.*, 2003). ATM also activates the non-receptor tyrosine kinase c-Abl that stabilizes Tp53 and phosphorylates Ser465 of Rad51 (Kharbanda, *et al.*, 1998; Chen, *et al.*, 1999). This signals the recruitment of other enzymes (*e.g.*, BRCA1-related proteins, MRN complex, *etc.*) to facilitate homologous recombinational repair (HRR) between a damaged strand and a sister chromatid template.

Tp53 and BRCA1 have both been shown to colocalize with centrosomes, indicating a critical role for these proteins in centrosome and mitotic spindle dynamics (Hsu and White, 1998; Hsu, *et al.*, 2001; Tarapore, *et al.*, 2001; Tritarelli, *et al.*, 2004). Mitotic spindle formation progress also feeds into the Tp53 pathway through the casein

kinases CK1 δ , CK1 ϵ , and CK2 (Appel, *et al.*, 1995; Dumaz, *et al.*, 1999; Sayed, *et al.*, 2001; Tritarelli, *et al.*, 2004). These kinases are required for the faithful segregation of chromosomes and are intimately associated with microtubules and centrosomes. Following spindle damage, these kinases are activated and subsequently colocalize with and stabilize Tp53 by phosphorylating serines 6, 9 and 392 and threonine 18. It has also been shown that *Tp53*^{-/-} cells develop aneuploidy following spindle poison treatment, a clear sign that Tp53 is essential for mitotic delay following spindle apparatus disruption (Khan, *et al.*, 2000; Andreassen, *et al.*, 2001; Stewart, *et al.*, 2001). Centrosome status is likewise monitored by the Polo-like kinases, PLK1 and PLK3, which also play a role in the G2-M phase transition by modulating the phosphorylation state of Cdc25C (Smits, *et al.*, 2000; Wang, *et al.* 2002). PLK1 is thought to stimulate Cdc25C activity by phosphorylation, and following DNA damage, is inactivated by ATM/ATR-mediated phosphorylation and targeted for ubiquitin-mediated degradation. PLK3, on the other hand, is stimulated by activated ATM-mediated phosphorylation and, in turn, phosphorylates Ser216 of Cdc25C thereby inhibiting its function.

Direct evidence for the repair of DNA damage in cells during the G2-phase delay is somewhat limited. Colocalization of many DNA damage sensor (*e.g.*, γ -H2AX, 53BP1), transducer (ATM, ATR), and effector proteins (NER proteins, Rad51 and other HRR proteins, *etc.*) into DNA damage foci has been documented in G2-phase cells (Cortez, *et al.*, 2001; Thompson and Limoli, 2003). In an interesting Kao, *et al.* (2001) report, BUdR incorporation into such foci was measured in double-thymidine/aphidicolin-synchronized cultures of radioresistant HeLa, HT29, and OVCAR and radiosensitive A2780 tumor cell lines following moderate doses (2–3 Gy) of

radiation. It was assumed that BUdR is directly utilized in DNA repair activities occurring in these foci, many of them occurring *in pairs* indicating involvement of both sister chromatids. G2-phase cells were identified by co-immunofluorescent staining of CENP-F, a G2-specific kinetochore protein, and showed minimal G2-phase BUdR incorporation in unirradiated cultures. The radioresistant cells demonstrated higher numbers of such foci that could also be followed through the subsequent mitosis into the G1 and S phases of the daughter cells (persistent repair foci). This procedure is analogous to the measurement of unscheduled (or radioresistant) DNA synthesis (UDS/RDS) in irradiated S-phase cells using BUdR or ^3H -TdR DNA base analogs. However, co-staining against CENP-F provides unambiguous identification of G2-phase cells, and the punctuate nature of the repair foci is noticeably different than the more diffuse whole nuclear staining of BUdR-incorporated S-phase cells.

Radiation-induced DSBs in G2-phase chromosomes and in S-phase chromosomes following DNA replication are repaired by both NHEJ and HRR repair mechanisms (the latter with the assistance of the Fanconi anemia repair complex; Taniguchi, *et al.*, 2002; Wang and D'Andrea, 2004; Tebbs, *et al.*, 2005), as discussed in Section 2.2.2. The availability of a nearby, undamaged repair template in the duplicated sister chromatid, held by cohesin complexes, promotes error-free HRR. The choice of repair pathway may not be directed by anything more than faster HRR complex kinetics in the more chromatin-dense nuclear environment of G2-phase cells (Rothkamm, *et al.*, 2003; Krüger, *et al.*, 2004). The passage of an individual electron track will likely generate a DSB in the DNA double helix of one chromatid only, resulting in a chromatid-type aberration. Much less frequently, both chromatids may be damaged by a single electron

track and result in an isochromatid break. These “iso-breaks” are identical in form to low frequency terminal deletions generated in G1 chromosomes when observed in the first post-irradiation mitosis. However, following G2-phase irradiation, such dual-chromatid aberrations are assumed to result from DSBs generated in the duplicated G2 chromosome as well.

The degree to which cells will delay following radiation exposure is both radiation dose and cell-type dependent, with mutations in critical proteins described above (*e.g.*, ATM/ATR) prompting cells into mitosis with damaged chromosomes that are likely to produce lethal acentric fragment losses in the daughter cells (Bedford, 1991; Schneiderman, *et al.*, 1996; Savage, 2004). However, many types of cells that experience a G2-phase delay may eventually overcome this block and enter into mitosis after moderate doses of DNA damage, a phenomenon known as “adaptation” (Andreassen, *et al.*, 2003; Yoo, *et al.*, 2004). Often such cells will fail in critical stages of mitosis, most notably the final stage of karyokinesis (nuclear cleavage) and cytokinesis (cellular cleavage) which results in cells reforming a single nuclear envelope with tetraploid DNA content and double the normal G1 chromosome number (Borel, *et al.*, 2002).

An additional Tp53-dependent checkpoint at the following G1–S checkpoint, the “tetraploidy checkpoint,” is thought to exist to prevent such cells from undertaking an additional rounds of DNA replication (becoming either more aneuploid or octoploid; Lanni and Jacks, 1998; Andreassen, *et al.*, 2001, 2003; Borel, *et al.*, 2002; Meraldi, *et al.*, 2002). Such tetraploid fibroblasts may form metabolically active giant cells, but engagement of this checkpoint in lymphoblasts results in apoptosis. However, this tetraploidy checkpoint is also capable of becoming “adapted”, especially in G1/S-phase

checkpoint-deficient *Rb1*^{-/-} and tetraploidy checkpoint-deficient *tp53*^{-/-} mutant cells, as evidenced by mitotic tetraploid (8N) cells (Lanni and Jacks, 1998; Andreassen, *et al.*, 2001, 2003; Borel, *et al.*, 2002; Meraldi, *et al.*, 2002; Margolis, *et al.*, 2003). Tetraploid cells are no more radiosensitive than their euploid counterparts; although burdened with twice the load of aberrations per unit dose, they are likely better able to tolerate otherwise lethal chromosome fragment losses due to the redundancy of essential genes (see Bedford, 1991). This phenomenon is further discussed in Chapter 6.

Cells irradiated in mitosis may suffer a mitotic checkpoint delay if damage is induced prior to the metaphase-anaphase transition point. This “spindle-assembly” checkpoint is engaged if chromosomes are not properly aligned on the metaphase plate due to disruption of chromosome or mitotic spindle integrity (*e.g.*, through the use of the spindle “poisons”), insufficient spindle tension and attachment to the centromeric kinetochore protein complexes, or absence of proper kinetochore function (reviewed in Jallepalli and Lengauer, 2001 and Andreassen, *et al.*, 2003). Mitotic spindle “poisons” include both microtubule hyper-polymerizing agents (taxanes, elutherobins, epothilones, laulimalide, sarcodictyins, docodermolide) and microtubule de-polymerizing agents (*Vinca* alkaloids, cryptophycins, halichondrins, estramustine, nocodazole, and colchicines; Jallepalli and Lengauer, 2001; Wood, *et al.*, 2001; Peterson and Mitchison, 2002; Castedo, *et al.*, 2004).

Damage induced after transition into anaphase is insufficient to induce a mitotic checkpoint delay, and cells complete cytokinesis and karyokinesis unimpeded (Mikhailov, *et al.*, 2002). Radiation exposure during mitosis may engage the mitotic checkpoint if cells are in prophase/early pro-metaphase, but chromosome aberrations are

less frequently observed (Cornforth, 1998; Durante, *et al.*, 1999), possibly due to the degree of chromosome compaction and condensation in mitotic cells (*i.e.*, induced DSBs remain tightly bound in this chromatin state) or heavily damaged cells fail to complete mitosis properly, reforming the un-furrowed nuclear envelope around a now tetraploid G1-phase arrested cell. The high degree of chromatin compaction in mitotic cells results in significantly higher rates of DNA single and double-strand break induction, which underlies the increased radiosensitivity of mitotic cells as compared to G1-phase cells (Radford and Broadhurst, 1986; Chapman, *et al.*, 1999; Roos, *et al.*, 2002; Stobbe, *et al.*, 2002).

Agents that interfere with the mechanisms of the G2-phase delay, such as caffeine and okadaic acid, increase the radiosensitivity of treated cells (Kaufmann, *et al.*, 2003). Caffeine, originally thought to target the ATM and ATR kinases (*e.g.*, Zhou, *et al.*, 2000), has been more recently shown to target a component (or components) of the homologous recombinational repair (HRR) machinery (Wang, X., *et al.*, 2003; Wang, H., *et al.*, 2003; Wang, *et al.*, 2004). Cortez, *et al.* (2003) convincingly demonstrated caffeine treatment alone results in *hyper*phosphorylation of the ATM and ATR kinases and their downstream kinase effectors, CHK1 and CHK2. Although the direct protein target of caffeine-induced radiosensitization in the HRR pathway remains unknown, it is my personal opinion that BRCA1 seems to be the most likely candidate; BRCA1-deficient lymphoblastoid cells experience only a minimal degree of caffeine sensitization in measurements of radiation-induced micronuclei (Speit, *et al.*, 2000). CHO cells deficient in *xrcc2* and *xrcc3* (two of the Rad51 paralogs) likewise show reduced caffeine sensitization (Wang, *et al.*, 2004) but to a lesser extent. Interestingly, caffeine was shown

to be ineffective in reducing radiation-induced G2-phase delay in normal human fibroblasts, in sharp contrast to human tumor cell lines (HeLa, MCF7, and OVG1; Jha, *et al.*, 2002 and HL60; Ning and Knox, 1999). These findings support the growing evidence for redundant (and apparently caffeine-*insensitive*) pathways of G2-phase delay induction in normal cells (see DeSimone, *et al.*, 2003).

2.3.3 Telomere Structure and Function

Telomeres are specialized nucleoprotein structures that cap the ends of chromosomes preventing both critical sequence losses during replication and illegitimate repair and recombination. Telomeres suffer from small losses of repetitive sequence during every replication due to the inability of DNA polymerases to process the tract of the final 3' to 5' (lagging strand) Okazaki fragment (Watson, 1972; Shay and Wright, 2000). As telomeres shorten below critical lengths (6–8 kb), coding sequences may become exposed concurrently with the loss of telomere protein binding sites and proper telomere structure, and cells may be incapable of preventing such telomeres from becoming involved in recombination or repair events (Harley, *et al.*, 1990; Slijepcevic, *et al.*, 1997). This “crisis” of proliferative senescence and growth arrest may be overcome by the activation of telomerase (Counter, *et al.*, 1992; Bodnar, *et al.*, 1998; Herbig, *et al.*, 2004) or an alternative lengthening of telomere (ALT) process mediated by homologous recombinational mechanisms (Bryan, *et al.*, 1995, 1997; Henson, *et al.*, 2002); resulting in the “immortalization” of cells, a hallmark step in the tumorigenic process (Hanahan and Weinberg, 2000; Maser and DePinho, 2002). Since cells are thought to require ~3-6 mutations to become fully malignant (according to the somatic mutation model), this

extension of proliferative capacity may allow for enough additional divisions to achieve such a phenotype (Kim, *et al.*, 1994; Hanahan and Weinberg, 2000). Different cell types execute different senescence pathways; for example, senescent human fibroblasts enter a permanent cell cycle arrest while senescent lymphoid cell types execute an apoptotic programmed cell death program which removes these cells from the population (Hayflick, 1965; Nagasawa, *et al.*, 1995; Williams, *et al.*, 1997; Shay and Wright, 2000; Campisi, 2003).

Telomeres vary in length from 2–30 kb in humans and 20–150 kb in rodents and are composed of long, double-stranded tandem arrays of (TTAGGG/CCCTAA)_n followed by a 3' single-stranded (ss) (TTAGGG)_n (“G-rich”) tail of a few hundred nucleotides at most (Moyzis, *et al.*, 1988; de Lange, *et al.*, 1990; Lejnine, *et al.*, 1995; Makarov, *et al.*, 1997; Wright, *et al.*, 1997). Nuclear matrix binding sites are also interspersed every kb or so within the telomeric sequences, and telomeres form distinct nucleosomal regions with abundant matrix interactions (de Lange, 1992; Luderus, *et al.*, 1996; Pandita, 2002). With the assistance of a number of telomere maintenance proteins, the 3' single-stranded tail is capable of invading the upstream double-stranded telomere repeat tracts and forming a “t-loop” structure which involves non-Watson/Crick or Hoogsteen G:G base pairing (Watson, 1972; Balagurumoorthy and Brahmachari, 1994; Griffith, *et al.*, 1999). Such a structure may negatively regulate improper elongation by telomerase.

Telomerase is a ribonucleoprotein (RNP) reverse transcriptase with two essential components: telomere template RNA (TERC, coded by *hTR* on human chromosome 3q26.2) and a catalytic subunit (TERT, coded by *hTERT* on human chromosome

5p15.33; Lingner, *et al.*, 1997). Telomerase activity is high in germ and rapid turnover (*e.g.*, GI, hematopoietic) cell lineages and in many transformed and tumor cell lines (Greenberg, *et al.*, 1998; Martin-Rivera, *et al.*, 1998; Liu, *et al.*, 1999; Liu, *et al.*, 2004). Activity is minimal in most somatic tissues as the TERT catalytic subunit mRNA is alternatively spliced to inactive isoforms (Liu, *et al.*, 1999). The TERC template RNA contains a 5'-CUAACCCUAAC-3' template motif used to add (TTAGGG) repeats and a 3' H/ACA motif found in other small nuclear RNAs (snoRNAs) associated with pseudouridine modifications during ribosomal RNA (rRNA) maturation (Mitchell, *et al.*, 1999; Antal, *et al.*, 2002). Telomerase-interacting proteins include TEP1, telomere associated protein 1 (Harrington, *et al.*, 1997); dyskerin, involved in H/ACA motif binding and TERT recruitment, mutation of which (or *hTR*) is responsible for the syndrome dyskeratosis congenita (Mitchell, *et al.*, 1999; Vulliamy, *et al.*, 2001); and molecular chaperones, such as Hsp90 and p23, which assist in folding the telomerase protein subunits into an active complex (Holt, *et al.*, 1999).

Telomeres are intimately associated with other proteins besides telomerase that function to maintain telomere length and assist in the formation of the t-loop structure. Pin2 and TRF1 exist as alternate splice forms of the *Pin2/TRF1* gene and bind as homodimers and heterodimers to limit telomere expansion (Shen, *et al.*, 1997; Griffith, *et al.*, 1998; Bianchi, *et al.*, 1999). Pin2 has a 20 amino acid deletion and is 5-10X more abundant, and is cell cycle-regulated peaking in expression in G2-M. These proteins contain a destruction box motif that targets them for degradation during mitosis. Overexpression of wild-type *Pin2/TRF1* results in reduction of telomere length while overexpression of dominant-negative *Pin2/TRF1* mutants result in increased telomere

length. Pin2/TRF1 protein levels detected by Western immunoblotting and immunohistochemistry were significantly lower in 51 breast cancer tumor samples versus 10 normal tissue controls, suggesting a proliferative advantage for cells maintaining longer telomeres by down-regulating Pin2/TRF1 activity or expression (Kishi, *et al.*, 2001a). Also, Pin2/TRF1 and an interacting partner EB-1, a microtubule plus-end binding protein, specifically localize to the mitotic spindle and affect microtubule polymerization *in vitro* during normal mitotic progression and after ATM-mediated pathway activation following DNA damage (Nakamura, *et al.*, 2002).

A second telomere binding protein, TRF2, assists in the formation and stabilization of the t-loop structure preventing unwanted telomerase and DNA repair protein involvement (Bailey, *et al.*, 2001; Stansel, *et al.*, 2001; Wang, *et al.*, 2004). Overexpression of wild-type *TRF2*, like *Pin2/TRF1*, decreases telomere length. However, overexpression of dominant negative *TRF2* results in loss of the 3' single-stranded overhang and the formation of telomeric chromosome fusions (van Steensel, *et al.*, 1998). This activates an ATM/p53-dependent apoptotic pathway and results in early proliferative senescence. Other telomere maintenance proteins include TIN2, which interacts with the homodimerization domain of Pin2/TRF1 and stabilizes higher order telomere structure (Kim, *et al.*, 1999); tankyrase-1 and 2, with N-terminal interactions with TRF1 and TIN2 and homology to the poly-ADP ribose polymerase (PARP) catalytic domain (Smith, *et al.*, 1998; Cook, *et al.*, 2002; Ye and de Lange, 2004); and, Pot1, the critical protein that binds the 3' single-stranded overhang preventing telomerase binding (Baumann and Cech, 2001; Loayza and de Lange, 2003), and an ever-growing list of additional interacting protein partners (see Goytisolo and Blasco, 2002).

Many DNA DSB repair proteins also reside at telomeres when not mobilized to the site of a DSB. The NHEJ proteins DNA-PKcs, Ku70 and Ku80 bind telomeric sequences and modulate telomere-related replication and capping activities (Boulton and Jackson, 1996; Hsu, *et al.*, 1999; Bailey, *et al.*, 1999, 2001, 2004; Samper, *et al.*, 2000; Gilley, *et al.*, 2001). The Mre11/Rad50/Nbs1 (MRN) complex associates with Pin2/TRF1, TRF2 and telomerase, and mutant Nbs1 results in shorter telomeres (Wu, *et al.*, 2000; D'Amours and Jackson, 2002; Bai and Murnane, 2003). Mice deficient for the HR protein Rad54 have significantly shorter telomeres than wild-type controls, and MEFs derived from these mice had significantly higher frequencies of spontaneous telomeric fusions (Jaco, *et al.*, 2003). A novel Rad50 interacting protein, RINT-1, has recently been identified and demonstrated to be involved with G2/M checkpoint control (Xiao, *et al.*, 2001). Deletion of this locus region is associated with AML, breast and ovarian tumors. Another family of G2/M checkpoint control proteins, the 14-3-3 σ proteins (involved with sequestering ATM/ATR-phosphorylated Cdc25C and preventing full CDK1/ClnB activation), are involved with modulating telomere-matrix interactions and the length of the 3' ss tail (Dhar, *et al.*, 2000) and the nuclear localization of telomerase (Seimiya, *et al.*, 2000). Expression of these proteins has been shown to be lost in 94% of breast tumors, and 14-3-3 σ ^{-/-} cells show loss of telomeric repeats, telomere fusions, and terminal non-reciprocal translocations.

ATM has essential functions at the telomere as well. It has been shown to regulate overall length of telomeres and interactions with matrix binding sites (Smilenov, *et al.*, 1997, 1999; Pandita, 2002). ATM also interacts with the N-terminal region of Pin2/TRF1, inhibiting it by phosphorylating Ser219 following IR exposure (Kishi, *et al.*,

2001b). The Werner and Bloom syndrome helicases, WRN and BLM, have also been shown to interact with telomeric DNA sequences and TRF1 and TRF2 (Stavropolous, *et al.*, 2002; Opresko, *et al.*, 2002, 2004; Lillard-Wetherell, *et al.*, 2004), and the WRN helicase associates directly with telomeric DNA in ALT cells and co-localizes with TRF1/PCNA complexes during DNA replication in these cells (Opresko, *et al.*, 2004).

The Tp53 protein has likewise been shown to interact at the mammalian telomere and with telomeric DNA sequences. Introduction of plasmids bearing telomere repeats resulted in stabilization and transcriptional activation of Tp53 in H1299 NSCLC cells, MCF-7 breast cancer cells, HepG2 hepatoblastoma cells, HCT116 colon cancer cells, and WI-38 normal human fibroblasts, while transfection of control plasmids without telomere repeat sequences into these cell lines did not result in Tp53 activation (Milyavsky, *et al.*, 2001). Electron microscopy experiments demonstrated the localization of Tp53 protein to the 3' single-stranded overhang and the t-loop junction of a plasmid-based telomere model system *in vitro*, and TRF2-catalyzed t-loop formation was increased 2-fold in the presence of Tp53 (Stansel, *et al.*, 2002).

The sequence erosion or uncapping of a telomere induced by a deficiency in a critical telomere maintenance protein caused by senescence, genetic mutation, or telomere protein inhibitor treatment induces a robust DNA repair response in mammalian cells. This is evidenced by the co-localization of numerous DNA repair and the telomere-binding proteins discussed above at senescence-associated foci ("SAFs," similar to ionizing radiation-induced foci or "IRIFs") located at telomere sequences in the nucleus (Takai, *et al.*, 2003; d'Adda di Fagagna, *et al.*, 2003; Herbig, *et al.*, 2004; Bradshaw, *et al.*, 2005). Interestingly, the telomere-binding protein TRF2 was recently

shown to colocalize with γ -H2AX at sites of 390-nm UV-A laser microbeam-induced DNA damage at genomic locations outside the telomere (Bradshaw, *et al.*, 2005), indicating these proteins may function in global genome DNA repair pathways as well as serving telomere maintenance functions.

Telomeric fusions resulting from telomeric sequence erosion or protein uncapping generate dicentric (or polycentric) chromosomes that have difficulty segregating properly to daughter cells during mitosis (Slijepcevic, *et al.*, 1997; Bailey, *et al.*, 1999, 2004; Goytisolo, *et al.*, 2001; Goytisolo and Blasco, 2002). Chromosomes are pulled to opposite poles under tension and form anaphase bridges, which ultimately breaks the chromosome during telophase and cytokinesis. This leaves the daughter cells with broken chromosomes, which subsequently undergo NHEJ-mediated DSB repair. Such chromosomes may be involved in subsequent bridge breakage-fusion events in following mitoses, forming complex non-reciprocal translocations (Albertson, *et al.*, 2003; Maser and DePinho, 2003; Pihan and Doxsey, 2003). Such translocations may deregulate oncogene sequences leading to massive gene dosage changes and/or the formation of chimeric protein products with novel functions. Of special note for Chapter 6, telomeric fusions were not observed in C57BL/6TacfBR-KOp53N4 HO *tp53*^{-/-} mouse fibroblasts, either spontaneously or after acute ¹³⁷Cs γ -rays doses of 25–340 cGy (Bailey, *et al.*, 1999, 2004).

2.3.4. The G2 Chromosomal Radiosensitivity Assay

The differential response of cells from different genetic backgrounds to G2-phase irradiation has been most clearly illustrated by a (mostly) standardized cytogenetic assay

known as the G2 chromosomal radiosensitivity assay (or simply, “the G2 assay”). The G2 assay measures the numbers of chromatid-type aberrations (chromatid gaps, breaks, and exchanges) induced by radiation in G2-phase cells collected only hours later at the first post-irradiation mitosis. This assay provides a rapid and fairly discriminatory means of measuring the relative radiosensitivity of cells within a matter of hours following irradiation, and is considered by the ICRP to be a useful measure of individual radiosensitivity (ICRP, 1999). The G2 assay has been applied to a large number of radiation sensitivity and cancer predisposition syndromes, and, by demonstrating strong correlation to the degree of radiosensitivity in these clinically diverse conditions, has found possible use as a cancer “biomarker” and may find future use as a tool to custom tailor individual patient radiation therapy regimens.

Briefly, asynchronously-cycling cultures of fibroblasts or mitogen-stimulated [usually phytohemagglutinin (PHA), a plant lectin] peripheral blood T-lymphocytes are irradiated (70–72 hours post-stimulation) with ≤ 1 Gy of X or γ -rays, allowed 0.5–1 hours for cells in mitosis at the time of irradiation to transit into G1, and the G2-phase cells at the time of irradiation are then blocked by 1–4 hours colcemid treatment at the metaphase/anaphase transition point in mitosis. Following colcemid treatment, cultures are either directly fixed onto the culture substrate (glass or plastic microscope chamber slides or Leighton tubes) or collected with standard cytogenetic techniques (hypotonic buffer and fixative treatments).

A procedural workshop on the G2 assay was held in 2001 at the University of St. Andrews to establish guidelines for the use of this assay, in order to standardize the methodology to achieve reproducible results and allow inter-laboratory comparisons

(Bryant, *et al.*, 2002). The majority of the research groups at this meeting use the Scott methodology, known as the PICR protocol (for the Paterson Institute for Cancer Research in Manchester, UK), which is adapted from the work of Sanford, *et al.* (1989) at the U.S. National Cancer Institute (NCI). Differences between the various protocols involve the use of lymphocytes versus fibroblasts, 37°C versus 0°C hypotonic treatments (to limit further rejoining of gaps and breaks after collection), the length of time between blood draw and culturing, and temperature used for blood storage and transport if cultures could not be initiated immediately (Bryant, *et al.*, 2002).

Differences in the nomenclature used to describe the types of chromatid-type aberrations observed in these metaphase preparations is also a potential source of confusion among the G2 assay research groups [this is considered by both Savage (2004) and Bryant (2004) to be the longest standing debate in radiation cytogenetics]. The Sanford and Scott groups identify chromosome “gaps” as fully discontinuous chromatid fragments greater than the width of a chromatid but aligned with the chromatid axis (they also refer to these as “non-displaced breaks”); chromatid “breaks” are discontinuous chromatid fragments that are not aligned with the main chromatid axis (ISCN, 1978). The Ghent group (Vral *et al.*, 2002a, 2002b) use a single category that includes all complete chromatid arm discontinuities, however narrow they may be. The chromosome aberration nomenclature of Savage (1973) and Bender (1974) was utilized by the remaining groups and is also employed by this laboratory for cytogenetic analyses, including the G2 assay measurements of the Rb family member and CIMR control fibroblast strains reported in Chapter 4.

Using the Savage criteria, chromatid “breaks” are defined as fully discontinuous chromatid fragments either displaced at a distance larger than the width of the chromatid arm or no longer aligned with the original chromatid axis. Chromatid “gaps” are defined as fully discontinuous chromatid fragments displaced at a distance smaller than the width of the chromatid arm. Isochromatid breaks are defined as disconnected chromatid fragments affecting both chromatid arms that may or may not be displaced from the original chromatid axis, and may also become involved in “sister union” events. Chromatid fragments that are not fully severed from the chromatid are considered achromatic lesions and are not scored. Direct comparison of data sets between various research groups can be accomplished by noting the different nomenclatures used and strictly matching the aberration and cell types actually scored (Sanford/Scott “gaps” and “breaks” would be both considered as a Savage “break”).

It should be noted that fully discontinuous chromatid arm deletions (whether one calls them gaps or breaks) are completely separated from the mononemic DNA strand of the centric chromosome. The degree (distance) of detachment from the chromosome therefore relies essentially on the extent of cohesin complex disruption between the sister chromatids and the cellular fixation and slide preparation protocols. Acentric fragments generated by asymmetrical chromatid-type exchanges are routinely observed in nearby proper spatial orientation to the original chromosomes. However, being fully detached, all such fragments may be destined to form micronuclei in the daughter cells and become lost in further mitoses. This high probability of mitotic death (failure of continued proliferation) is due to the loss of critical genes on these fragments (Bedford, 1991; Savage, 2004). Paz-y-Mino, *et al.* (2002) found significantly better correlation ($R=0.78$

versus $R=0.5$) to the total number of chromatid gaps and breaks (*i.e.*, regardless of the distance between the acentric fragment and original chromatid) rather than breaks alone, and the amount of DNA damage measured in the tail moment of a matched series of single-cell gel electrophoresis (Comet assay) experiments of irradiated lymphocytes. As such, both the numbers of chromatid gaps, chromatid breaks (to allow comparison to published data sets in the literature), and combined gaps and breaks (which probably more realistically measures the frequency of potential acentric fragment loss) are recorded in the G2 chromosomal radiosensitivity assay measurements of the Rb family member and CIMR control fibroblast strains presented in Chapter 4.

Chromatid-type exchanges of both symmetrical (no acentric fragments) and asymmetrical forms (with acentric fragments) are also observed, but at a much lower frequency, consistent with the short mitotic collection window (<2 hours) used in these types of experiments. Extending the window between irradiation and mitotic collections results in increasing frequencies of chromatid exchanges, as more time is available for unrestituted chromatid break ends on different chromosomes to illegitimately recombine. Sister chromatid exchange measurements are not possible with the traditional G2 assay, as two cell cycles must be completed with BUdR incorporation to achieve the differential Harlequin staining used to reveal the chromatid-type intrachanges.

As discussed previously, cells may undergo a G2-phase delay following irradiation to an extent depending on the genetic status of the cells being examined. Radiosensitive strains may experience a greater delay and the kinetics of repair may be substantially altered if key protein pathways are disrupted by mutation of critical genes (Nagasawa, *et al.*, 1994; Scott, *et al.*, 1994; Scott, *et al.*, 2003). BUdR labeling of S-

phase cells followed by G2-phase irradiation offers a method of tracking the window of labeled G2-phase cells as they progress into mitosis (*e.g.*, Nagasawa, *et al.*, 1994; Palitti, *et al.*, 1999). Yields of aberrations could then be compared for equivalent labeled mitotic indices that may need to be collected at different time intervals. The G2 assay metaphase chromosome collections of the Rb family and CIMR control fibroblast strains for the G2 assays described in Chapter 4 consistently yielded sufficient mitotic indices for aberration scoring purposes. Since the majority of these strains are derived from apparently normal individuals, they were processed identically to the normal radiosensitivity strains used as reference controls, and the use of BUdR labeling was deemed unnecessary. No evidence of significant differences in radiation-induced G2-phase delay response in Rb patient cells, compared to normal controls, has been reported in the literature to date (Zampetti-Bosseler and Scott, 1980; Sanford, *et al.*, 1996).

Direct comparison of data sets from various research groups also illustrates a large degree of inter-laboratory variation in the scoring results. Overshadowing this source of variability in G2 assay results is the larger degree of *intra*-individual variability observed (Vral, *et al.*, 2002; Smart, *et al.*, 2003; Vral, *et al.*, 2004), making comparisons of *inter*-individual difficult. Repeated measurements of G2-phase chromatid damage of the same cell strains can be strongly influenced by culture conditions employed (such as different batches of serum), the type of low LET radiation employed (X- versus γ -rays), cell density at the time of irradiation, and the individual performing the measurements. A large range of apparently normal control responses, often overlapping with the radiosensitive groups, makes definitive assignment of radiation sensitivity difficult. The Scott PICR group recommends a cutoff placed at the 90% percentile of the reference

normal range, which the mean of the potential radiosensitive strains must exceed (Scott, *et al.*, 1996, 1999; Scott, 2004).

While low-LET ionizing radiations are employed for the G2 assay, a number of other clastogenic agents and DNA repair inhibitors have been employed either as surrogates to obviate the use of radiation sources or to more generally probe the induction of G2-phase DNA damage and the kinetics of its repair on the cytogenetic level (Hsu, *et al.*, 1985, 1989; Cloos, *et al.*, 1999). The radiomimetic agent most often employed as a surrogate for radiation in the G2 assay are the bleomycins, a group of related glycopeptide antibiotics used in combination with other chemotherapeutic agents (cisplatin and etoposide) in the treatment of lymphomas, squamous cell carcinomas, and germ-cell tumors (Chen and Stubbe, 2005). The bleomycins bind both oxygen and the transition metals Fe (II) and Cu (I), and in the presence of a reducing agent, can catalyze the formation of single-stranded and double-stranded DNA damage at a ratio of approximately 6–20:1 depending on the specific DNA sequence (Chen and Stubbe, 2005). Adema, *et al.* (2003) reported that treatment of various lymphoblastoid cell lines with 5 mU/ml bleomycin gave a very strong correlation to the levels of chromatid-type aberrations measured after 30 cGy irradiation.

As mentioned earlier, the G2 assay has been applied to a large number of radiation sensitivity and cancer predisposition syndromes (summarized in ICRP, 1999). Beginning in the late 1970s with the seminal work of Taylor (1978), Bigelow *et al.* (1979), and Natarajan (1979), the measurement of G2-phase chromatid aberrations in these syndromes following treatment with low LET radiation, bleomycin, ultraviolet (UV-A and UV-B) or visible spectrum fluorescent light (which produces ROS)

consistently demonstrated much higher aberration frequencies compared to normal controls. These conditions included ataxia-telangiectasia, Bloom syndrome, familial polyposis, Fanconi anemia, Gardner syndrome, xeroderma pigmentosum (except the XP-A complementation group), and a number of familial cancer syndromes, such as Li-Fraumeni syndrome, Wilms tumor, and dysplastic nevus syndrome. The high frequencies of chromatid-type aberrations noted in all these groups have been attributed to deficiencies in DNA repair or related signaling pathways, contributing to the high rates of cancers seen in these groups (Parshad, *et al.*, 1983, 1985; Hsu, *et al.*, 1985; Gantt, *et al.*, 1986; Sanford, *et al.*, 1989; Terzoudi, *et al.*, 2000).

The Sanford laboratory and the Redpath/Bedford laboratories sought to correlate the degree of G2 chromosomal radiosensitivity with the malignant phenotype in non-tumorigenic and tumorigenic segregates of somatic cell hybrids generated from HeLa cells and normal human fibroblasts (Sanford, *et al.*, 1986; Redpath, *et al.*, 1992). While the work of Sanford (1986) demonstrated significantly higher G2-phase chromatid aberration frequencies segregating with the tumorigenic phenotype, the studies of Redpath and Bedford (1992) using the same cell lines did not detect such enhanced G2 radiosensitivity, which the authors partially attribute to technical differences in experimental protocols. Tumor cells, in general, consistently display significantly higher G2 assay indices than non-transformed cells (Darroudi, *et al.*, 1995; Jha and Bedford, unpublished results), perhaps as a reflection of their inherent genetic deregulation.

Currently, there is much recent interest in the utility of the G2 assay to positively identify ataxia-telangiectasia heterozygotes (Bender, *et al.*, 1985; Scott, *et al.*, 1996) and radiosensitive breast cancer cases (reviewed in Scott, 2004). The A-T heterozygote

carrier frequency in the human population is estimated as 1%; however, the frequency of A-T heterozygotes in breast cancer cases is 3.8% (Easton, 1994) and thus *ATM* heterozygosity is considered a genetic risk factor for breast cancer (Savitsky, *et al.*, 1995). According to a report by Thompson, *et al.* (2005), this heterozygosity imparts a 2.23-fold excess relative risk (95% CI, 1.16–4.28) in the general population and a 4.94-fold excess relative risk (95% CI, 1.90–12.9) in those <50 years of age for breast cancer, the only cancer to be found significantly elevated in such individuals. Aside from the earliest G2 assay report performed with A-T heterozygotes (Bender, *et al.*, 1985), three independent research groups have confirmed a significant radiosensitive response in heterozygote cell strains (both fibroblasts and lymphocytes; Parshad, *et al.*, 1985; Shiloh, *et al.*, 1989; Mitchell and Scott, 1997; Tchirkov, *et al.*, 1997).

Scott, *et al.* (1999), Baeyens, *et al.* (2002), and Scott (2004) report that 25–45% of lymphocytes obtained from breast cancer cases demonstrate G2-phase chromosomal hyper-radiosensitivity or higher G1-phase micronuclei frequencies using the 90th percentile cutoff of the distribution of normal responses ($n > 200$ strains). Taken as a marker of heritable low penetrance predisposition to cancer, the Scott group (Burrill, *et al.*, 2000) has also identified 27% of unaffected first-degree relatives with high G1-phase micronuclei frequencies. The relationship of cellular radiosensitivity, tumor recurrence and survival data in this group of breast cancer patients, however, revealed these cytogenetic studies were *not* strong prognostic indicators of patient outcome (Roberts, *et al.*, 2003), although this may be due to a limited sample size. G2 assays of young cancer patients (multiple types) reported in Baria, *et al.* (2002) demonstrated enhanced radiosensitivity in 14 of 32 (44%) of lymphocytes strains examined.

2.4. Effects of Low Dose-Rate Irradiation on Mammalian Cells

2.4.1. Definition of Low Dose-Rate Irradiation

Along with the overall dose, linear energy transfer, and particular species of ionizing radiation, the *rate* at which radiation doses are delivered is a critical determinant of biological and cellular responses. The most effective timing for dose delivery in radiation therapy to achieve maximum therapeutic benefit has been argued for more than a century (Paterson, 1952; Ellis, 1968; Elkind, 1977; Hall, 2000); higher doses and dose rates may be more effective for tumor cell killing, but dose protraction spares such damage and potential long-term consequences in the surrounding normal tissues. A large body of research exists for effects of whole-body exposure to low dose-rate ionizing radiation in many higher organisms, including plants (*Tradescantia*, *Vicia faba*, barley) and animals (fish, turtles, mice, rats, rabbits, dogs, donkeys, monkeys and humans; NCRP, 1980; NRC BEIR, 1990; UNSCEAR, 2000). Reports dating from the 1920s onward demonstrated many groups appreciated dose rate as a significant factor in cellular biological responses (Hall, 2000). Early endpoints studied included mitotic division delay in chicken tissue cultures (Canti and Spear, 1927) and cleavage delay in *Arbacia* sea urchin eggs (Henshaw, *et al.*, 1933). This review will focus primarily on low dose-rate effects in rodent and human *in vitro* cellular models.

Each of the human body's approximately 10^{14} cells is exposed to a background dose rate of $\sim 2.5 \mu\text{Gy/day}$ ($\sim 0.1 \mu\text{Gy/h}$) of low-LET ionizing radiation from cosmic, terrestrial, and internal sources (NCRP, 1987; NRC BEIR, 1990; UNSCEAR, 2000). This background radiation dose rate and the low doses delivered to the general population

is of great practical value in the context of radiation protection and risk analysis, providing the baseline value for cancer incidence for studies of radiation-exposed human cohorts. While the distinction is arbitrary, for the purposes of radiation protection, UNSCEAR recommends that dose rates of ≤ 3 mGy/h be considered “low dose-rate,” those ≥ 1 Gy/min (60 Gy/h) are “high dose-rate”, and the “intermediate dose-rate” region lies between. Most of the literature on low and intermediate dose-rate effects exists for dose rates of 1 mGy/h to ≤ 1 cGy/min (≤ 60 cGy/h); where the greatest range of effect on biological systems is observed (NCRP, 1980; NRC BEIR, 1990; Amdur and Bedford, 1994; Vilenchik and Knudson, 2000, 2003). Dose-rate exposures in ≤ 60 cGy/h region, including the technically “intermediate” dose rates (0.5–15 cGy/h) used in the studies in Chapters 5 and 6, will all be considered “low dose-rate” in the following discussions.

As the dose-rate is lowered, the density of particle track traversals per unit time in the cell nucleus decreases and likewise, the probability that two independent chromosome breaks may interact in time and space to form a lethal or possibly mutagenic aberration in a “misrepair” event also decreases as more time is allowed for individual breaks to be repaired (Sax, 1940; Catcheside, *et al.*, 1946; Lea, 1947; reviewed in Savage, 1998). Dose-rate effects are reflected in the disappearance of the βD^2 , the *inter-track* or *multi-track*, component of the linear-quadratic survival equation (LQ, $\ln(S) = -\alpha D - \beta D^2$) and often a reduction in the biological effect being measured. The βD^2 component is predicted to become insignificant at or below the “limiting” low dose-rate, with biological response dictated solely by αD , or *intra-track*, effects (Wells and Bedford, 1983; Bedford, 1991; Cornforth, *et al.*, 2002). Radiation response is not affected by further decreases in dose rate below the limiting low dose-rate, being linear and

dependent *only* on the total dose (number of particle tracks) received during the exposure period, *i.e.*, the component of radiation damage described by the α term.

The ratio of the slope of the acute, high dose-rate (HDR) radiation dose-response curve extrapolated to the origin, α_{HDRe} , to the slope of the linear low dose-rate (LDR) radiation dose response curve, α_{LDR} , for points in the low dose region is known as the dose-rate effectiveness factor (DREF). Alternatively, if LDR response data are unavailable, the slope of the line extrapolated through the low-dose region of the HDR response curve, α_{HDRi} , may be used to calculate a dose-effectiveness factor (DEF). Collectively, these two related parameters are referred to as the dose and dose-rate effectiveness factor (DDREF; NRC BEIR, 1990). The BEIR V (NRC BEIR, 1990) and UNSCEAR (2000) reports recommend an average DDREF factor of ~2–2.5 for the extrapolation of high dose-rate data to low dose-rate and for doses ≤ 20 cGy. The reader is directed to these extensive reviews of human epidemiological (primarily Japanese A-bomb survivors, nuclear industry workers, and radiation therapy patients) and laboratory animal *in vivo* radiation exposure studies from which these review panels have derived their DDREF estimate (NRC BEIR, 1990; UNSCEAR, 2000).

2.4.2. Dose Fractionation and Sub-lethal Damage Repair

The original mechanistic background for low dose-rate effects in mammalian cells stems from studies of fractionated radiation exposures. The first such study of the survival of mammalian cells by Elkind and Sutton (1959) compared the single-fraction acute dose radiation survival of V79 Chinese hamster cells to that following doses split into two separate fractions and delivery times. This and further experiments

demonstrated an increase in survival when doses were separated by as little as 30 minutes, suggesting cells were repairing “sub-lethal” damage and recovering during this time (Elkind and Sutton, 1960; Elkind and Whitmore, 1967; Cornforth and Bedford, 1987; Bedford, 1991). Sublethal damage is a measure of the change in biological response to a second radiation dose after a previous radiation exposure. Single-dose acute survival responses are characterized by a shoulder region in the low dose region (≤ 3 Gy), implying that some kind of damage accumulation process is involved, so by delaying the time between radiation exposures, survival may increase due to the repair of such “sublethal damage.”

Sublethal damage, as the name implies, is also not necessarily immediately lethal to cells but constitute forms of damage (*e.g.*, DNA and chromosome breaks) that may be repairable if sufficient time is allowed (Sax, 1940; Lea, 1947; Little, 1969; Bedford and Cornforth, 1987; Bedford, 1991). However, further interactions with other sites of damage, at higher doses and dose rates, will lead multiple sites of (and multiple possibilities for) lesion interaction, more cell killing, or “fixation” of the damage as a permanent mutation by a misrepair event and subsequent DNA replication and cell division in a survivor (Cornforth and Bedford, 1987; Bedford, 1991; Cornforth, 1998). Sublethal damage, therefore, reflects the overall *density* of radiation damage and the kinetics of lesion repair (acute dose shoulders, dose-rate effects), while potentially lethal damage (discussed in Section 2.2.6) represents modulation of the initial *induction* and/or subsequent *expression* of lesions (*i.e.*, the translation of DSBs to chromatin breaks) by affecting either surrounding chromatin status (non-isotonic salt solution treatments before or after irradiation) or DNA repair function and cellular signaling functions (chemical

inhibitors, temperature, and time, in the form of post-irradiation confluent holding). Both phenomena are reflections of innate cellular radiation-induced damage production and resolution.

Lajtha and Oliver (1961) used Elkind and Sutton's split-dose data to infer the response of cells during *continuous* low dose-rate radiation exposures. Such exposures represent the limiting extreme of hyper-fractionation (Bedford, 1991; Amdur and Bedford, 1994; Fritz, *et al.*, 1996) with the lower limit of the dose per fraction constrained to the actual number of track traversals per unit time. Two important predictions were made. First, they hypothesized that as the dose-rate was lowered, survival would increase and the slope of the survival curve would become progressively less steep as cells have more time to repair sub-lethal damage during the extended exposure periods. Second, they predicted a change in the shape of the survival curve from linear-quadratic (sigmoid) for acute doses to a simple exponential (linear) for protracted irradiations. The shoulder region of acute dose survival curves, reflective of sub-lethal damage accumulation and repair induction processes, would be expected to be absent as cells have sufficient time to repair the low frequency damage (Lloyd and Edwards, 1983; Wells and Bedford, 1983; Bedford and Cornforth, 1987; Savage, 1998); this has been repeatedly demonstrated (see below).

2.4.3. Low Dose-Rate Cell Survival and Chromosome Aberration Induction

Berry and Cohen (1962) reported the first experimental data set on the effects of low dose-rate γ -irradiation on mammalian cell survival. Tumor-bearing mice were exposed at low dose rates of 20 or 40 cGy/h or at an acute dose rate of 115 cGy/min.

Tumor cells were then removed from the mice, diluted and plated for colony formation; the measured survival matched the predictions of Lajtha and Oliver (1961) in that survival was increased in the low dose-rate irradiated tumor cells. Bedford and Hall further substantiated the predictions of Lajtha and Oliver in a series of *in vitro* survival experiments with HeLa cervical carcinoma cells exposed to a wide range of dose rates of ^{222}Rn γ -rays (Bedford and Hall, 1963) and later ^{60}Co γ -rays (Hall and Bedford, 1964). The dose rate effect was most dramatic between 100 and 1 cGy/min, above and below which, respectively, only small changes in survival were observed. Since then, cellular dose-rate effects have been well documented for many of the classic radiobiological endpoints: survival (colony formation, apoptosis, senescence), chromosome aberration induction, and mutation induction in other cellular systems.

Wells and Bedford (1983) demonstrated the existence of the *limiting* low dose-rate for the survival of density-inhibited C3H 10T_{1/2} mouse cells (to prevent complications of proliferation and differential cell cycle radiosensitivities). They reported a pronounced dose-rate effect between 5.56 Gy/h (556 cGy/h) and 29 cGy/h, after which little further reduction in effect per unit dose at the lower dose-rates (17 and 6 cGy/h) was observed. Bedford and Cornforth (1987) reported similar results with multiple dose fractionation regimens in normal human fibroblasts. Experiments with log-phase cultures exposed to this range of low dose rates demonstrated varying levels of cell cycle redistribution and proliferative capability, the latter of which was severely compromised at dose rates >50 cGy/h. Earlier experiments by Mitchell *et al.* (1979b) with cells derived from a variety of species (hamster, mouse, rat kangaroo, Indian muntjac, pig, human) and later experiments by Stackhouse and Bedford (1993, 1994)

with the CHO *irs-20* mutant reported similar results although the dose rates needed to prevent proliferation varied by species. This failure to proliferate was linked to cell cycle redistribution and the buildup of cells delayed at the G2–M transition point (Bedford and Mitchell, 1973, 1979; Mitchell, *et al.*, 1979a, 1979b; Stackhouse and Bedford, 1993, 1994), a sensitive phase of the cell cycle (Terasima and Tolmach, 1961; Sinclair, 1968; Dewey, *et al.*, 1972).

A fractionated brachytherapy scheme referred to as “pulsed” dose-rate irradiation has been optimized to mimic the effect of continuous low dose-rate irradiation, using small doses per fraction delivered on a daily (or longer) basis (Brenner and Hall, 1991; Hall, 1991). Such radiotherapy regimens offer potentially better dose distribution in the tumor treatment volume and radiation protection advantages for radiologists and radiological technicians (doses delivered in short fractions rather than continuously; Hall and Bedford, 1964; Hall, 1991). Brenner and Hall (1991) demonstrated that a pulsed regimen of 0.5 Gy delivered in a 10-minute duration pulse hourly was equivalent to continuous 50-cGy/h irradiation in 36 normal and malignant human cell strains. Later *in vitro* studies with rat 9L gliosarcoma cells (Armour, *et al.*, 1991), Chinese hamster V79 fibroblasts (Fritz, *et al.*, 1996), and other human normal and tumor cells (Chen, *et al.*, 1997; Pomp, *et al.*, 1999; Niedbala, *et al.*, 2001) reported shorter pulse durations were equally effective and that inherent radiosensitivity plays a large role in the response of cells to either low dose-rate or pulsed dose-rate radiation exposure.

Radiation cytogenetics has also provided a large body of evidence relating the cellular effects of dose-rate fractionation and LDR radiation exposures to the α and β parameters of the linear-quadratic model (Savage, 1979; Lloyd and Edwards, 1983;

Edwards, 1997; Cornforth, *et al.*, 2002; Loucas, *et al.*, 2004). Results for γ -ray-induced dicentrics from these studies (primarily in lymphocytes) indicate the α component changes little from an average value of $\sim 2 \times 10^{-4}$ dicentrics/cell per cGy (range $0.5\text{--}7 \times 10^{-4}$ dicentrics/cell per cGy) with acute, fractionated or low dose-rate γ -ray exposures. This value is approximately double for 30–250 kVp X-rays. The average β component is $5\text{--}6 \times 10^{-6}$ dicentric/cell per cGy²; the β component demonstrates less variability (range $4\text{--}8 \times 10^{-6}$ dicentric/cell per cGy²) than the α component for acute doses and experiences a ~ 5 -fold reduction as the dose rate is reduced (Lloyd and Edward, 1983). Dicentrics are often the aberration of choice in these studies as they are fairly unambiguous to score and have a low spontaneous frequency in unirradiated cells of $\sim 5\text{--}10 \times 10^{-4}$ per cell (1 in 1000–2000), approximately a 150-fold lower frequency than the yield of dicentrics predicted for an exposure of 1 Gy (Lloyd and Edwards, 1983; Edwards, 1997).

Examination of different cell types may significantly impact the observed distribution of radiation-induced chromosome aberrations. For example, lymphocytes have approximately 2–5-fold higher induction of chromosome interchanges than fibroblasts at the same dose (Ostashevsky, 2000; Cornforth, *et al.*, 2002). From the data of Loucas and Cornforth (2001) and Cornforth, *et al.* (2004), complex exchanges account for 40% of the breakpoint involvement after 4 Gy γ -ray exposures of PHA-stimulated T-lymphocytes, while only 25% of potential breakpoints were involved in complex exchanges after the same dose in fibroblasts. Likewise the degrees of exchange complexity are different for the two cell types: 40% of complex aberrations induced in lymphocytes after 4 Gy involved 5 or more breakpoints in their formation, accounting for <5% of the complex exchanges observed in fibroblasts after 3.6 Gy. This is caused by

differences in the shape and chromatin organization of the cell nucleus, spherical in lymphocytes and ellipsoid (“pancake-shaped”) in fibroblasts (see Figures 7.4 and 7.5). Interestingly, even after exposures with *sub*-limiting low dose rates, ~10% of induced radiation exchange breakpoints in fibroblasts are involved in complex aberrations; whether this is due to single-track action or compound-track action at different times remains conjectural (Cornforth, *et al.*, 2002; Loucas, *et al.*, 2004; further discussed in Chapter 7).

Radiation cytogenetics has been very useful for testing the validity of the mathematical theories underpinning cellular radiation response (specifically the LQ model) for acute and low dose-rate chronic radiation delivery. To avoid the complication of differential cell cycle radiosensitivities and proliferation during the long exposure periods associated with low dose-rate irradiation, such exposures are conducted with quiescent, non-cycling cultures, predominantly density-inhibited monolayers of fibroblasts and un-stimulated lymphocytes (Wells and Bedford, 1983; Bedford, 1991; Cornforth, *et al.*, 2002). Cytogenetic studies of low dose-rate chromosome aberration induction in human lymphocytes and fibroblasts have reported a limiting low dose-rate range of ~5–15 cGy/h (Scott, *et al.*, 1970; Liniecki, *et al.*, 1971; Purrot and Reader, 1976; Cornforth, *et al.*, 2002; Loucas, *et al.*, 2004). Species-specific (rodent versus human) differences in radiation response (acute and low dose-rate) have long been noted, rodents being more radioresistant than humans in general (Hall, 1991; see further discussions in Chapters 5 and 6).

2.4.4. Low Dose-Rate Exposures and the DNA Repair Connection

Individual components and suites of DNA damage response proteins can differ in mammalian cells of different genetic origins, greatly influencing their signaling threshold levels and capable rates of repair (Anderson and Lees-Miller, 1992; Lindahl, 1993). Therefore, the dose rate at which ionizing radiations are delivered can significantly alter the magnitude of dose rate effects observed for different biological endpoints. Low dose and dose-rate radiation exposures result in single-stranded DNA damage levels similar to those generated by cellular oxidative metabolism and can be easily repaired (Lindahl, 1993; Ward, 1998; Vilenchik and Knudson, 2003). However, the low frequency of more complex damage types and DSBs induced by direct radiation exposure are not routinely encountered in a majority of tissue types. Vilenchik and Knudson (2003) estimated that the level of endogenous DSBs generated in cycling human cells (primarily during replication-associated activities and the decatenation of sister chromatids prior to mitosis) is equivalent to levels induced at a continuous low dose rate of ~30 cGy/h. Induction of radiation-induced DSBs at 1 cGy/h may therefore fail to signal the appropriate DNA repair response pathways and potentially result in a mutation if the cell replicates. Increasing the dose rate an order of magnitude to 10 cGy/h (a DNA damage induction level now discernable from the endogenous “noise”) will register a robust induction of repair and reduced mutagenicity (Vilenchik and Knudson, 2001, 2003).

The radiobiological approaches described in the previous section have clearly demonstrated that the rate of DNA DSB damage induction and repair in dose fractionation and low dose-rate exposure studies is a critical determinant of chromosome aberration induction and cell killing. Likewise, the use of various human and rodent

DNA DSB repair-deficient cell strains has illustrated the essential contribution of normal DSB repair status to low dose-rate recovery. This is well illustrated by the LDR response of the A-T homozygote fibroblast strain GM02052 that shows minimal, if any, evidence of sparing during low dose-rate exposures (Cox, 1982; Nagasawa, *et al.*, 1992). Likewise, the resistant mouse lymphoma cell line L5178Y-R showed a pronounced dose-rate effect, while its radiosensitive counterpart, L5178Y-S, showed only a slight shift in survival (Courtenay, 1965; Beer, *et al.*, 1985; Evans, *et al.*, 1985; Furuno-Fukushi, *et al.*, 1988). Although the precise genetic mutation(s) responsible for the high sensitivity of L5178Y-S cells (acute dose D_0 of ~20–25 cGy) remains unknown, the cells are deficient in a number of DNA repair assays in a manner consistent with mutation in a core NHEJ protein (Nagasawa, *et al.*, 1980; Evans, *et al.*, 1987; Jeggo, 1990; Szumiel, 2005a, 2005b). The CHO DSB repair mutants *xrs-5*, *xrs-6* (Nagasawa, *et al.*, 1989; Hu and Hill, 1996), V-3 (Whitmore, *et al.*, 1989; Hu and Hill, 1996), *irs-2* (Thacker and Wilkinson, 1995), and *irs-20* (Stackhouse and Bedford, 1993a, 1993b, 1994) demonstrated slower repair kinetics and minimal dose-rate sparing, compared to ~2.5-fold increase in survival observed in their wild-type CHO K-1, AA8, and 10B2 counterparts. The large degree of dose-rate sparing is consistent with the large shoulder seen in wild-type CHO acute dose survival curves (same references).

Biochemical evidence of low dose and low dose-rate effects is also becoming increasingly available. Many “low” dose demonstrations of activation of key DNA DSB repair and signaling proteins involved in radiation response (*e.g.*, γ -H2AX, ATM, DNA-PK, Tp53, and CDKN1A/p21) use doses ≥ 25 –50 cGy (*e.g.*, Bakkenist and Kastan, 2003; Buscemi, *et al.*, 2004). However, evidence discussed previously of hypersensitive (HRS)

survival responses at *lower* doses (≤ 10 – 25 cGy; Marples, *et al.*, 2004); adaptive responses being stimulated by doses ≥ 1 – 10 mGy (an order of magnitude below the HRS range), but not by doses below that (Broome, *et al.*, 2002); and the lack of repair of long-lived γ -H2AX foci observed in human fibroblasts exposed to only 2 mGy (Rothkamm and Löbrich, 2003), implies the existence of a low dose threshold for DNA repair signaling for doses ≤ 1 – 5 mGy. The 2 mGy dose in the study by Rothkamm and Löbrich (2003) resulted in a $\sim 10\%$ reduction in relative survival compared to unirradiated controls.

More recently, reports using low dose-*rate* exposures observed significantly moderated activation of the ATM protein (via phosphorylation of serine-1981) and its downstream protein target Nbs1 (via phosphorylation of serine-343; Collis, *et al.*, 2004) and almost no activation of Tp53 (via phosphorylation of serine-15; Ishizaki, *et al.*, 2004) after continuous LDR irradiation of 9.4 and 1.8 cGy/h, respectively, in normal, malignant, and *hTERT*-immortalized human cells by Western analysis. Cytologically, the formation of γ -H2AX foci (by DSB-activated, ATM/ATR/DNA-PK-mediated phosphorylation of H2AX serine-139) detected by immunofluorescence was likewise significantly attenuated in both studies. In another report by Sugihara, *et al.* (2004), NIH 3T3 mouse cells (wild-type *tp53*) transfected with a *tp53*-promoter-driven GFP-reporter plasmid demonstrated activation after 72 hours of low dose-rate γ -irradiation at 5 cGy/h but not at 1 cGy/h or 0.1 cGy/h.

2.4.5. “Inverse” Dose Rate Effects and Low Dose-Rate Changes in Gene Expression

An “inverse dose-rate effect” for survival and mutagenesis also illustrates a potential threshold for DNA DSB repair signaling. An inverse dose rate effect for the survival of HeLa cells irradiated at low dose-rate was first observed by Mitchell, *et al.* (1979a); cell survival was *lower* for a dose rate of 37 cGy/h compared to 154 cGy/h. This was also observed in confluent and asynchronous cultures of human PC3 prostate carcinoma and T98G and A7 glioblastoma cells irradiated at dose rates below 100 cGy/h (Mitchell, *et al.*, 2002). The inverse dose-rate effect for asynchronous cultures of cells is likely to result from *dose-rate-specific* effects on cell cycle progression and blockage of cells in more radiosensitive phases of the cell cycle in terms of killing and mutation induction (Burki, 1980; Jostes, *et al.*, 1980; Chuang and Liber, 1996).

Inverse dose rate effects for the induction of mutations at the *tk* and *hprt* loci have been documented in human WTK1 and TK6 lymphoblastoid cell lines (Amundson and Chen, 1996), Chinese hamster V79-S cells (Crompton, *et al.*, 1990), L5178Y mouse lymphoma cells (Ueno, *et al.*, 1986; Furuno-Fukushi, *et al.*, 1988), and mouse T-lymphocytes (Lorenz, *et al.*, 1993). The induction of somatic *hprt* mutations in these cell lines and germline specific-locus mutations in rodents by low dose-rate irradiation is reviewed and discussed in Vilenchik and Knudson (2000). Consistently, doses delivered at ≤ 1 cGy/h are more effective for mutation induction (in a number of experimental systems and genetic loci) than doses delivered at 10 or 100 cGy/h. Somewhat surprisingly, the radiosensitive L5178Y-S mouse lymphoma strain appears to be less mutable (*hypomutable*) at the *hprt* locus following exposure to both low and high dose-rate irradiation than its resistant counterpart, L5178Y-R (Evans, *et al.*, 1985).

Exposure of cultured cells or even whole animals to LDR radiation also affords a strategy for identifying genes responsive to DNA damage incurred at these dose rates by other genetic techniques such as microarray analysis (Amundson, *et al.*, 2003a) or RT-PCR analysis (de Toledo, *et al.*, 1995) and reporter protein (GFP-tagged) studies (Sugihara, *et al.*, 2004). Prior to these more modern molecular techniques, measurements of LDR irradiation-induced changes in gene expression were typically accomplished by labor-intensive mRNA-based Northern blot analyses (Melkonyan, *et al.*, 1995; Ross, *et al.*, 1997). Northern analyses also require the use of ^{32}P -dCTP for probe-labeling reactions; ^{32}P emits a high-energy β -particle (1.71 MeV maximum and ~690 keV average energies) upon decay with a half-life of 14.3 days and is considered to be a significant radiological hazard for laboratory employees. The advent of more sophisticated microarray analysis techniques allowed researchers to “interrogate” a much larger selection of potentially radiation-responsive genes more quickly and without the use of radioactive materials in the laboratory (Amundson, *et al.*, 2003a).

In another report by Amundson *et al.* (2003b), gene expression patterns were monitored by microarray analysis following irradiation in the ML-1 human myeloid leukemia cell line. Low dose-rate exposures at 16.8 cGy/h resulted in an approximate 2–8-fold induction of radiation-inducible stress genes *GADD45A*, *CDKN1A*, and *MDM2*. Maximal induction of these and other genes, primarily related to apoptosis and cell cycle regulatory pathways, occurred roughly two hours following the completion of irradiation, regardless of the dose-rate examined. Sugihara, *et al.* (2004) observed a number of tp53-responsive genes (including *ccng1*, *mdm2*, *cdkn1a*, *dcxr*, *sip27*, *epfx1*) upregulated in NIH 3T3 cells following exposures of 5 cGy/h or greater by microarray analysis. It

would be interesting to examine microarray profiles for human cell types other than those of lymphoid origin (*i.e.*, cells which do not undergo apoptosis and are more relatable to the pathogenesis of radiation-induced carcinomas and sarcomas) with lower dose-rate exposures (<10 cGy/h) at the border of potential DSB signaling thresholds (see previous section).

In a recent report by Kovalchuk *et al.* (2004) examining the global gene expression profiles of male and female mice exposed to either acute or chronic low-dose radiation, a significant sex difference was noted in the expression of nuclear and G protein-coupled receptors and members of the PKC, TGF, and WNT signaling pathways, with one gene, *WNT5A*, elevated only in chronically exposed (5 cGy/day) female mice. Also many genes up-regulated (>3-fold) in irradiated female mice were down-regulated in male mice or vice versa, while some gene expression changes in one sex were not observed in the other. While many of these differences could be attributed to sex-specific hormonal pathways, complicated by effects on many different tissue types within the whole animal, these results suggest that sex-related radiation responses may merit further study.

2.4.6. Low Dose-Rate Irradiation Survival for Genetic Screening Purposes

The apparent lack of dose-rate effects in DNA DSB repair mutants (*e.g.*, A-T cells, rodent NHEJ mutants) and variability observed in normal individuals (Little and Nove, 1990; Weeks, *et al.*, 1991; Geara, *et al.*, 1992; Nagasawa, *et al.*, 1992; Elyan, *et al.*, 1993; Jones, *et al.*, 1995; Badie, *et al.*, 1996; Burnet, *et al.*, 1996; Dikomey, *et al.*, 2003) has attracted many groups to exploit this phenomenon for the purposes of genetic

testing (akin to the G2 chromosomal radiosensitivity assay in Section 2.3.4). A classic example of this approach will be briefly reviewed, the identification of A-T heterozygote strains using confluent cultures of fibroblasts exposed to low dose-rate irradiation. Chapter 5 likewise begins with a discussion of the continuous low dose-rate irradiation strategy used to identify radiosensitive mutants in asynchronous cultures, which resulted in the discovery of the CHO 10B2 *prkdc*-mutated cell line, *irs*-20 (Stackhouse and Bedford, 1993a, 1993b; Priestly, *et al.*, 1998).

The attraction of unambiguously identifying A-T heterozygote carrier individuals was introduced at the end of Section 2.3.4. Such individuals may represent up to ~4% of breast cancer cases, and ATM heterozygosity is associated with a ~5-fold excess relative risk of this disease (Easton, 1994; Thompson, *et al.*, 2005). A report by Weeks, *et al.* (1991) attempted to use low dose-rate ^{137}Cs γ -irradiation to positively identify A-T heterozygote individuals in a large Amish A-T family. While fibroblasts from the A-T heterozygote group were moderately radiosensitive, differences between their radiation response and that of normal family members were not statistically significant. Likewise, a report by Nagasawa, *et al.* (1992) did not observe any significant difference in the survival response of A-T heterozygote fibroblast strains GM03396 and GM03488 compared to 6 normal fibroblast strains in low dose-rate ^{137}Cs γ -ray exposures of density-inhibited G0/G1-phase cultures.

Cytogenetic and molecular techniques have been better able to delineate the response of A-T heterozygote strains using low dose-rate IR exposures. The induction of chromosomal aberrations in A-T heterozygote lymphoblastoid cell lines (and lymphocytes from “over-responder” breast cancer patients) by low dose-rate radiation

exposures was significantly higher than normal controls; this difference was not significant after high dose-rate exposures (Waghray, *et al.*, 1990; Jones, *et al.*, 1995). DNA DSB rejoining studies (assayed by PFGE) following low dose-rate irradiation in A-T heterozygote cells have demonstrated an initial fast-rejoining phase of repair similar to normal cells, but a significantly delayed slow-rejoining phase of repair, resulting in higher numbers of unrepaired breaks (Blöcher, *et al.*, 1991). More recently, excellent discrimination of A-T heterozygote cell lines has been demonstrated in this laboratory by examining the repair kinetics of γ -H2AX foci, a DSB marker, in confluent cultures exposed to LDR ^{137}Cs γ -irradiation (T. Kato, personal communication). A similar degree of differential radiosensitivity is observed using continuous low dose-rate exposures of low-density asynchronous cultures of mouse embryo fibroblast cells assayed for colony formation (reported in Chapter 6).

CHAPTER 3

Effects of G0/G1-phase Irradiation on Rb Family Member and Apparently Normal CIMR Fibroblast Strains

3.1. Abstract

Acute dose irradiation survival measurements for confluent cultures of the 14 Rb family member and 2 radiosensitive apparently normal CIMR control (GM04505 and GM08447) fibroblast strains, allowed approximately 24 hours of confluent-holding post-irradiation potentially lethal damage (PLD) repair, are presented. These results confirm in this laboratory the moderate to hyper-radiosensitive classifications of the Rb family member fibroblast strains made in Fitzek, *et al.* (2002). While the Fitzek report identified two proband strains (MF-7R and MF-14R) from the Rb families with survival responses within their laboratory's range of apparently normal CIMR fibroblast strains; all 14 Rb family member and both radiosensitive apparently normal CIMR fibroblast strains examined in this report were significantly more radiosensitive (2–4-fold, $P < 0.05$) than this laboratory's range of typical apparently normal CIMR fibroblast survival responses using the PLD repair protocol. As previously reported for other bilateral Rb patient fibroblast strains, the degree of PLD repair recovery, measured as the ratio of the initial slopes of the asynchronous culture and confluent culture plus PLD repair survival curves, was reduced (although not significantly) in affected $RbI^{+/-}$ individuals compared to their unaffected first-degree relatives.

3.2. Introduction

Potentially lethal damage (PLD) repair following irradiation occurs when cells are allowed to “rest” and repair radiation-induced DNA damage (*i.e.*, prior to subculture or serum stimulation, which stimulates re-entry into the cell cycle) before being assayed for a relevant radiobiological parameter (colony formation, chromosome aberration induction, *etc.*; reviewed in Section 2.2.6 in Chapter 2). Reports by Arlett and Priestly (1983) and Weichselbaum, *et al.* (1985) indicated that some fibroblast strains derived from patients with bilateral or 13q-deletion retinoblastoma (Rb) may be defective in PLD repair after 6–24 hours of post-irradiation confluent holding, although other reports by the latter group (Nagasawa, *et al.*, 1992) have found little evidence of this phenotype. Likewise, surveys of PLD repair capability in apparently normal fibroblast strains have revealed that ~25–35% were defective in post-irradiation recovery, as measured by overall survival and re-entry into the cell cycle (Fertil, *et al.*, 1988; Nagasawa and Little, 1988; Malaise, *et al.*, 1989).

The failure of moderately radiosensitive, PLD repair-deficient cells to display an increase in post-irradiation survival broadens the range of differential radiosensitivity when compared to normal, repair-deficient cells, which may demonstrate ≥ 2 –5-fold increases in survival due to PLD repair (Arlett and Priestly, 1983; Weichselbaum, *et al.*, 1985; Cornforth and Bedford, 1987; Fertil, *et al.*, 1988; Hall, *et al.*, 1988; Nagasawa and Little, 1988; Malaise, *et al.*, 1989; Nagasawa, *et al.*, 1992). For this reason (and to evaluate their radiation response in this laboratory), the radiation survival of confluent cultures of fibroblast strains derived from the 14 Rb family members (Fitzek, *et al.*, 2002) and two radiosensitive apparently normal CIMR controls (GM04505A and GM08447,

identified as radiosensitive in the continuous LDR irradiation colony formation experiments described in Chapter 5) allowed 24 hours of post-irradiation PLD repair (by confluent-holding) was measured. If the moderately radiosensitive strains were defective in PLD repair, a better accentuation of the degree of radiosensitivity, compared to this laboratory's validated reference apparently normal CIMR strains (AG01521, AG01522, GM02149, GM08333, and GM08429), would be expected.

3.3. Materials and Methods

3.3.1 Cell Culture and Irradiations

Human fibroblast cultures were initiated from liquid nitrogen stocks at the lowest passage number available (all $\leq$ P20). All fibroblasts strains were grown in α MEM cell culture medium (GIBCO/Invitrogen) supplemented with 15% FBS (Sigma), 100 μ g/ml streptomycin sulfate, 100 U/ml penicillin G (both Sigma), and 1X GlutaMAX™-I (*L*-alanyl-*L*-glutamine, GIBCO/Invitrogen) in a 37° C incubator supplied with 95% air/5% CO₂ mixture. Cultures were routinely passaged at 1:4 dilutions prior to density-inhibited contact inhibition. A single lot of FBS (Sigma lot no. 32HK8401) was used in all the experiments to reduce experimental variability.

Cultures of the fibroblast strains identified in Table 3.1 were initiated from liquid nitrogen-frozen stocks by rapid thawing of the frozen ampule in a 37°C water bath. One to two ml of the thawed fibroblast suspensions in 10% DMSO/90% cell culture cryomedium were supplemented with 5 ml of fresh cell culture medium and centrifuged at 1000 rpm for five minutes. The supernatant was then aspirated and the cell pellet broken up and resuspended in one ml of fresh cell culture medium. These suspensions

were then aliquoted into T-25 tissue culture flasks (Greiner Bio-One) filled with 5 ml of pre-equilibrated (by placement of the medium-filled flask in the 37°C incubator for a minimum of one hour). The cell culture medium was replaced the next day with 5 ml of fresh cell culture medium. Cultures were passaged until sufficient cell numbers were available for experimental analysis. Identically numbered experiments (*e.g.*, PFW126A, -126B, -126C, *etc.*) were completed contemporaneously with cultures recovered from different frozen ampules.

For the acute irradiation survival plus PLD repair experiments, fibroblast cultures in T-25s were allowed to grow to confluency, usually within two to three days following subculture. The cell culture medium would then be replaced twice at three day intervals to achieve a >95% G0/G1 population (as monitored by long-term BUdR incorporation experiments). One day after the final medium change, the flasks were capped tightly and transported to the MRB470 irradiation facility. Cells were irradiated at room temperature with graded doses of 0 (sham) to 400 cGy of ¹³⁷Cs 662 keV gamma rays at a dose rate of 250 cGy/min, supplied by a J. L. Shepard and Associates Mark I-68A beam irradiator with an approximate activity of 3170 Ci (as of September 2004). The flasks were then returned to the 37°C incubator, with caps loosened, to allow approximately 24 hours of confluent-holding PLD repair.

The following day (22–26 hours post-irradiation), the medium was aspirated and the cells were detached with 0.25% trypsin/EDTA (GIBCO/Invitrogen) in Ca²⁺/Mg²⁺-free PBS (Dulbecco's PBS-A formulation) for three minutes at 37°C. 500 µl of the well-mixed single cell suspension was diluted into 9.5 ml of PBS and counted with a Coulter Z-1 cell counter (Beckman-Coulter, Inc.). Cells were then plated at low density in T-25

flasks containing pH-equilibrated medium (placed for a minimum of 1 hour in the incubator) to achieve approximately 50–100 viable colonies for each dose point following two weeks of growth. After plating, the flasks were returned to the incubator and the medium was fully replaced after the first week of growth. After two weeks, the flasks were aspirated, rinsed with 0.9% sodium chloride and fixed with 100% ethanol. The flasks were then stained with a 0.1% crystal violet/methanol solution and colonies with >50 viable cells were counted as survivors.

3.3.2. Data and Statistical Analyses

Estimates for absolute survival were calculated as the average number of colonies counted per number of cells plated per dose point. The logarithm of these survival estimates were averaged for 3–4 independent experiments per fibroblast strain, and fitted with either a linear ($y=ax+c$) or linear-quadratic ($y=\alpha x^2+\beta x+c$) least-squares-weighted regression function where y is the average absolute survival after a dose x (SigmaPlot). The calculated intercept (c) was used as the zero-dose plating efficiency (PE) to calculate the average relative surviving fraction (*i.e.*, the absolute surviving fractions corrected for the zero dose plating efficiencies). Standard errors of the mean (SEM) for 3–4 independent experiments were calculated by combining the standard deviations of the absolute surviving fractions by the square root of the sum-of-the-squares method (Bevington and Robinson, 1992) and dividing this value by the square root of the number of experiments, and constitute the error bars for Figures 3.1–3.6. SEM values for the calculated intercept (c)/PE, relative surviving fraction at 2 Gy (SF_2), slope (a) and $1D_0$ (=

1/a) values reported in Table 3.1 and Figures 3.1–3.6 were provided by the software regression analyses (SigmaPlot).

The survival curve fit parameters obtained with the linear-quadratic function were not significantly different from those obtained with fitting the survival data with the exponential function. Consistent with the low doses ($\leq 3\text{--}4$ Gy) of radiation used in these survival experiments, values for β were very small ($\leq 10^{-7}$) and values for α quickly approached a . As such, only parameters derived from linear-exponential ($y=ax+c$) fitting of the survival data (e.g., ${}_1D_0$, SF₂) are reported in Table 3.1. Statistical differences in the survival data were tested by Student *t*-tests for two independent sample distributions (SigmaPlot; Tarone, *et al.*, 1983). Correlations between the different culture and radiosensitivity parameters (PE, D_0 , ${}_1D_0$) were assessed using the Pearson product-moment correlation coefficient, fit by the least-squares method to the respective data sets being compared (SigmaPlot).

3.4. Results

Acute dose irradiation survival results for confluent G0/G1-phase cultures of the 14 Rb family member and two radiosensitive apparently normal CIMR control (GM04505 and GM08447) fibroblast strains exposed to graded doses of cesium-137 gamma rays and allowed approximately 24 hours of confluent-holding, post-irradiation potentially lethal damage (PLD) repair are summarized in Table 3.1 and plotted in Figures 3.1–3.6. The measured and exponential fit ($S=e^{-aD}$) regression curve-calculated plating efficiencies (PE) and radiosensitivity parameters [relative surviving fraction at 2 Gy (SF₂), slope a , and ${}_1D_0 = 1/a$] are listed in Table 3.1. The 14 Rb family member

Table 3.1. Results of acute irradiation with 22–26 hour PLD repair survival experiments for the 14 Rb family member fibroblast strains and two radiosensitive apparently normal CIMR fibroblast strains, GM04505 and GM08447. The pooled experimental average $\pm$ SEM and range of plating efficiencies (PE), and the average and log-transformed average $\pm$ SEM of the relative surviving fraction at 2 Gy (SF_2) values for each fibroblast strain are reported. Parameters obtained from exponential-fit ($S=e^{-aD}$) regression curve fitting of the pooled log-transformed absolute survival data, (PE_{EXP} , SF_{2EXP} , a , D_0) $\pm$ SEM, are also reported. The Rb family member fibroblast strains are ranked from least (1) to most (14) radiosensitive.

Table 3.1.

Fibroblast Strain	No. Expts.	Average PE + SEM _{PE} (Range)	Average Relative SF ₂ ± SEM _{SF2} (Log-Ave. Relative SF ₂ ± SEM _{SF2})	Exponential Fit: $S = e^{-aD}$				Rb Family _{1D₀} Rank
				PE _{EXP} ± SEM _{PE EXP} ^{a,b}	SF _{2 EXP} ± SEM _{SF2 EXP} ^a	a ± SEM _a (cGy ⁻¹)	_{1D₀} (= 1/ a) ± SEM _{1D0} ^c (cGy)	
Retinoblastoma Family Members								
MF-2M Rb Family I	3	0.112 ± 0.017 (0.078–0.136)	0.559 ± 0.043 (0.545 ± 0.021)	0.116 ± 0.008	0.442 ± 0.034	0.0041 ± 0.0004 cGy ⁻¹	244 ± 26 cGy	1
MF-3R Rb Family I	3	0.181 ± 0.013 (0.156–0.196)	0.404 ± 0.027 (0.400 ± 0.013)	0.190 ± 0.012	0.334 ± 0.026	0.0055 ± 0.0004 cGy ⁻¹	183 ± 14 cGy	4
MF-4F Rb Family I	3	0.214 ± 0.016 (0.197–0.247)	0.350 ± 0.029 (0.344 ± 0.017)	0.194 ± 0.011	0.348 ± 0.027	0.0053 ± 0.0004 cGy ⁻¹	190 ± 15 cGy	3
MF-6F Rb Family II	4	0.033 ± 0.001 (0.029–0.035)	0.260 ± 0.091 (0.240 ± 0.045)	0.025 ± 0.004	0.271 ± 0.054	0.0065 ± 0.0011 cGy ⁻¹	153 ± 32 cGy	10
MF-7R Rb Family II	3	0.081 ± 0.003 (0.072–0.084)	0.393 ± 0.058 (0.381 ± 0.035)	0.081 ± 0.006	0.333 ± 0.032	0.0055 ± 0.0005 cGy ⁻¹	181 ± 19 cGy	5
MF-10R Rb Family III	3	0.166 ± 0.040 (0.112–0.244)	0.349 ± 0.045 (0.344 ± 0.026)	0.165 ± 0.016	0.266 ± 0.030	0.0066 ± 0.0006 cGy ⁻¹	151 ± 16 cGy	11
MF-11F Rb Family III	3	0.048 ± 0.024 (0.011–0.094)	0.291 ± 0.038 (0.266 ± 0.017)	0.029 ± 0.007	0.315 ± 0.082	0.0058 ± 0.0015 cGy ⁻¹	173 ± 60 cGy	6
MF-12M Rb Family III	3	0.083 ± 0.012 (0.059–0.100)	0.429 ± 0.055 (0.388 ± 0.024)	0.074 ± 0.008	0.352 ± 0.046	0.0052 ± 0.0007 cGy ⁻¹	191 ± 31 cGy	2

Table 3.1. Continued.

Fibroblast Strain	No. Expts.	Average PE + SEM _{PE} (Range)	Average Relative SF ₂ ± SEM _{SF2} (Log-Ave. Relative SF ₂ ± SEM _{SF2})	Exponential Fit: $S = e^{-aD}$				Rb Family _{1D₀} Rank
				PE _{EXP} ± SEM _{PE EXP} ^{a,b}	SF _{2 EXP} ± SEM _{SF2 EXP} ^a	a ± SEM _{a} (cGy ⁻¹)	_{1D₀} (= 1/ a) ± SEM _{1D0} ^c (cGy)	
Retinoblastoma Family Members								
MF-13M Rb Family IV	3	0.141 ± 0.003 (0.136–0.147)	0.222 ± 0.017 (0.219 ± 0.010)	0.135 ± 0.003	0.222 ± 0.004	0.0075 ± 0.0001 cGy ⁻¹	133 ± 2 cGy	14
MF-14R Rb Family IV	3	0.201 ± 0.004 (0.193–0.206)	0.337 ± 0.028 (0.336 ± 0.015)	0.212 ± 0.011	0.293 ± 0.017	0.0061 ± 0.0003 cGy ⁻¹	163 ± 8 cGy	7
MF-15F Rb Family IV	3	0.167 ± 0.013 (0.142–0.182)	0.338 ± 0.033 (0.322 ± 0.017)	0.161 ± 0.008	0.292 ± 0.017	0.0062 ± 0.0003 cGy ⁻¹	163 ± 8 cGy	8
MF-16M Rb Family V	3	0.098 ± 0.025 (0.050–0.136)	0.304 ± 0.036 (0.298 ± 0.016)	0.096 ± 0.004	0.244 ± 0.010	0.0071 ± 0.0002 cGy ⁻¹	142 ± 4 cGy	13
MF-17R Rb Family V	3	0.104 ± 0.031 (0.044–0.146)	0.270 ± 0.059 (0.263 ± 0.031)	0.082 ± 0.006	0.262 ± 0.020	0.0067 ± 0.0004 cGy ⁻¹	149 ± 10 cGy	12
MF-18F Rb Family V	3	0.221 ± 0.012 (0.206–0.244)	0.347 ± 0.024 (0.346 ± 0.013)	0.233 ± 0.011	0.273 ± 0.011	0.0065 ± 0.0002 cGy ⁻¹	154 ± 5 cGy	9

Table 3.1. Continued.

Fibroblast Strain	No. Expts.	Average PE + SEM _{PE} (Range)	Average Relative SF ₂ ± SEM _{SF2} (Log-Ave. Relative SF ₂ ± SEM _{SF2})	Exponential Fit: $S = e^{-aD}$			
				PE _{EXP} ± SEM _{PE EXP} ^{a,b}	SF _{2 EXP} ± SEM _{SF2 EXP} ^a	$a \pm \text{SEM}_a$ (cGy ⁻¹)	₁ D ₀ (= 1/ a) ± SEM _{1D0} ^c (cGy)
Sensitive apparently normal CIMR controls							
GM04505	3	0.118 ± 0.007 (0.111–0.131)	0.307 ± 0.042 (0.307 ± 0.020)	0.109 ± 0.005	0.318 ± 0.012	0.0057 ± 0.0002 cGy ⁻¹	175 ± 6 cGy
GM08447	3	0.139 ± 0.008 (0.127–0.154)	0.187 ± 0.038 (0.185 ± 0.021)	0.122 ± 0.006	0.230 ± 0.013	0.0074 ± 0.0003 cGy ⁻¹	136 ± 6 cGy
Bedford Laboratory range of resistant apparently normal CIMR controls (AG01521, AG01522, GM02149, GM08333, GM08429) ^d							
Lower limit		0.45 (0.10–0.60)	0.50–0.75	—	0.540 ± 0.034	0.0031 cGy ⁻¹	325 cGy
Upper Limit					0.727 ± 0.030	0.0016 cGy ⁻¹	630 cGy

^a The calculation of PE_{EXP} ± SEM_{PE EXP} and SF_{2 EXP} ± SEM_{SF2 EXP} result in asymmetrical SEM distributions for both parameters, therefore the larger value of the SEM range was reported. SF₂ = relative surviving fraction at 2 Gy.

^b The plating efficiency estimate PE_{EXP} is the calculated ordinate intercept for the linear (exponential) fit of log-smoothed absolute survival data averaged for 3–4 independent experiments.

^c The calculation of D₀ ± SEM_{D0} as inverse of $a \pm \text{SEM}_a$ results in an asymmetrical SEM_{D0} distribution, therefore the larger value of the absolute value of $[D_0 - (1/(a - \text{SEM}_a))]$ was reported.

^d Data for the Bedford Laboratory range of apparently normal CIMR control fibroblast strains are derived from Cornforth, 1987; Hwang, 1990; Muhlmann-Diaz, 1993; Dugan, 2002; and multiple experiments per strain performed myself from 2000–2004.

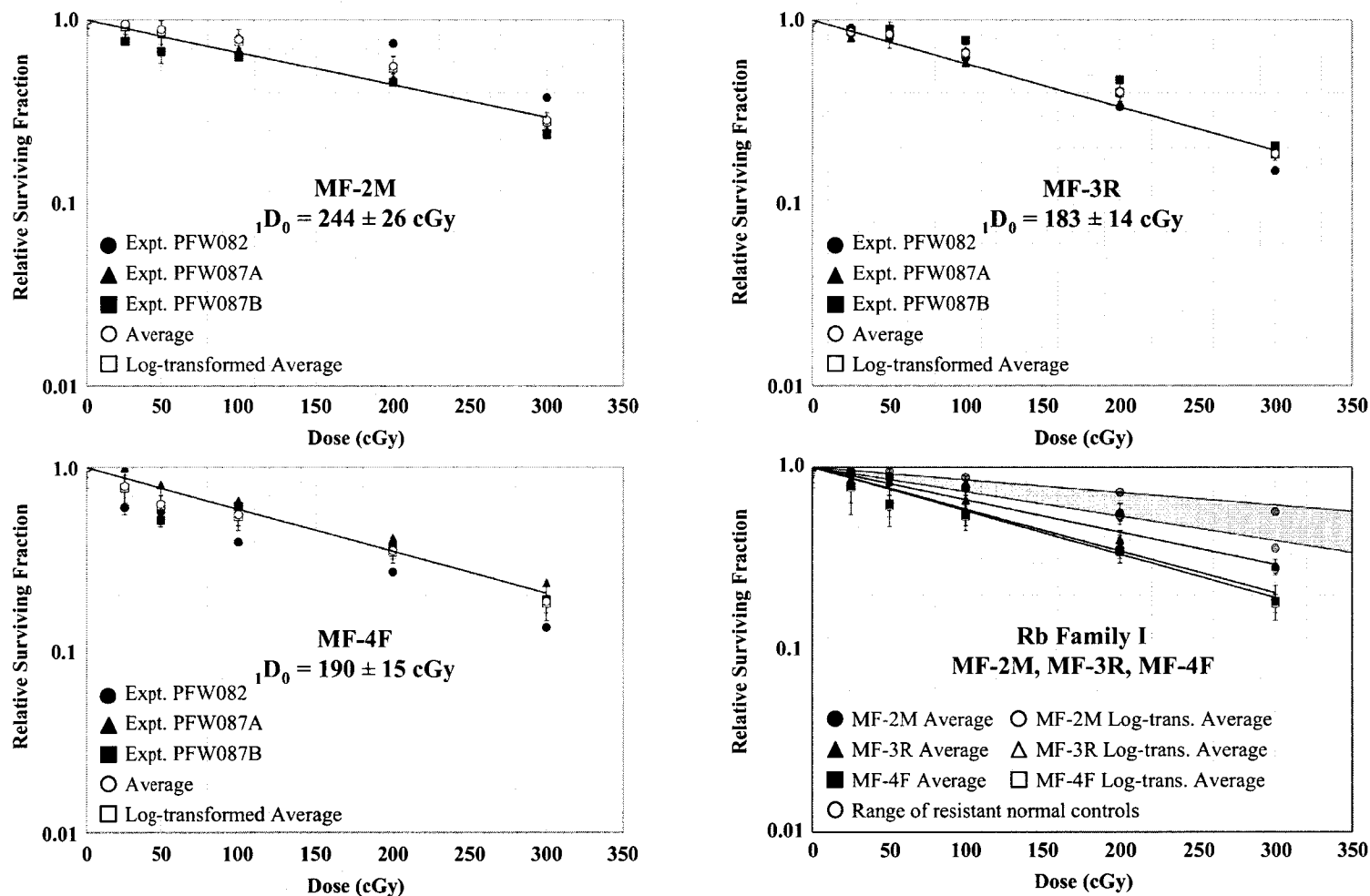


Figure 3.1. Acute dose plus 22–26 hours PLDR survival experiment plots, exponential-fit ($S = e^{-aD}$) regression curves, and calculated $1D_0 (= 1/a)$ $\pm$ SEM values for fibroblast strains obtained from members of Rb Family I: MF-2M ($Rb1^{+/+}$), MF-3R ($Rb1^{+/+}$), and MF-4F ($Rb1^{+/+}$).

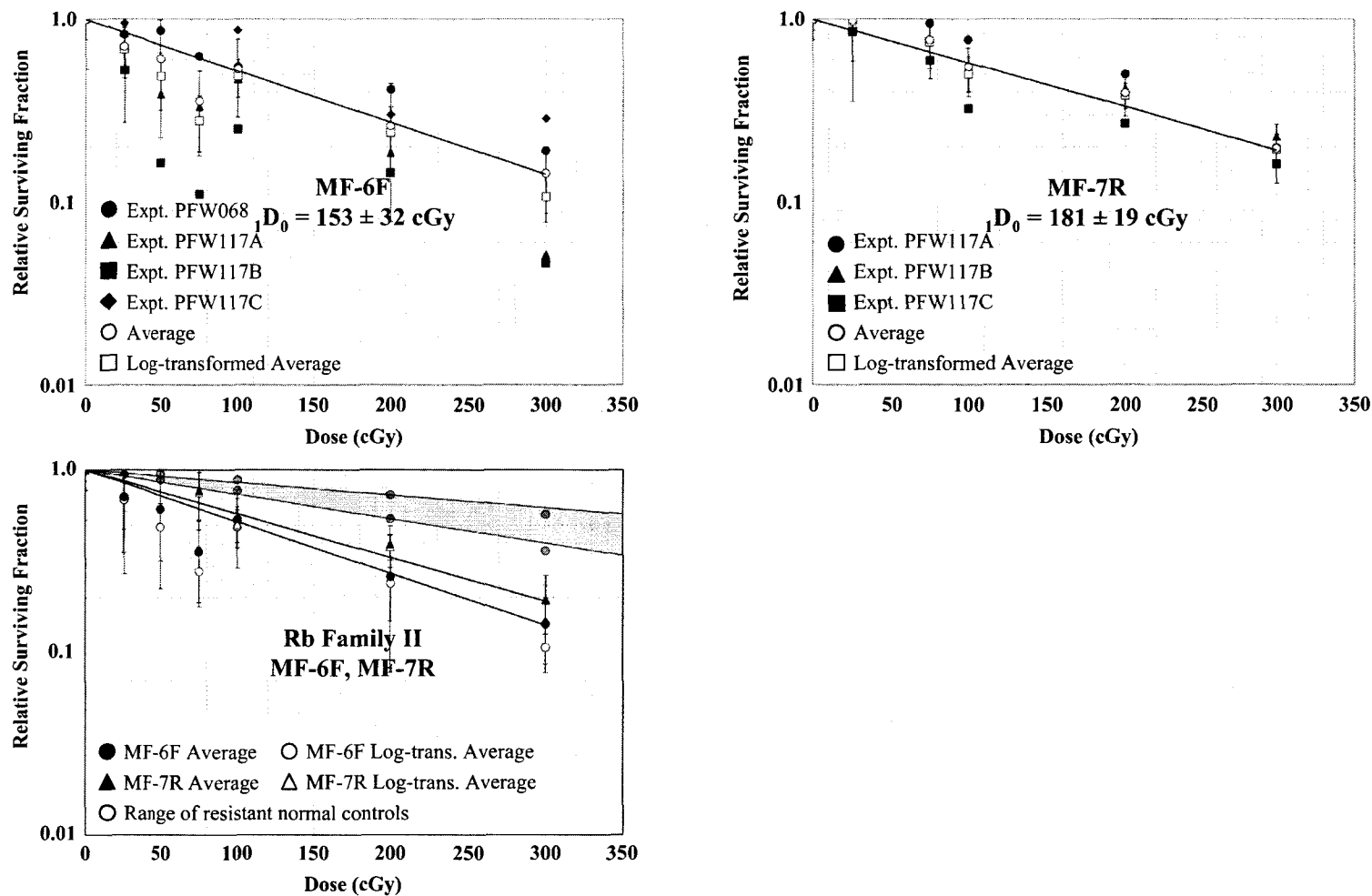


Figure 3.2. Acute dose plus 22–26 hours PLDR survival experiment plots, exponential-fit ($S = e^{-aD}$) regression curves, and calculated D_0 ($= 1/a$) $\pm$ SEM values for fibroblast strains obtained from members of Rb Family II: MF-6F ($RbI^{+/+}$) and MF-7R ($RbI^{+/-}$).

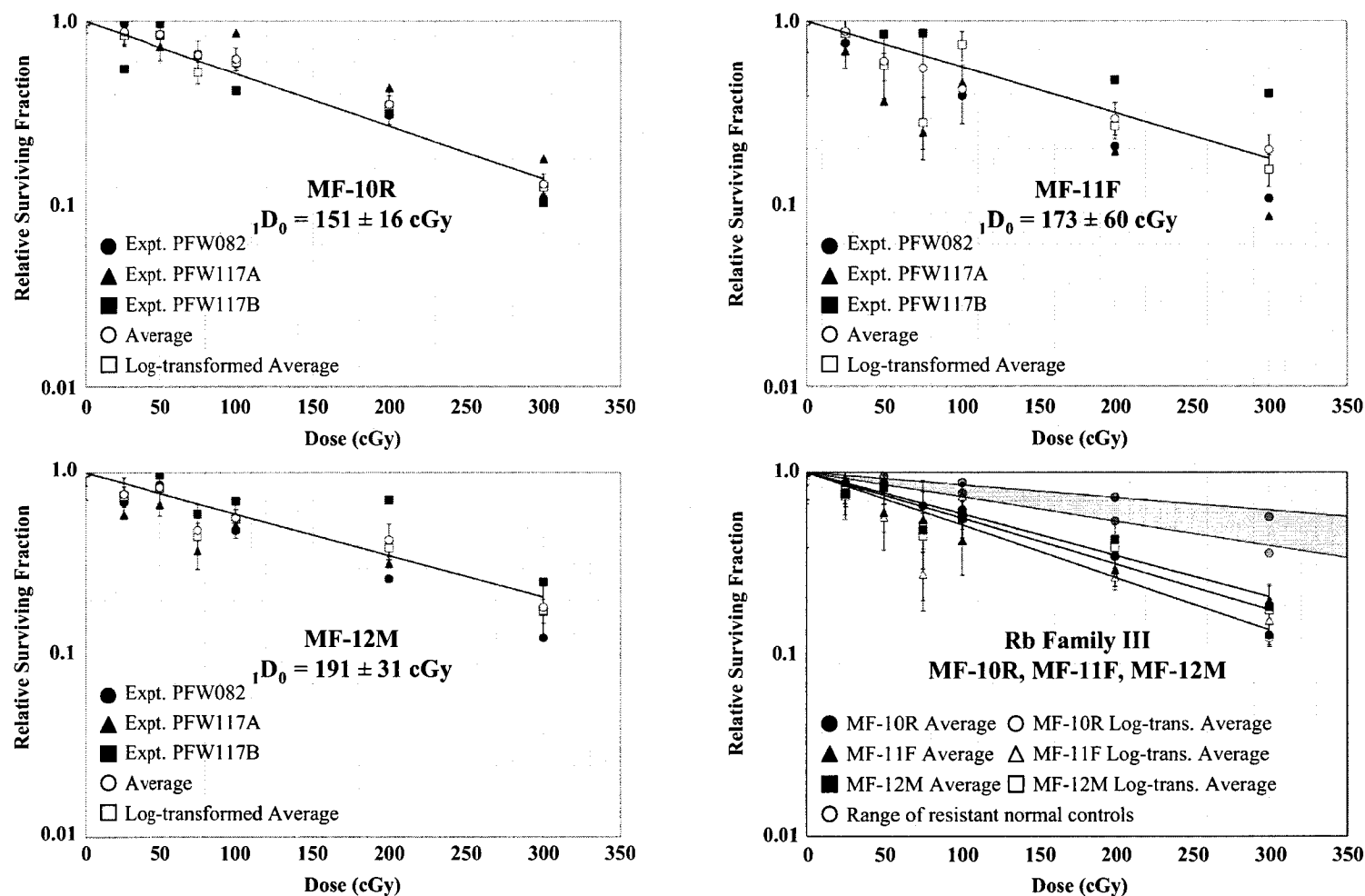


Figure 3.3. Acute dose plus 22–26 hours PLDR survival experiment plots, exponential-fit ($S = e^{-aD}$) regression curves, and calculated $1D_0$ ($= 1/a$) $\pm$ SEM values for fibroblast strains obtained from members of Rb Family III: MF-10R ($Rb1^{+/-}$), MF-11F ($Rb1^{+/-}$), and MF-12M ($Rb1^{+/-}$).

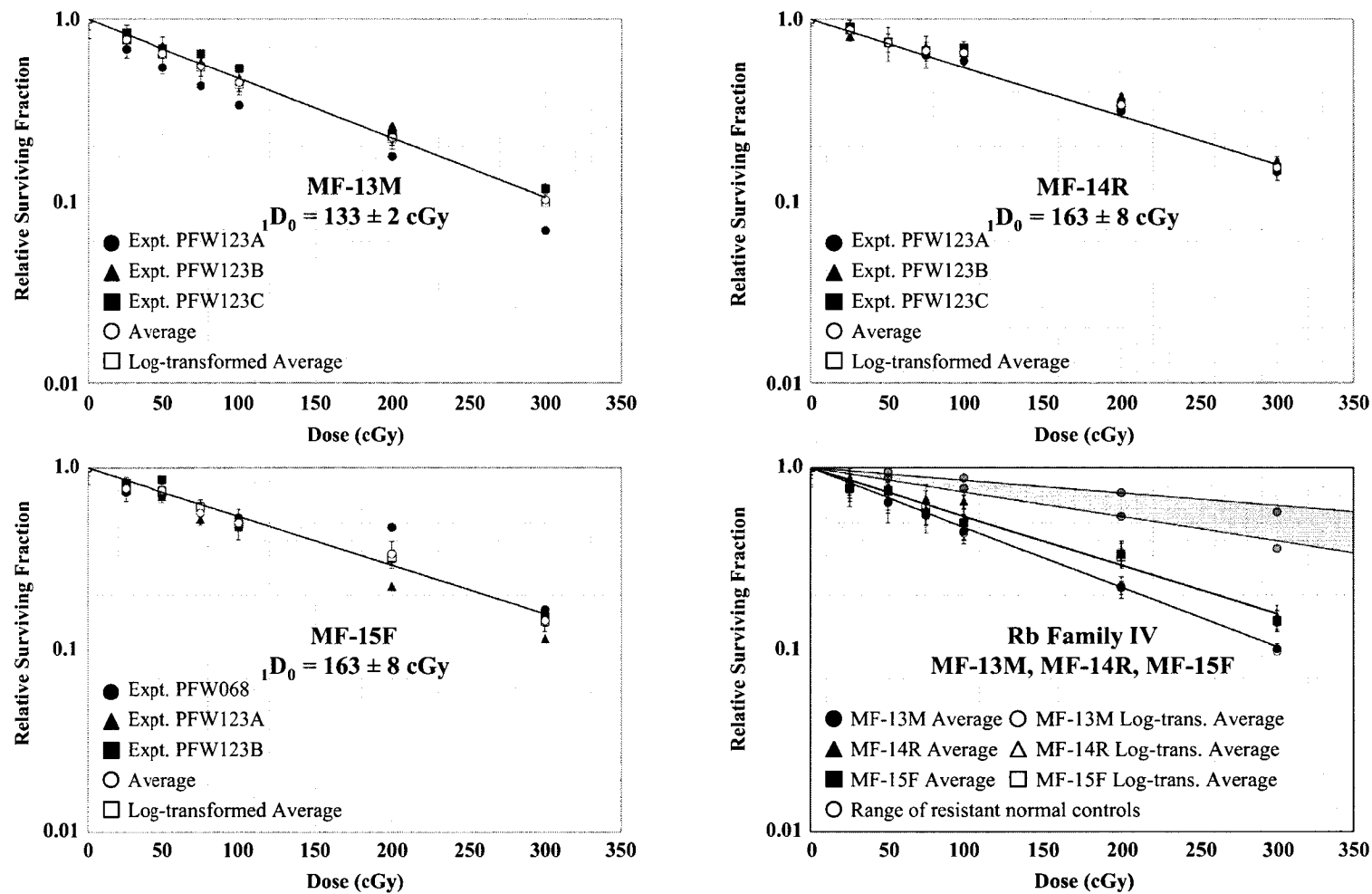


Figure 3.4. Acute dose plus 22–26 hours PLDR survival experiment plots, exponential-fit ($S = e^{-aD}$) regression curves, and calculated $1D_0$ ($= 1/a$) $\pm$ SEM values for fibroblast strains obtained from members of Rb Family IV: MF-13M ($Rb1^{+/+}$), MF-14R ($Rb1^{+/+}$), and MF-15F ($Rb1^{+/+}$).

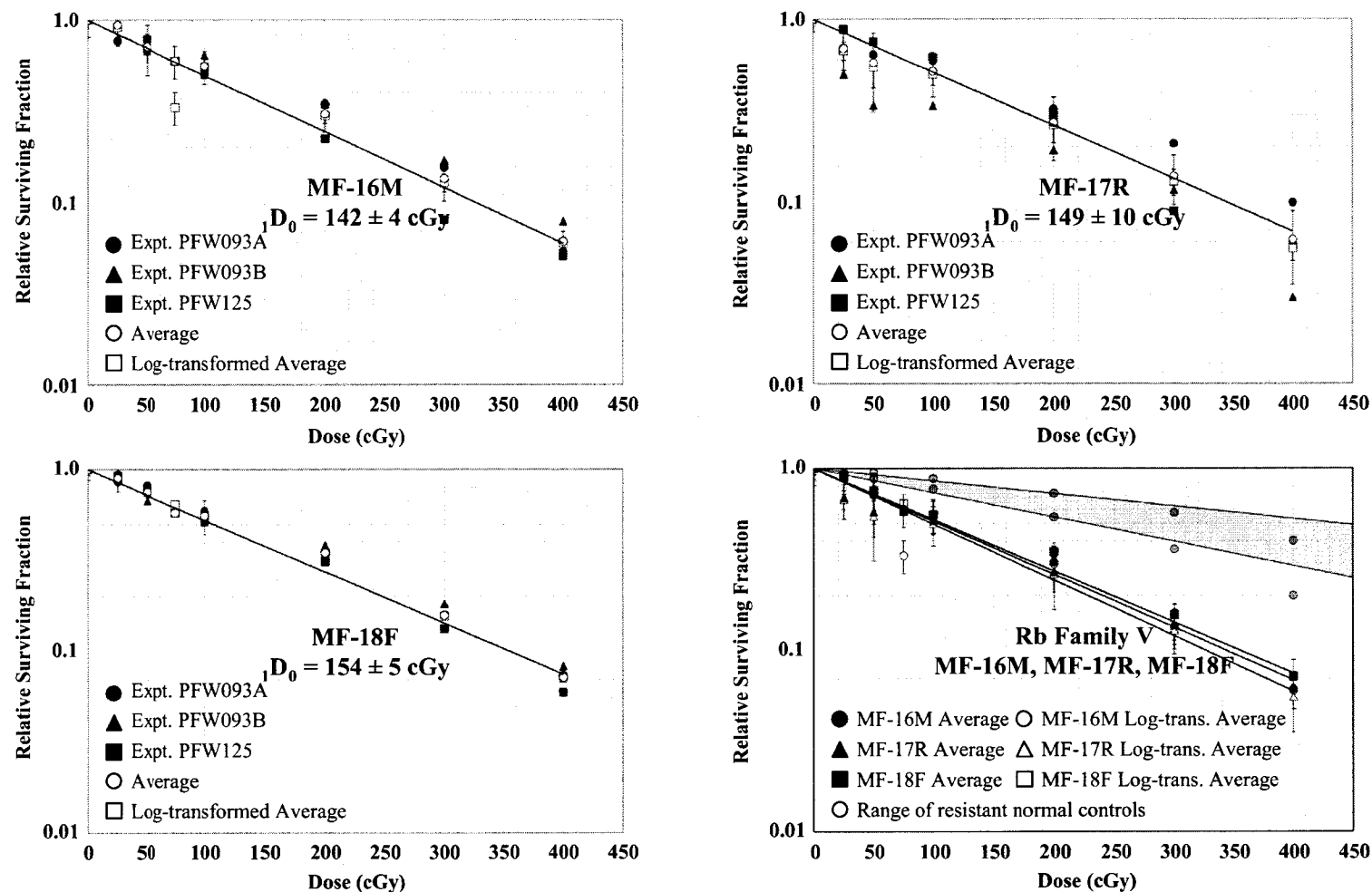


Figure 3.5. Acute dose plus 22–26 hours PLDR survival experiment plots, exponential-fit ($S = e^{-aD}$) regression curves, and calculated $D_0 (= 1/a)$ $\pm$ SEM values for fibroblast strains obtained from members of Rb Family V: MF-16M ($Rb1^{+/+}$), MF-17R ($Rb1^{+/-}$), and MF-18F ($Rb1^{+/+}$).

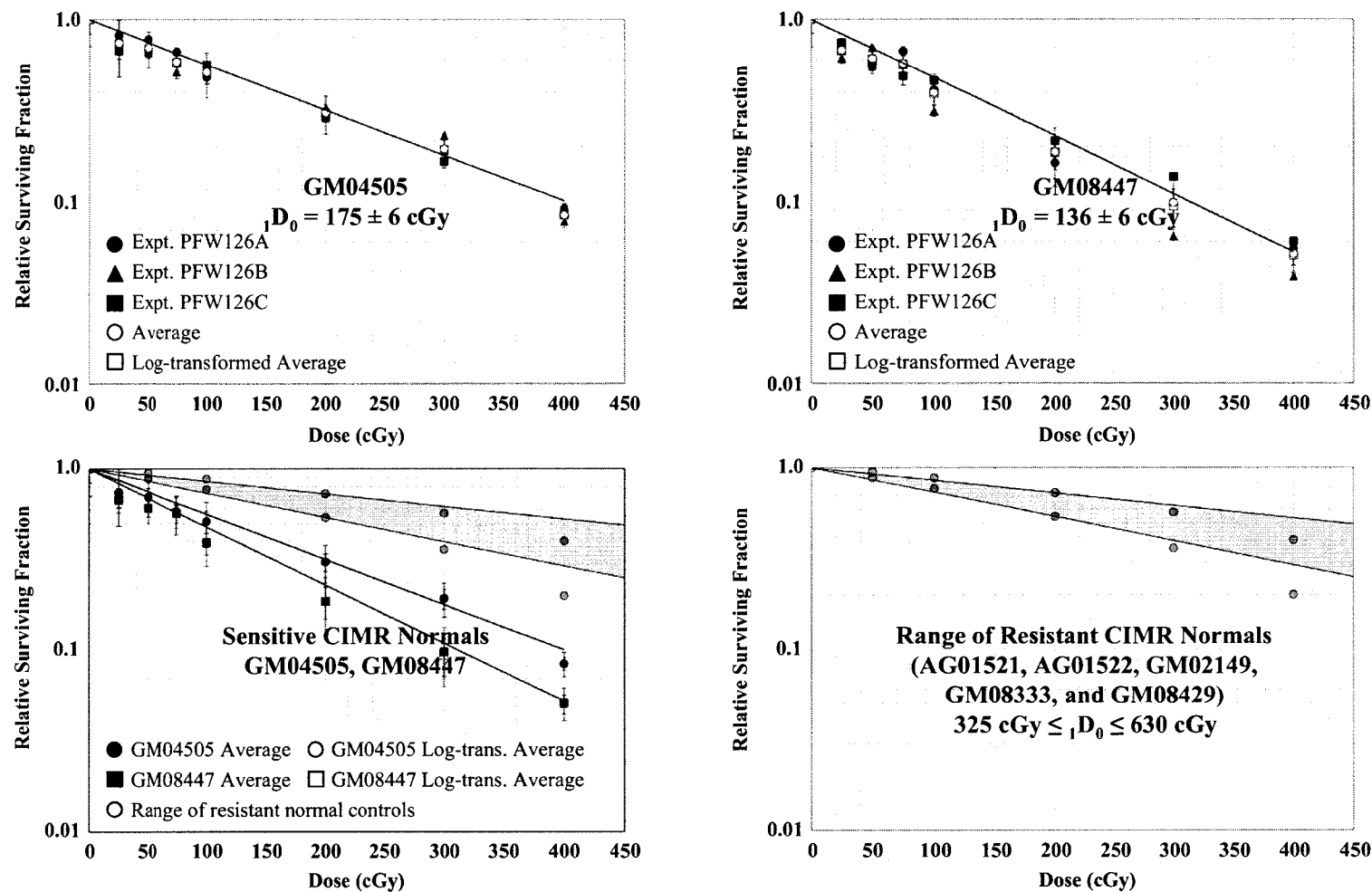


Figure 3.6. Acute dose plus 22–26 hours PLDR survival experiment plots, exponential-fit ($S = e^{-aD}$) regression curves, and calculated $1D_0$ ($= 1/a$) $\pm$ SEM values for fibroblast strains obtained from the CIMR: GM04505, GM08447, and the Bedford laboratory range of resistant strains.

fibroblast strains are also ranked from least (1) to most (14) radiosensitive in this assay in Table 3.1. Results for three to four independent survival experiments per fibroblast strain are plotted in the first panels of Figures 3.1–3.6. In the final panel, the average survival ($\pm$ SEM) for the members of that respective family or group is compared to the range of response for this laboratory's group of validated apparently normal CIMR control fibroblast strains (AG01521, AG01522, GM02149, GM08333, GM08429), indicated by the grey-shaded area. The data for these “radioresistant” apparently normal CIMR controls has been collected from a number of investigators (including myself) in this laboratory over the last twenty years (Cornforth, 1987; Hwang, 1990; Mulhmann-Diaz, 1993; Dugan, 2002; and this volume). The 14 Rb family member and two radiosensitive apparently normal CIMR fibroblast strains are all significantly ($P < 0.05$) more radiosensitive than the group of five radioresistant apparently normal CIMR fibroblast strains.

The average ratio of the survival D_0 values for the apparently normal CIMR fibroblast strains in the Fitzek report (D_0 of ~ 125 cGy, omitting the moderately radiosensitive strains AG05565 and AG05658 with D_0 values of ~ 90 – 95 cGy) compared to the 14 Rb family member fibroblast strains (52 – 98 cGy, listed in Table 1.1) was ~ 1.8 (range of 1.3 – 2.4). The average ratio of the survival D_0 values for the same 14 Rb family member fibroblast strains (133 – 244 cGy, listed in Table 3.1) compared to the lower range of apparently normal CIMR fibroblast strains (~ 325 cGy) after 24 hours of PLD repair in this laboratory is ~ 2.0 (range of 1.3 – 2.4), similar to that of the Fitzek report. However, when compared to the upper range (~ 630 cGy, the more typical response) of this laboratory's range of apparently normal CIMR fibroblast responses after

24 hours of PLD repair, this ratio of ${}_1D_0$ values rises to ~3.9 (range of 2.6–4.7). Thus the post-irradiation confluent-holding PLD repair protocol was successful in generating a greater degree of differential survival between the radiosensitive Rb family member fibroblast strains and the apparently normal CIMR fibroblast strains than could be achieved with asynchronous cultures irradiated ~18 hours after plating at low density in the Fitzek report.

Histograms of the acute irradiation survival curve ${}_1D_0$ (the reciprocal of the initial slope of the survival curve, a) and relative surviving fraction at 2 Gy (SF_2) values for the 14 Rb family member, two radiosensitive apparently normal CIMR (GM04505A and GM08447), and five radioresistant apparently normal CIMR control (AG01521, AG01522, GM02149, GM08333, and GM08429) fibroblast strains are shown in Figures 3.7 and 3.8 (the “MF,” “AG,” and “GM” prefixes have been omitted). Both distributions are bimodal, with all or most (in the case of MF-2M in Figure 3.8) of the Rb family member and radiosensitive CIMR fibroblast strains noticeably separated from the radioresistant CIMR fibroblast strains.

The ratios of D_0 values reported by Fitzek, *et al.* (2002; listed in Table 1.1) and ${}_1D_0$ values reported in Table 3.1 for the 14 Rb family member fibroblast strains are plotted in Figure 3.9. Differences in the acute irradiation survival assay cell culture protocols (asynchronous cultures versus confluent cultures allowed 24 hours of PLD repair) resulted in an ~2.4-fold average increase (range of 1.7–3.0) in the dose required to reduce relative survival to 37%. Unexpectedly, given the very strong (*i.e.*, highly significant) correlations between these radiosensitivity parameters on an individual strain basis noted in large surveys of fibroblast strains irradiated asynchronously, in confluent

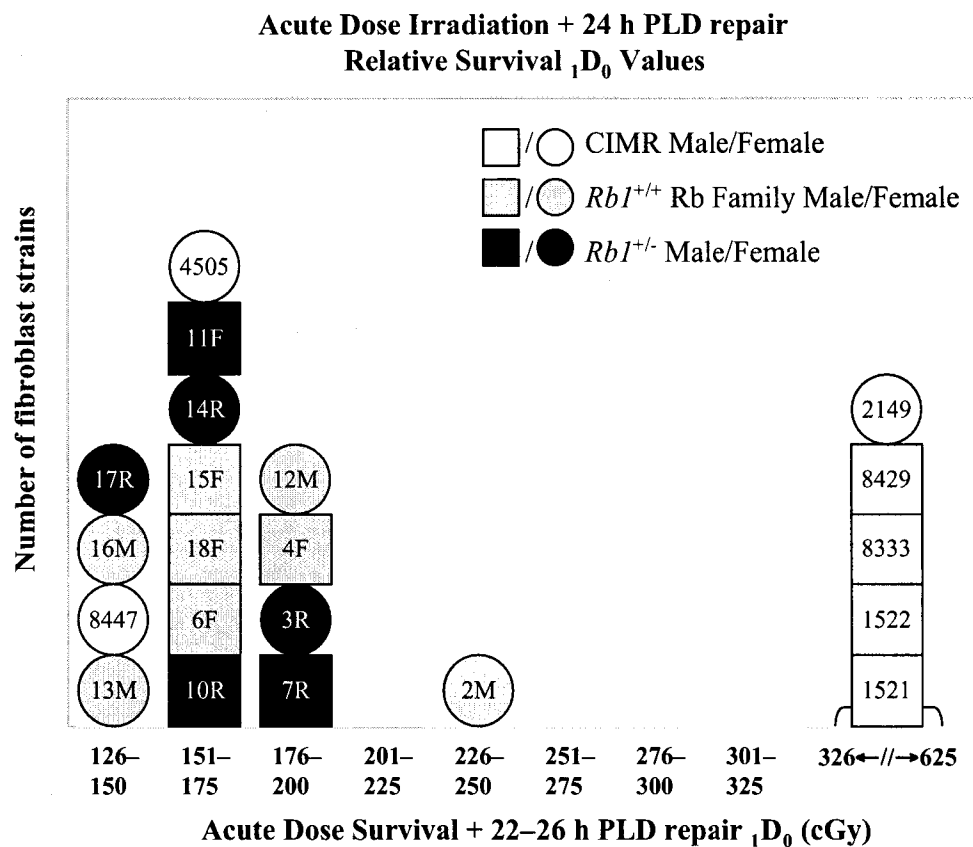


Figure 3.7. Distribution of acute dose irradiation + 22-26 hours of PLDR survival $_1D_0$ values for the 14 Rb family member and 7 apparently normal CIMR control fibroblast strains (see Table 3.1 and Figures 3.1-3.6).

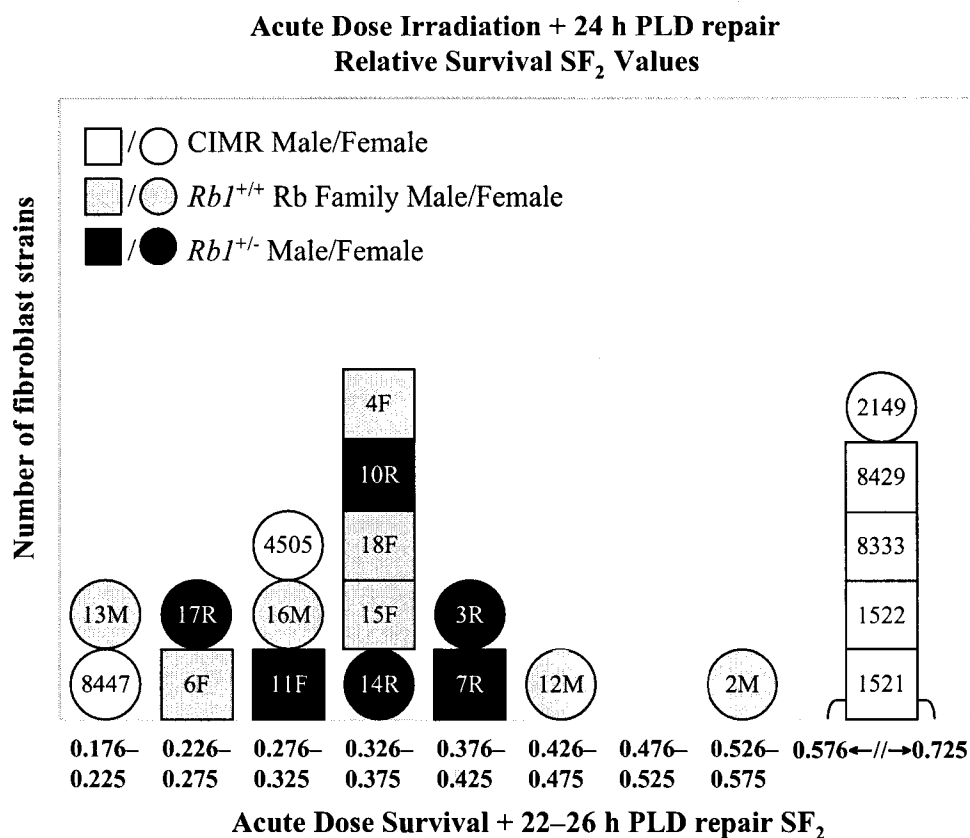


Figure 3.8. Distribution of acute dose irradiation + 22-26 hours of PLDR surviving fraction at 2 Gy (SF_2) values for the 14 Rb family member and 7 apparently normal CIMR control fibroblast strains (see Table 3.1 and Figures 3.1-3.6).

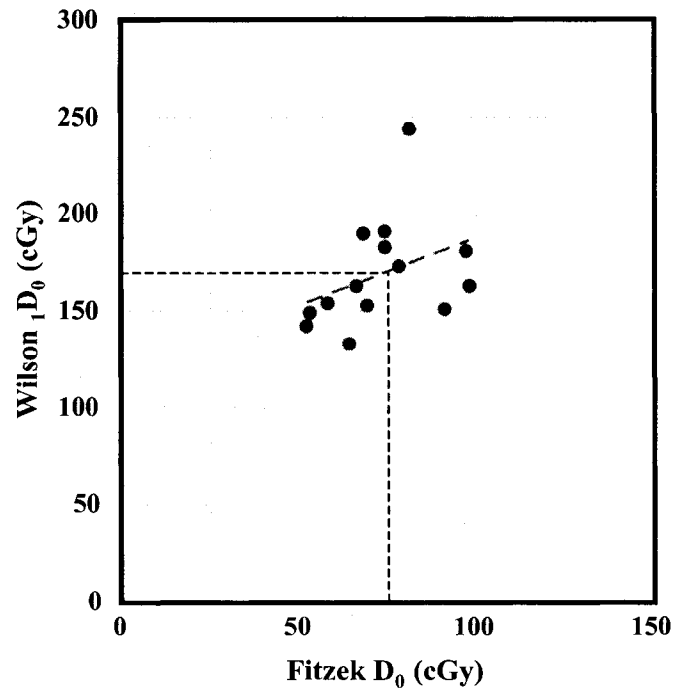


Figure 3.9. Plot of Fitzek, *et al.* (2002) acute dose irradiation survival D_0 values compared to Wilson acute dose irradiation + 22–26 hours of PLDR survival ${}_1D_0$ values for the 14 Rb family member fibroblast strains (see Tables 1.1 and 3.1). Differences in survival assay cell culture protocols (asynchronous cultures versus confluent cultures allowed time for PLDR, respectively) resulted in an ~2.4-fold (range of 1.7–3.0) average increase in ${}_1D_0$ values compared to D_0 values (indicated by dashed lines). The two acute irradiation survival parameters are not significantly correlated ($P = 0.195$).

cultures immediately plated for colony formation, and in confluent cultures delayed in plating 24 hours for PLD repair (Fertil, *et al.*, 1988; Malaise, *et al.*, 1989), the two acute irradiation survival parameters in this study are not significantly correlated ($P = 0.195$).

The average ratio of Wilson ${}_1D_0$ and Fitzek D_0 values for the 6 affected $RbI^{+/-}$ family member fibroblast strains (all affected proband children and the affected father, MF-11F) was 2.1 (range of 1.7–2.8). The average ratio of ${}_1D_0$ and D_0 values for the 8 unaffected, apparently normal $RbI^{+/+}$ family member fibroblast strains was 2.6 (range of 2.1–3.0). Although this difference was not quite significant ($P = 0.074$), it lends support to the notion that Rb patient fibroblasts *may* be somewhat deficient in the DNA repair and cell cycle control mechanisms underlying the PLD repair phenomenon (Arlett and Priestly, 1983; Weichselbaum, *et al.*, 1985).

The relationship between the two acute irradiation survival parameters, D_0 and ${}_1D_0$, and the zero-dose plating efficiency (PE) values for the 14 Rb family member fibroblast strains recorded in Fitzek, *et al.* (2002; see Table 1.1) and Table 3.1, respectively, is plotted in Figure 3.10. The two radiation survival parameters and the associated PE values for the individual fibroblast strains are not significantly correlated with each other ($P = 0.075$ for the Fitzek data set and $P = 0.842$ for the Wilson data set). Cross-comparisons of the PE data sets available for the 14 Rb family member fibroblast strains (from the Fitzek report, this chapter, and from the continuous LDR irradiation colony formation assays performed with Dr. Hatsumi Nagasawa in Chapter 5; see Tables 1.1, 3.1, and 5.2) are plotted in Figure 3.11. The Wilson and Nagasawa PE values are significantly correlated ($P = 0.0095$), showing a ~1:1 correspondence. The Wilson and

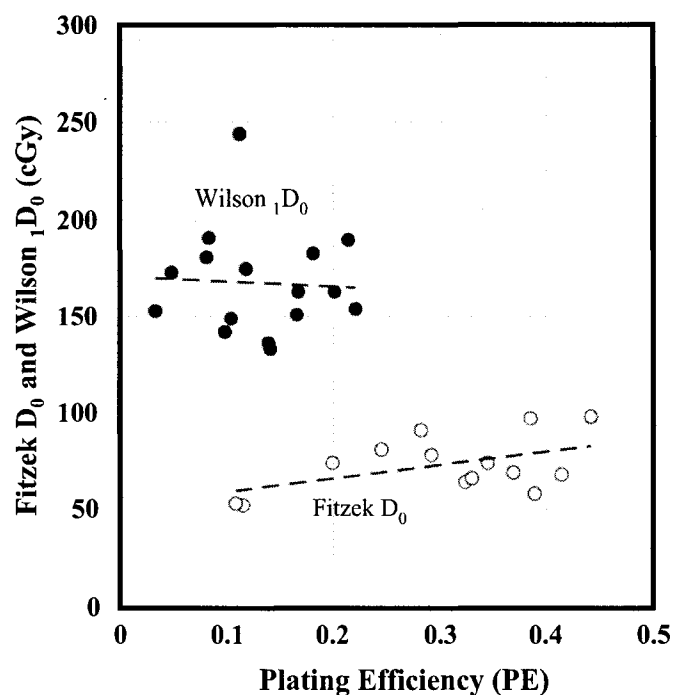


Figure 3.10. Plot of Fitzek, *et al.* (2002) acute dose irradiation survival D_0 values (open circles) and Wilson acute dose irradiation + 22–26 hours PLDR survival ${}_1D_0$ values (filled circles) versus reported and measured plating efficiency (PE) values, respectively, for the 14 Rb family member fibroblast strains (see Tables 1.1 and 3.1). The two acute irradiation survival parameters and associated PE values are not significantly correlated ($P = 0.075$ and 0.842 , respectively).

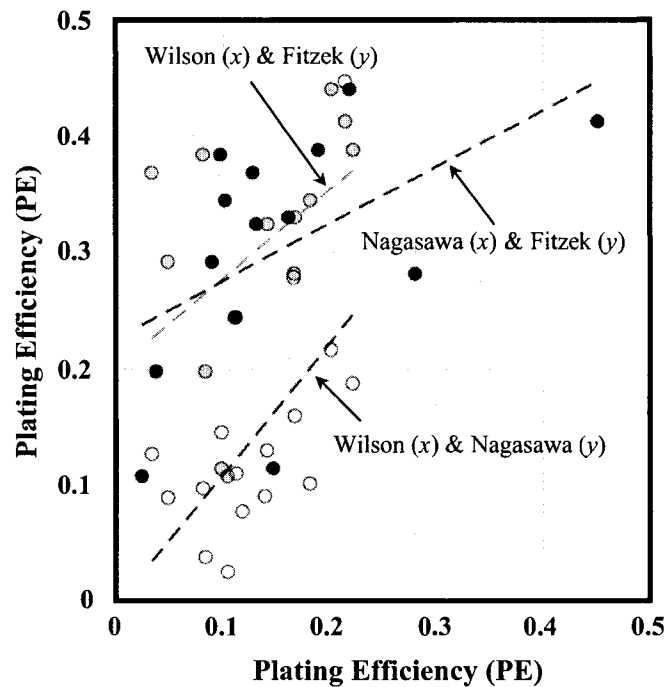


Figure 3.11. Plot of cross-comparisons of Fitzek, *et al.* (2002), Wilson (this chapter), and Wilson/Nagasawa (Chapter 5) plating efficiency (PE) values for the 14 Rb family member fibroblast strains (see Tables 1.1, 3.1, and 5.1). Open circles represent Wilson (*x*-axis) versus Nagasawa (*y*-axis) PE value comparisons; grey-filled circles represent Wilson (*x*-axis) versus Fitzek (*y*-axis) PE value comparisons; and black-filled circles represent Nagasawa (*x*-axis) versus Fitzek (*y*-axis) PE value comparisons. The Wilson and Nagasawa PE values are significantly correlated ($P = 0.0095$), however neither set is significantly correlated with the Fitzek PE values ($P = 0.107$ and 0.061 , respectively).

Fitzek and the Nagasawa and Fitzek PE values are not quite significantly correlated ($P = 0.107$ and 0.061 , respectively).

3.5. Discussion

The current PLD repair protocol allowed an approximate two-fold better discrimination of the degree of radiosensitivity in the Rb family member fibroblast strains compared to the reference apparently normal CIMR control strains than the asynchronous protocol employed by Fitzek, *et al.* (2002), supporting our initial hypothesis of the utility of this protocol (see Figures 3.1–3.8 and Table 3.1). As expected, radiation survival increased following 24-hour confluent holding for PLD repair, with a repair recovery ratio (ratio of the D_0 and ${}_1D_0$ values) of ~ 2.4 (Figure 3.9). Although the reported radiosensitivity parameters (D_0 and ${}_1D_0$) were obtained by different protocols (asynchronous phase irradiation versus plateau phase irradiation with 24 hour PLD repair), Fertil, *et al.* (1988) and Malaise, *et al.* (1989) concluded that survival responses measured by both techniques are highly significantly correlated on an individual strain basis, reflective of the inherent repair capacity of the cells examined. It was surprising to learn that this was not the case in this study ($P = 0.195$, see Figure 3.9).

The PLD repair recovery ratio for the 14 Rb family member fibroblast strains ranged from 1.7–3.0, well within the published range of PLD recovery (≥ 2 –5) in the literature for human apparently normal fibroblast strains (Arlett and Priestly, 1983; Weichselbaum, *et al.*, 1985; Cornforth and Bedford, 1987; Fertil, *et al.*, 1988; Hall, *et al.*, 1988; Nagasawa and Little, 1988; Malaise, *et al.*, 1989; Nagasawa, *et al.*, 1992). Interestingly, the PLD repair recovery ratios for fibroblast strains derived from the

affected $RbI^{+/-}$ individuals (average of 2.1, range of 1.7–2.8) was approximately 20% lower than this ratio for the unaffected $RbI^{+/+}$ individuals (average of 2.6, range of 2.1–3.0), although this difference was not quite significant ($P = 0.074$). This lends support to the hypothesis that bilateral Rb patient cells may be somewhat deficient in PLD repair mechanisms (Arlett and Priestly, 1983; Weichselbaum, *et al.*, 1985).

Fertil and Deschavanne (1999) published a report examining the relationship of plating efficiency (PE) to the measured radiosensitivity of 446 human cell lines. They have concluded that, despite a wide range of cell types and experimental protocols employed and a number of reports dismissing such a relationship (including Little, *et al.*, 1988), there is significant evidence that PE is correlated with individual cellular radiosensitivity parameters (α , SF_2 , *etc.*). This is not well supported by Figure 3.10 for the 14 Rb family fibroblast strains, as neither data set demonstrated any significant correlation of PE and D_0 or $1D_0$ values. The Fitzek data set correlation was nearly significant ($P = 0.075$), much more so than the current PLD repair data set ($P = 0.842$), suggesting PE may be a much more critical parameter for asynchronous cultures attempting to cycle and divide immediately after irradiation than for confluent cultures allowed a “resting” period in which to repair PLD damage before being subcultured for colony formation ability. This concept is further discussed in Chapter 5 in discussions of effects of plating efficiency during continuous LDR irradiation exposures.

The range of PE values is broader in the Fitzek data set and noticeably higher than the PE values reported in Table 3.1. Cross-comparisons of the PE values for each Rb family fibroblast strain measured in the Fitzek report, by myself, or by Dr. Hatsumi Nagasawa (in conjunction with the LDR studies presented in Chapter 7) were plotted in

Figure 3.12. There was a significant ($P = 0.0095$), almost 1:1 concordance between the Wilson and Nagasawa PE values, while both sets of PE values were ~2-fold lower than and not quite significantly correlated ($P = 0.107$ and $P = 0.061$, respectively) with the Fitzek PE values. In discussions with Dr. Nagasawa, this has been attributed to inter-laboratory and researcher-specific differences in cell handling and culture protocols.

Despite this, the acute irradiation survival responses of the individual fibroblast strains were consistent; generally, the same trends of radiosensitivity were observed for both experimental protocols employed (discussed further in Chapter 7, see Table 7.1). Furthermore, measurements of radiation survival following PLD repair in these strains seem to indicate that they are somewhat deficient in molecular mechanisms underlying this repair response, allowing for better differentiation of their radiosensitive nature when compared to the normal control strains. Unfortunately, the degree of differential survival between the radiosensitive Rb family member fibroblast strains and the apparently normal CIMR control strains is still insufficient for radiation survival-based genetic complementation and correction strategies (see Chapter 7). The high frequency of false-positive clones following transfection of a potential correcting cDNA sequence and screening by acute irradiation survival would limit the usefulness of such a strategy. The results presented in this chapter provide an additional reference data set of acute irradiation survival, along with Fitzek, *et al.* (2002), with which to test the radiation response of a potentially corrected Rb family member fibroblast strain generated from more promising genetic complementation/correction strategies employing continuous LDR irradiation colony formation (see Chapters 5 and 7).

CHAPTER 4

Effects of G2-phase Irradiation on Rb Family Member and Apparently Normal and *RbI*^{+/-} CIMR Fibroblast Strains

4.1. Abstract

G2-phase chromosomal radiosensitivity assay data for asynchronous cultures of fibroblast strains derived from the 14 Rb family members, two radiosensitive apparently normal CIMR controls (GM04505A and GM08447), two radioresistant apparently normal CIMR controls (AG01521 and GM02149), and a radioresistant *RbI*^{+/-} CIMR control (GM06419) are presented. The 14 Rb family member and two radiosensitive CIMR control fibroblast strains had significantly higher (~1.5-fold, $P < 0.05$) chromatid-type aberration frequencies (CAF) in the first post-irradiation mitosis after 50 and 100 cGy doses of G2-phase gamma irradiation, compared to the three other radioresistant CIMR control strains. Significantly high spontaneous levels of tetraploidy and endoreduplication were observed in some of the Rb family member fibroblast strains but were not observed in the apparently normal CIMR fibroblast strains, and were not correlated with the frequencies of radiation-induced chromosome aberrations. Results from these experiments confirm, by a different radiation-induced damage endpoint, the moderate radiosensitivity classifications of the Rb family member fibroblast strains made in Fitzek, *et al.* (2002) and provide a rapid assay to determine the success of a genetic complementation/correction strategy performed with these cells.

4.2. Introduction

Measurements of G2-phase chromosomal radiosensitivity in retinoblastoma patients and family members were reported in Chaum, *et al.* (1984) and two reports by Sanford, *et al.* (1989, 1996) using bleomycin and radiation, respectively, as the genotoxic agent. A retinoblastoma tumor cell line examined in Darroudi, *et al.* (1995) did not demonstrate any increased frequency of G2 aberrations after X-irradiation compared to normal, untransformed control fibroblasts. Likewise, the spontaneous and bleomycin-induced chromatid-type aberration frequencies in Chaum, *et al.* (1984) did not differ significantly from those of normal control lymphocytes.

Sanford, *et al.* (1987) reported a mean chromatid aberration frequency (CAF) of 3.22 per cell (range 1.00–5.06) for 8 bilateral (including one familial unilateral) Rb fibroblast strains, a sporadic unilateral Rb fibroblast strain with a CAF of 4.10, and a mean CAF of 0.39 per cell (range 0.16–2.04) for 29 normal control fibroblast strains after a dose of 53 cGy. The “gaps” and “breaks” recorded in Sanford’s study are equivalent to the Savage (1973) and Bender (1974) “break” definition used in this current study. In a 1996 report, Sanford, *et al.* reported a mean CAF of 2.20 per cell (range ~1.30–3.20) for 13 additional bilateral Rb fibroblast strains, a mean CAF of 0.74 (range 0.40–2.10) for 6 additional unilateral Rb fibroblast strains, and a mean CAF of 0.39 per cell (range 0.30–0.46) for 8 additional normal control fibroblast strains after a dose of 53 cGy. Of the 6 unilateral Rb fibroblast strains examined, four had mean CAF values similar to the normal control strains (~0.40), while the other two had high CAF values (~1.30 and 2.10), reminiscent of the bilateral Rb strains. Interestingly, certain unaffected first-degree relatives displayed similarly high CAF values (≥ 1.10 per cell) as the bilateral Rb cases.

The range of normal response, CAF = 0.30–0.46, in this second Sanford report is admittedly quite narrow and questionably low. Likewise, if two outliers in the first Sanford report (F664, CAF = 2.04 and 3216T, CAF = 1.18) were omitted, the normal CAF range is only 0.16–0.42. This seems at odds with the Little and Deschavanne surveys of radiosensitivity discussed in Chapter 1, where ~30% of randomly chosen normal fibroblast strains could be considered moderately radiosensitive. One would expect 10 or so strains (out of 37 total) in the combined Sanford normal fibroblast strain cohort to have CAF values similar to the lower end of the bilateral Rb responses observed, but only two are observed.

Revaluation of the *same* normal fibroblast strains by Scott, *et al.* (1996) demonstrated higher CAF frequencies (average of ~2.2, range 1.1–3.3), different kinetics of repair, and more inter-experimental variability; and now 12 of the 53 normal strains (23%) appeared to be moderately radiosensitive. Similarly, in the second Sanford report, an apparently normal CIMR fibroblast strain, GM00969D, was compared to a bilateral Rb strain, GM05887, in terms of mitotic progression after G2-phase irradiation (mitotic index measurements) and kinetics of repair of chromatid aberrations; no significant differences were reported. GM00969D was *consistently* moderately radiosensitive in the low dose-rate studies reported in Chapter 5 and Weeks, *et al.* (1991). This emphasizes the need for cross-validation of reference normal control strains obtained from the national cell banks. Differences between the bilateral Rb fibroblast strain GM05887 and a better choice of a reference normal fibroblast strain may have demonstrated significant differences for these endpoints.

In the present study, the G2 chromosomal radiosensitivity of fibroblasts derived from the 14 Rb family members (both apparently normal *Rb1*^{+/+} and affected *Rb1*^{+/-} individuals), two radiosensitive apparently normal CIMR controls (GM04505A and GM08447), and three radioresistant CIMR controls (apparently normal AG01521 and GM02149, and *Rb1*^{+/-} GM06419) was measured for 0, 50, and 100 cGy doses of acute, HDR ¹³⁷Cs gamma irradiation. The frequencies of tetraploid and endoreduplicated metaphase spreads in the total cell population were also measured.

4.3. Materials and Methods

4.3.1. Cell Culture and Irradiations

The G2 chromosomal radiosensitivity assay procedure employed in these series of experiments have been modified from that of the Sanford NCI/Scott PCR protocol (Scott, *et al.*, 2002). All fibroblasts strains were grown in α MEM cell culture medium (GIBCO/Invitrogen) supplemented with 15% FBS, 100 μ g/ml streptomycin sulfate, 100 U/ml penicillin G (all from Sigma), and 1X GlutaMAX™-I (L-alanyl-L-glutamine, GIBCO/Invitrogen) in a 37° C incubator supplied with 95% air/5% CO₂ mixture. Cultures were routinely passaged at 1:4 dilutions prior to density-inhibited contact inhibition. A single lot of FBS (Sigma lot no. 32HK8401) was used in all the experiments to reduce experimental variability.

Low-passage ($\leq$ P20) fibroblasts cultures of the fibroblast strains identified in Table 4.1 were initiated from liquid nitrogen-frozen stocks by rapid thawing of the frozen ampule in a 37°C water bath. One to two ml of the thawed fibroblast suspensions in 10% DMSO/90% cell culture cryomedium were supplemented with 5 ml of fresh cell culture

medium and centrifuged at 1000 rpm for five minutes. The supernatant was then aspirated and the cell pellet broken up and resuspended in one ml of fresh cell culture medium. These suspensions were then aliquoted into T-25 tissue culture flasks (Greiner Bio-One) filled with 5 ml of pre-equilibrated (by placement of the medium-filled flask in the 37°C incubator for a minimum of one hour). The cell culture medium was replaced the next day with 5 ml of fresh cell culture medium. Cultures were passaged until sufficient cell numbers were available for experimental analysis and identically numbered experiments (*e.g.*, PFW126A, -126B, -126C, *etc.*) were completed contemporaneously with cultures recovered from different frozen ampules.

Six to nine T-75 tissue culture flasks (Greiner Bio-One) were prepared for the G2 assay by replacing the cell culture medium 24 hours prior to irradiation in cultures that had maintained asynchronous log-phase growth for a minimum of five days. On the day of irradiation, the six to nine flasks were randomly segregated into 0, 50, or 100 cGy dose groups, tightly capped and transported to the MRB470 irradiation facility in a large Tupperware[®] container maintained at 37°C by large T-225 culture flasks filled with 37°C water placed inside. The T-75 cell culture flasks were irradiated at 37°C (by placing a 37°C water-filled flask on the irradiation sample holder) with 0 (sham), 50 or 100 cGy of ¹³⁷Cs gamma rays at a dose rate of 250 cGy/min, supplied by a J. L. Shepard and Associates Mark I-68A beam irradiator with an approximate activity of 3170 Ci (as of September 2004). The flasks were transported back in the 37°C Tupperware container and returned to the incubator with the caps loosened. Following 30 minutes, 0.1 µg/ml colcemid (GIBCO/Invitrogen) was added to the cultures and the flasks incubated for another 60 minutes.

4.3.2. Cytogenetic Slide Preparations

Ninety minutes post-irradiation, the cell cultures were processed by standard cytogenetic techniques. The cell culture medium was removed into 15 ml conical tubes (Greiner Bio-One) to ensure collection of any detached and rounded mitotic cells and the cells were detached with 0.25% trypsin/EDTA (GIBCO/Invitrogen) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (Dulbecco's PBS-A formulation) for three minutes at 37°C. The detached single cell suspensions were then transferred into the 15 ml conical tubes with the cell culture medium and centrifuged for 4 minutes at 1000 rpm at room temperature. After pouring off the supernatant, the cell pellet was gently broken up and resuspended in 10 ml of 37°C 75mM KCl hypotonic buffer. The tubes were then incubated for 7.5 minutes in a 37°C water bath, after which 2 ml of freshly prepared 3:1 methanol:acetic acid (Carnoy's fixative) was added and the tubes gently inverted to mix the solutions. The tubes were incubated for 2.5 minutes at room temperature and then centrifuged for four minutes at 1000 rpm. The supernatant was poured off into a hazardous waste container and the cell pellet was very gently broken. The cell suspensions were then fixed by the dropwise addition of fresh fixative to a volume of 3–4 ml and mixed by air-bubbling the suspensions with the pipette. The cell suspensions were then placed into the refrigerator or cold room.

Days or weeks later, the cell suspensions in fixative (now containing methyl acetate) were allowed to return to room temperature prior to being centrifuged at 1000 rpm for five minutes. The supernatant was poured off into a hazardous waste container, and the cell pellet was gently broken up, fixed by the dropwise addition of freshly prepared Carnoy's fixative to a volume of 3–4 ml and mixed by air-bubbling the

suspensions with the pipette. After five minutes, the suspension was again centrifuged and the fixation process repeated. Following the final centrifugation, the suspensions were resuspended in 0.5–1.5 ml of fresh fixative depending upon the volume of the cell pellet and 60 μ l of cell suspension was dropped onto clean, cold and wet microscope slides (VWR). After air-drying, the slides were placed in a desiccation chamber in the 37°C warm room overnight. The following day, the slides were stained in a 10% Giemsa solution (Gurr[®] R66, BDH Chemicals, Ltd.) prepared with a pH 6.8 buffered solution (Sorenson's) for 15 minutes at room temperature, washed vigorously in McIlvaine's rinsing buffer (approximately 18 mM citric acid/16 mM disodium phosphate prepared in distilled water) and distilled water, and dried with an air jet. Following an additional overnight stay in the 37°C desiccation chamber, the slides were sealed at room temperature with Cytoseal[™] 60 mounting medium (Microm International) and a 24-mm x 60-mm No. 1 glass coverslip (VWR).

4.3.3. Chromosome Aberration Scoring

Approximately fifty to eighty-five metaphase spreads per experiment were then scored under 2000X total magnification (100X objective with 10X oculars and a 2X optivar lens) per dose point for three independent experiments. Only metaphase spreads with good chromosome morphology and minimal overlap and cytoplasmic interference were scored; the modal chromosome number for the cell strains in these experiments was 46 chromosomes and ranged from 45–47. The frequencies of tetraploid (8N DNA content with unpaired chromosome homologs, see Figure 4.1 bottom left panel) and endoreduplicated (8N DNA content with paired chromosome homologs, see Figure 4.1

bottom right panel) metaphase spreads were recorded but not scored for aberrations. The frequencies of both aneuploid forms were calculated as $n_{TET} \div (n + n_{TET})$ and $n_{ENDO} \div (n + n_{ENDO})$, respectively, where n is the number of diploid cells scored for chromosome aberrations.

As discussed previously, the Savage (1973) and Bender (1974) chromosome aberration classification scheme was used. Chromatid gaps were defined as fully discontinuous chromatid fragments that were detached at a distance *less* than the width of the chromatid arm. Chromatid breaks were defined as fully discontinuous chromatid fragments either displaced at a distance *greater* than the width of the chromatid from the original chromosome or were no longer aligned with the original chromatid axis. Chromatid fragments that did not appear to be severed from the chromatid were considered achromatic lesions and not scored. Chromatid-type exchanges of both symmetrical (not generating acentric fragments) and asymmetrical forms (generating acentric fragments) were also observed at a low frequency. Examples of these aberration types are shown in Figure 4.1.

4.3.4. Data and Statistical Analyses

For illustrative purposes, Poisson distributions with means equal to the total and background-corrected (0 cGy) chromatid break and combined chromatid gap and break frequencies were plotted with the experimental data histograms in Figures 4.7–4.13. Goodness-of-fit testing between the calculated Poisson distributions and the experimental data sets was accomplished using the χ^2 -test. Standard errors of the mean (SEM) for 2–3 independent experiments were calculated by combining the standard deviations of the

aberration frequencies by the square root of the sum-of-the-squares method (Bevington and Robinson, 1992) and dividing this value by the square root of the number of experiments, and constitute the error bars for Tables 4.1–4.6 and Figures 4.2–4.6.

Statistical differences of the various G2 assay aberration frequencies (chromatid breaks, combined chromatid gaps and breaks, exchanges, tetraploidy, and endoreduplication) between the individual Rb family member and the sensitive normal CIMR (GM04505A and GM08447) fibroblast strains, and the *average* resistant normal CIMR fibroblast strain response (averaged for AG01521, GM02149 and GM06419) were tested using Student *t*-tests for two independent sample distributions and Wilcoxon Rank-Sum tests (Mann-Whitney *U*-tests) (SigmaPlot). The Student *t*-test assumes the data sets are normally distributed while the Wilcoxon Rank-Sum test does not require the data sets to be normally distributed (although it must be ranked). As there was little difference between the results of these two statistical methods, the results of the Student *t*-tests are reported in this chapter. Correlations between the different radiosensitivity parameters (PE, D_0 , ${}_1D_0$, G2 assay aberration frequencies) were assessed using the Pearson product-moment correlation coefficient fit by the least-squares method to the respective data sets being compared (SigmaPlot).

4.4. Results

Examples of chromatid-type aberrations observed in the human fibroblast metaphase spreads and recorded in the G2 chromosomal radiosensitivity assay for the 19 strains examined in this chapter are shown in Figure 4.1. Chromatid gaps, breaks, and iso-gaps/breaks (affecting both chromatid arms) were the most common aberration types

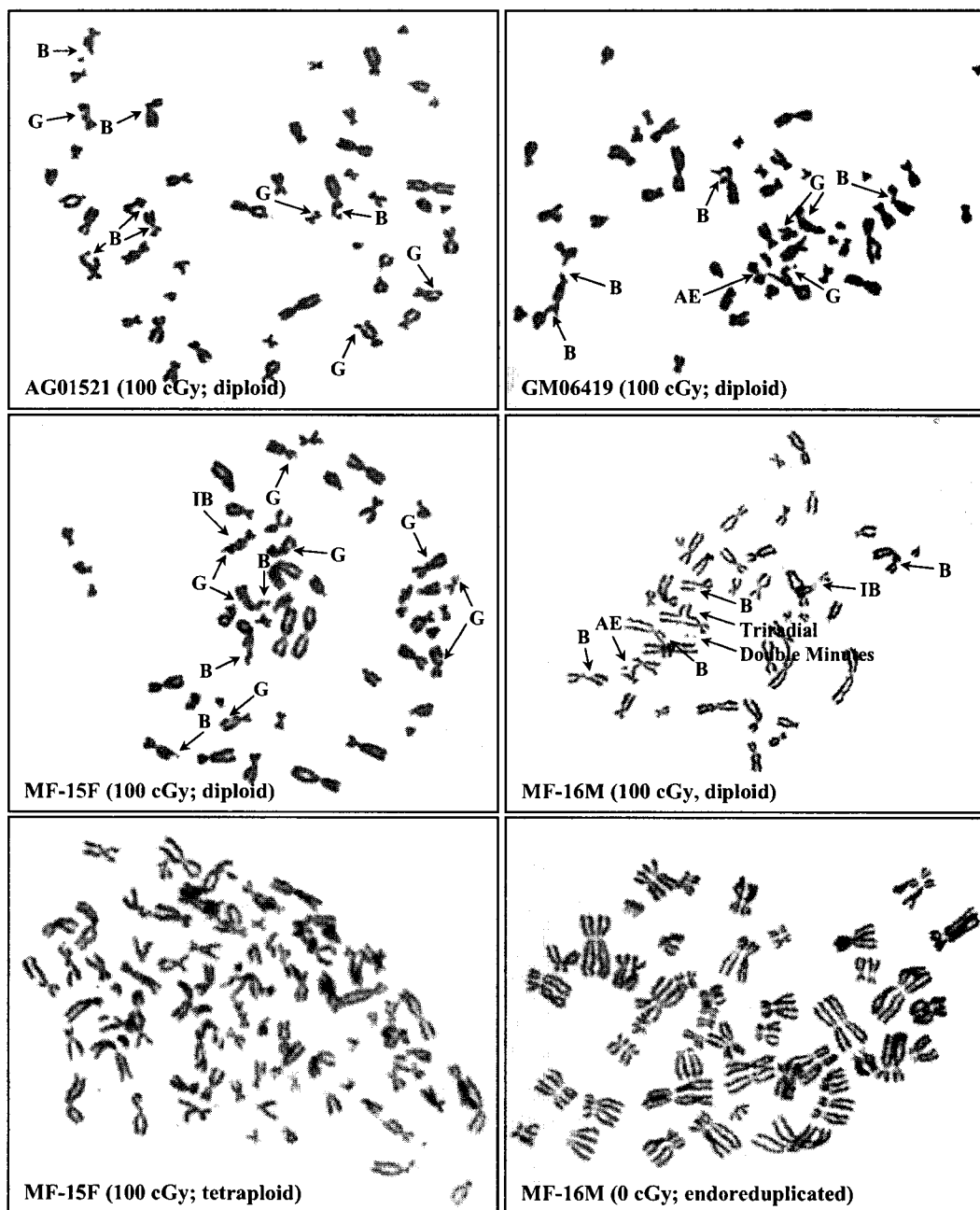


Figure 4.1. Examples of human fibroblast metaphase spreads obtained in the first post-irradiation mitosis after G₂-phase irradiation in the G₂ chromosomal radiosensitivity assay. The first four panels demonstrate the types of chromatid-type aberrations induced by radiation: chromatid gaps (G), breaks (B), iso-breaks (IB), and exchanges (asymmetrical or symmetrical, AE/SE). The bottom two panels demonstrate an irradiated tetraploid cell (left) and an unirradiated endoreduplicated cell (right).

observed, while chromatid-type exchanges (both symmetrical and asymmetrical forms) and tetraploid/endoreduplicated metaphase spreads were much less frequently observed. Chromatid-type aberration frequencies (CAF) and the frequencies of tetraploidy and endoreduplication were recorded separately, *i.e.*, the chromatid-type aberration frequencies presented are per diploid cell alone and the tetraploidy/endoreduplication frequencies are per total cell in culture (regardless of ploidy). In most cases, a total of >150–250 diploid metaphase spreads per dose point in 2–3 independent experiments were examined for each fibroblast strain; the two radiosensitive apparently normal CIMR strains (GM04505A and GM08447) were only examined in one experiment each.

The average chromosome aberration frequencies ($\pm$ SEM, both chromatid-type and chromosome-type) per cell for 0, 50 and 100 cGy doses of cesium-137 gamma rays for the 19 human fibroblast strains examined in these experiments are summarized in Tables 4.1–4.3, respectively. The chromatid break, gap, and combined break and gap frequencies per cell are plotted in Figures 4.2–4.4, respectively. Figure 4.5 is a plot of the frequency of radiation-induced chromatid exchanges [both symmetrical (no acentric fragment produced) and asymmetrical (acentric fragment produced) types] for the 50 and 100 cGy doses; exchanges were not observed in unirradiated cultures.

The “induced” aberration frequencies presented in Tables 4.2 and 4.3 and Figures 4.3–4.5 have been corrected for the spontaneous (0 cGy) background levels of these aberrations types (Table 4.1 and Figure 4.2). Significant differences ($P < 0.05$ for Table 4.1, 4.4, and 4.5 and Figures 4.2 and 4.6; $P < 0.01$ for Tables 4.2–4.3 and Figures 4.3–4.4) between the individual fibroblast strain chromatid break and combined chromatid gap and break frequencies, and the average aberration frequencies of the resistant,

Table 4.1. G2 chromosomal radiosensitivity assay results for Rb family member and CIMR fibroblast strains – spontaneous (0 cGy).

Fibroblast Strain	No. Expts.	Spontaneous Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Rb Family G2 Assay Rank ^a
		Breaks	Gaps	Breaks and Gaps	Exchanges		
<i>Retinoblastoma Family Members</i>							
MF-2M Rb Family I	3	0.090 $\pm$ 0.057 (0–0.203)	0.220 $\pm$ 0.087 (0.048–0.392)	0.309 $\pm$ 0.107 (0.097–0.521)	0	0	11
MF-3R Rb Family I	2	0.110 $\pm$ 0.057 (0–0.223)	0.263 $\pm$ 0.069 (0.126–0.373)	0.372 $\pm$ 0.102 (0.170–0.574)	0	0.006	5
MF-4F Rb Family I	2	0.079 $\pm$ 0.052 (0–0.182)	0.238 $\pm$ 0.073 (0.093–0.383)	0.318 $\pm$ 0.098 (0.124–0.512)	0	0	2
MF-6F Rb Family II	2	0.120 $\pm$ 0.072 (0–0.263)	0.450 $\pm$ 0.118 (0.216–0.684)	0.570 $\pm$ 0.143* (0.287–0.853)	0	0	9
MF-7R Rb Family II	3	0.117 $\pm$ 0.086 (0–0.286)	0.353 $\pm$ 0.118 (0.120–0.586)	0.470 $\pm$ 0.145 (0.184–0.756)	0	0.008	3
MF-10R Rb Family III	2	0.080 $\pm$ 0.049 (0–0.177)	0.311 $\pm$ 0.087 (0.139–0.483)	0.391 $\pm$ 0.113 (0.167–0.615)	0	0	10
MF-11F Rb Family III	2	0.147 $\pm$ 0.079 (0–0.303)	0.270 $\pm$ 0.094 (0.084–0.456)	0.416 $\pm$ 0.139 (0.141–0.691)	0	0	13
MF-12M Rb Family III	3	0.103 $\pm$ 0.070 (0–0.242)	0.348 $\pm$ 0.101 (0.148–0.548)	0.451 $\pm$ 0.133 (0.188–0.714)	0	0	14

Table 4.1. Continued.

Fibroblast Strain	No. Expts.	Spontaneous Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Rb Family G2 Assay Rank ^a
		Breaks	Gaps	Breaks and Gaps	Exchanges		
<i>Retinoblastoma Family Members</i>							
MF-13M Rb Family IV	3	0.165 $\pm$ 0.098 (0–0.357)	0.333 $\pm$ 0.119 (0.099–0.568)	0.498 $\pm$ 0.171* (0.160–0.835)	0	0	12
MF-14R Rb Family IV	3	0.179 $\pm$ 0.169 (0–0.512)	0.316 $\pm$ 0.110 (0.099–0.533)	0.495 $\pm$ 0.154* (0.192–0.798)	0	0	1
MF-15F Rb Family IV	3	0.154 $\pm$ 0.098 (0–0.347)	0.364 $\pm$ 0.118 (0.131–0.596)	0.517 $\pm$ 0.156* (0.209–0.826)	0	0	7
MF-16M Rb Family V	3	0.159 $\pm$ 0.092 (0–0.340)	0.325 $\pm$ 0.108 (0.112–0.538)	0.484 $\pm$ 0.149* (0.191–0.777)	0	0.004	4
MF-17R Rb Family V	3	0.114 $\pm$ 0.077 (0–0.266)	0.211 $\pm$ 0.087 (0.039–0.383)	0.325 $\pm$ 0.126 (0.077–0.573)	0	0	6
MF-18F Rb Family V	3	0.073 $\pm$ 0.058 (0–0.188)	0.195 $\pm$ 0.080 (0.038–0.353)	0.268 $\pm$ 0.104 (0.063–0.474)	0	0	8

^a The Rb family member fibroblast strains are ranked by their induced chromatid break and combined chromatid gap/break frequencies per cell per Gray (averaged for both the 50 and 100 cGy data sets) from least (1) to most (14) radiosensitive (see Table 4.4).

Table 4.1. Continued.

Fibroblast Strain	No. Expts.	Spontaneous Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell
		Breaks	Gaps	Breaks and Gaps	Exchanges	
<i>Sensitive apparently normal CIMR controls</i>						
GM04505A	1	0.073 $\pm$ 0.029 (0.016–0.131)	0.232 $\pm$ 0.053 (0.126–0.337)	0.305 $\pm$ 0.057 (0.192–0.418)	0	0
GM08447	1	0.098 $\pm$ 0.045 (0.009–0.186)	0.244 $\pm$ 0.051 (0.143–0.345)	0.341 $\pm$ 0.074 (0.194–0.489)	0	0
<i>Resistant apparently normal and Rb1^{+/-} CIMR controls</i>						
AG01521	3	0.161 $\pm$ 0.094 (0–0.345)	0.300 $\pm$ 0.099 (0.105–0.495)	0.461 $\pm$ 0.140 (0.185–0.736)	0	0
GM02149	3	0.085 $\pm$ 0.059 (0–0.202)	0.264 $\pm$ 0.092 (0.082–0.446)	0.350 $\pm$ 0.117 (0.118–0.581)	0	0.004
GM06419	3	0.080 $\pm$ 0.061 (0–0.201)	0.194 $\pm$ 0.083 (0.031–0.357)	0.274 $\pm$ 0.103 (0.071–0.478)	0	0

* Significant difference ($P < 0.05$) from average response (excluding chromatid gaps).

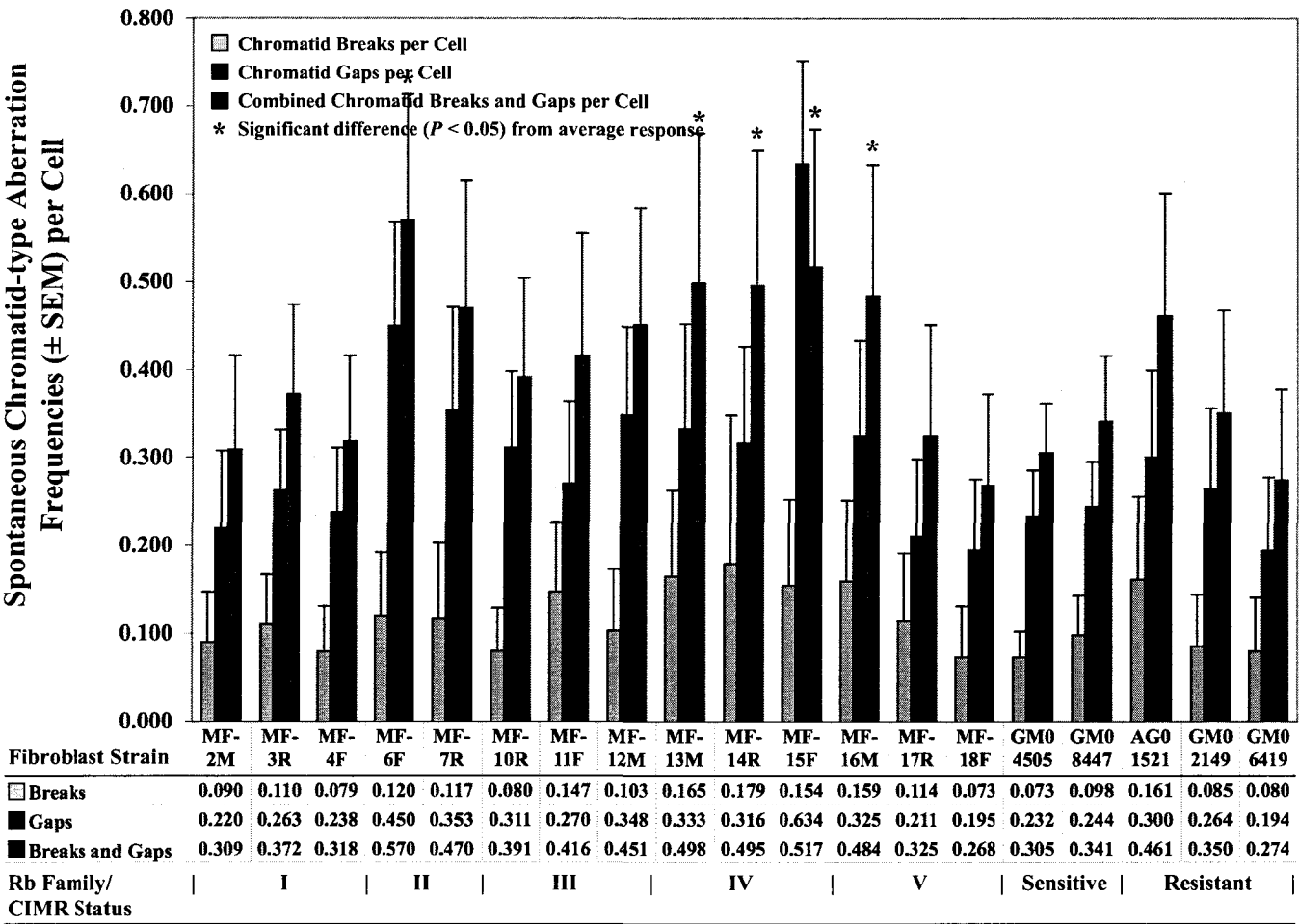


Figure 4.2. Spontaneous (0 cGy) chromatid gap and break frequencies ($\pm$ SEM) for the 14 Rb family member and 5 apparently normal and *Rb1*^{+/-} CIMR fibroblast strains examined in the G2 chromosomal radiosensitivity assay in this chapter (see Table 4.1).

Table 4.2. G2 chromosomal radiosensitivity assay results for Rb family member and CIMR fibroblast strains – 50 cGy.

Fibroblast Strain	No. Expts.	50 cGy-Induced Chromatid-Type Aberrations per Cell (± SEM)				Chromosome-Type Aberrations per Cell	Rb Family G2 Assay Rank ^a
		Breaks	Gaps	Breaks and Gaps	Exchanges		
Retinoblastoma Family Members							
MF-2M Rb Family I	3	2.429 ± 0.301* (1.833–3.025)	2.988 ± 0.318 (2.358–3.618)	5.428 ± 0.487* (4.464–6.392)	0.013 ± 0.007 (0–0.027)	0	11
MF-3R Rb Family I	2	1.933 ± 0.223* (1.491–2.375)	2.513 ± 0.172 (2.172–2.854)	4.445 ± 0.325* (3.802–5.089)	0.012 ± 0.012 (0–0.036)	0	5
MF-4F Rb Family I	2	1.663 ± 0.235 (1.198–2.128)	2.403 ± 0.193 (2.021–2.785)	4.064 ± 0.344 (3.383–4.745)	0.006 ± 0.012 (0–0.030)	0	2
MF-6F Rb Family II	2	2.215 ± 0.278* (1.665–2.765)	2.453 ± 0.253 (1.952–2.954)	4.668 ± 0.377* (3.922–5.414)	0	0	9
MF-7R Rb Family II	3	1.876 ± 0.300* (1.285–2.467)	2.426 ± 0.294 (1.846–3.006)	4.302 ± 0.456* (3.403–5.201)	0.008 ± 0.008 (0–0.024)	0.008	3
MF-10R Rb Family III	2	2.641 ± 0.259* (2.128–3.154)	2.293 ± 0.192 (1.913–2.673)	4.933 ± 0.377* (4.187–5.679)	0.012	0	10
MF-11F Rb Family III	2	2.635 ± 0.340* (1.962–3.308)	3.369 ± 0.296 (2.783–3.955)	6.003 ± 0.470* (5.072–6.934)	0.015 ± 0.015 (0–0.045)	0	13
MF-12M Rb Family III	3	2.560 ± 0.313* (1.940–3.180)	2.794 ± 0.241 (2.317–3.271)	5.354 ± 0.448* (4.467–6.241)	0.016 ± 0.008 (0–0.032)	0	14

Table 4.2. Continued.

Fibroblast Strain	No. Expts.	50 cGy-Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Rb Family G2 Assay Rank ^a
		Breaks	Gaps	Breaks and Gaps	Exchanges		
Retinoblastoma Family Members							
MF-13M Rb Family IV	3	2.516 $\pm$ 0.349* (1.829–3.204)	2.821 $\pm$ 0.278 (2.273–3.369)	5.173 $\pm$ 0.504* (4.180–6.165)	0.008 $\pm$ 0.004 (0–0.016)	0	12
MF-14R Rb Family IV	3	1.525 $\pm$ 0.292 (0.950–2.101)	2.192 $\pm$ 0.279 (1.643–2.740)	3.717 $\pm$ 0.450 (2.830–4.603)	0.008 $\pm$ 0.004 (0–0.016)	0	1
MF-15F Rb Family IV	3	2.049 $\pm$ 0.347* (1.366–2.732)	2.593 $\pm$ 0.327 (1.948–3.237)	4.642 $\pm$ 0.507* (3.643–5.640)	0.016 $\pm$ 0.011 (0–0.038)	0	7
MF-16M Rb Family V	3	2.094 $\pm$ 0.294* (1.514–2.673)	2.618 $\pm$ 0.249 (2.127–3.109)	4.711 $\pm$ 0.434* (3.857–5.565)	0.028 $\pm$ 0.018 (0–0.063)	0	4
MF-17R Rb Family V	3	2.089 $\pm$ 0.304* (1.491–2.688)	2.508 $\pm$ 0.265 (1.987–3.029)	4.598 $\pm$ 0.465* (3.682–5.514)	0.008 $\pm$ 0.004 (0–0.016)	0	6
MF-18F Rb Family V	3	2.114 $\pm$ 0.331* (1.461–2.766)	2.850 $\pm$ 0.273 (2.311–3.388)	4.963 $\pm$ 0.467* (4.044–5.882)	0.004 $\pm$ 0.004 (0–0.012)	0	8

^a The Rb family member fibroblast strains are ranked by their induced chromatid break and combined chromatid gap/break frequencies per cell per Gray (averaged for both the 50 and 100 cGy data sets) from least (1) to most (14) radiosensitive (see Table 4.4).

Table 4.2. Continued.

Fibroblast Strain	No. Expts.	50 cGy-Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell
		Breaks	Gaps	Breaks and Gaps	Exchanges	
<i>Sensitive apparently normal CIMR controls</i>						
GM04505A	1	2.574 $\pm$ 0.226* (2.125–3.023)	3.160 $\pm$ 0.147 (2.884–3.436)	5.734 $\pm$ 0.309* (5.123–6.345)	0	0
GM08447	1	2.061 $\pm$ 0.155* (1.765–2.357)	2.866 $\pm$ 0.143 (2.601–3.131)	4.927 $\pm$ 0.248* (4.456–5.398)	0.012	0
<i>Resistant apparently normal and Rb1^{+/-} CIMR controls</i>						
AG01521	3	1.571 $\pm$ 0.280 (1.019–2.123)	1.662 $\pm$ 0.273 (1.124–2.201)	3.449 $\pm$ 0.404 (2.653–4.245)	0.009 $\pm$ 0.004 (0.001–0.012)	0
GM02149	3	1.801 $\pm$ 0.259 (1.291–2.311)	2.618 $\pm$ 0.249 (2.128–3.108)	4.419 $\pm$ 0.409* (3.613–5.224)	0.008 $\pm$ 0.004 (0–0.016)	0
GM06419	3	1.319 $\pm$ 0.295 (0.736–1.901)	2.155 $\pm$ 0.282 (1.600–2.709)	3.473 $\pm$ 0.447 (2.593–4.353)	0	0

* Significant difference ($P < 0.01$) from average resistant CIMR response (excluding chromatid gaps).

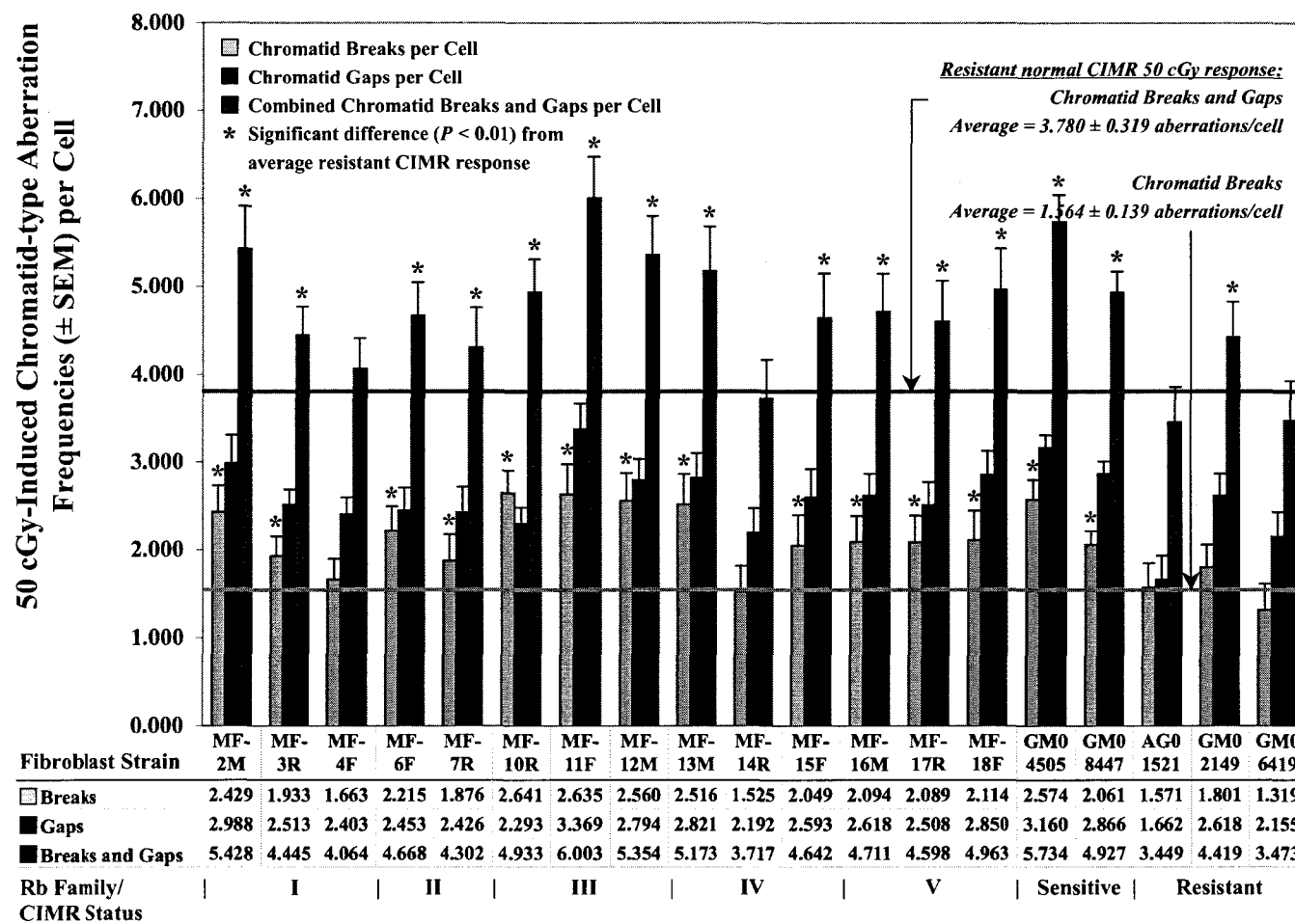


Figure 4.3. 50 cGy-induced chromatid gap and break frequencies ($\pm$ SEM) for the 14 Rb family member and 5 apparently normal and *Rb*^{+/-} CIMR fibroblast strains examined in the G2 chromosomal radiosensitivity assay in this chapter (see Table 4.2).

Table 4.3. G2 chromosomal radiosensitivity assay results for Rb family member and CIMR fibroblast strains – 100 cGy.

Fibroblast Strain	No. Expts.	100 cGy-Induced Chromatid-Type Aberrations per Cell (± SEM)				Chromosome-Type Aberrations per Cell	Rb Family G2 Assay Rank ^a
		Breaks	Gaps	Breaks and Gaps	Exchanges		
Retinoblastoma Family Members							
MF-2M Rb Family I	3	4.830 ± 0.475* (3.900–5.771)	5.048 ± 0.346 (4.363–5.733)	9.879 ± 0.674* (8.544–11.214)	0.016 ± 0.016 (0–0.048)	0	11
MF-3R Rb Family I	2	4.262 ± 0.352* (3.565–4.959)	4.974 ± 0.288 (4.404–5.544)	9.236 ± 0.486* (8.274–10.198)	0.043 ± 0.043 (0–0.128)	0	5
MF-4F Rb Family I	2	3.799 ± 0.331* (3.144–4.454)	4.671 ± 0.262 (4.152–5.190)	8.470 ± 0.500* (7.480–9.460)	0.031 ± 0.007 (0.018–0.044)	0	2
MF-6F Rb Family II	2	4.987 ± 0.488* (4.021–5.953)	4.917 ± 0.357 (4.210–4.624)	9.904 ± 0.702* (8.514–11.294)	0.012 ± 0.012 (0–0.036)	0	9
MF-7R Rb Family II	3	4.604 ± 0.482* (3.654–5.554)	4.092 ± 0.385 (3.333–4.851)	8.696 ± 0.673* (7.370–10.023)	0.009 ± 0.005 (0–0.018)	0	3
MF-10R Rb Family III	2	5.073 ± 0.369* (4.342–5.804)	4.750 ± 0.296 (4.164–5.336)	9.823 ± 0.522* (8.789–10.857)	0.031 ± 0.019 (0–0.068)	0	10
MF-11F Rb Family III	2	4.583 ± 0.593* (3.409–5.757)	5.065 ± 0.440 (4.194–5.936)	9.648 ± 0.769* (8.125–11.171)	0.010 ± 0.010 (0–0.030)	0.010	13
MF-12M Rb Family III	3	5.532 ± 0.476* (4.590–6.474)	4.803 ± 0.334 (4.142–5.464)	10.334 ± 0.650* (9.047–11.621)	0.008 ± 0.004 (0–0.016)	0	14

Table 4.3. Continued.

Fibroblast Strain	No. Expts.	100 cGy-Induced Chromatid-Type Aberrations per Cell (± SEM)				Chromosome-Type Aberrations per Cell	Rb Family G2 Assay Rank ^a
		Breaks	Gaps	Breaks and Gaps	Exchanges		
Retinoblastoma Family Members							
MF-13M Rb Family IV	3	5.088 ± 0.512* (4.080–6.096)	5.267 ± 0.369 (4.540–5.995)	10.355 ± 0.694* (8.987–11.723)	0.013 ± 0.001 (0.011–0.015)	0	12
MF-14R Rb Family IV	3	3.625 ± 0.467* (2.704–4.545)	4.416 ± 0.378 (3.671–5.161)	8.040 ± 0.613* (6.833–9.248)	0.004 ± 0.004 (0–0.012)	0	1
MF-15F Rb Family IV	3	4.633 ± 0.559* (3.532–5.733)	4.902 ± 0.386 (4.142–5.661)	9.535 ± 0.758* (8.042–11.027)	0.014 ± 0.002 (0.009–0.019)	0	7
MF-16M Rb Family V	3	4.118 ± 0.462* (3.207–5.028)	4.354 ± 0.337 (3.690–5.017)	8.472 ± 0.664* (7.164–9.779)	0.037	0	4
MF-17R Rb Family V	3	4.346 ± 0.459* (3.442–5.249)	4.317 ± 0.325 (3.677–4.957)	8.663 ± 0.646* (7.390–9.935)	0.037 ± 0.024 (0–0.085)	0	6
MF-18F Rb Family V	3	4.370 ± 0.466* (3.453–5.287)	5.653 ± 0.345 (4.974–6.332)	10.023 ± 0.660* (8.723–11.323)	0.008 ± 0.008 (0–0.024)	0	8

^a The Rb family member fibroblast strains are ranked by their induced chromatid break and combined chromatid gap/break frequencies per cell per Gray (averaged for both the 50 and 100 cGy data sets) from least (1) to most (14) radiosensitive (see Table 4.4).

Table 4.3. Continued.

Fibroblast Strain	No. Expts.	100 cGy-Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell
		Breaks	Gaps	Breaks and Gaps	Exchanges	
<i>Sensitive apparently normal CIMR controls</i>						
GM04505A	1	5.707 $\pm$ 0.344* (5.017–6.397)	4.748 $\pm$ 0.194 (4.374–5.122)	10.455 $\pm$ 0.447* (9.554–11.346)	0.020	0
GM08447	1	4.548 $\pm$ 0.259* (4.041–5.055)	4.110 $\pm$ 0.139 (3.853–4.367)	8.659 $\pm$ 0.331* (8.018–9.300)	0	0
<i>Resistant apparently normal and Rb1^{+/-} CIMR controls</i>						
AG01521	3	3.366 $\pm$ 0.382 (2.614–4.118)	3.367 $\pm$ 0.336 (2.975–4.300)	7.003 $\pm$ 0.564 (5.891–8.115)	0.016 $\pm$ 0.016 (0–0.048)	0
GM02149	3	3.256 $\pm$ 0.364 (2.539–3.973)	4.150 $\pm$ 0.297 (3.566–4.735)	7.407 $\pm$ 0.547 (6.329–8.484)	0.016 $\pm$ 0.004 (0.008–0.024)	0
GM06419	3	2.908 $\pm$ 0.396 (2.127–3.689)	4.314 $\pm$ 0.382 (3.562–5.065)	7.222 $\pm$ 0.630 (5.981–8.462)	0.005 $\pm$ 0.005 (0–0.015)	0

* Significant difference ($P < 0.01$) from average resistant CIMR response (excluding chromatid gaps).

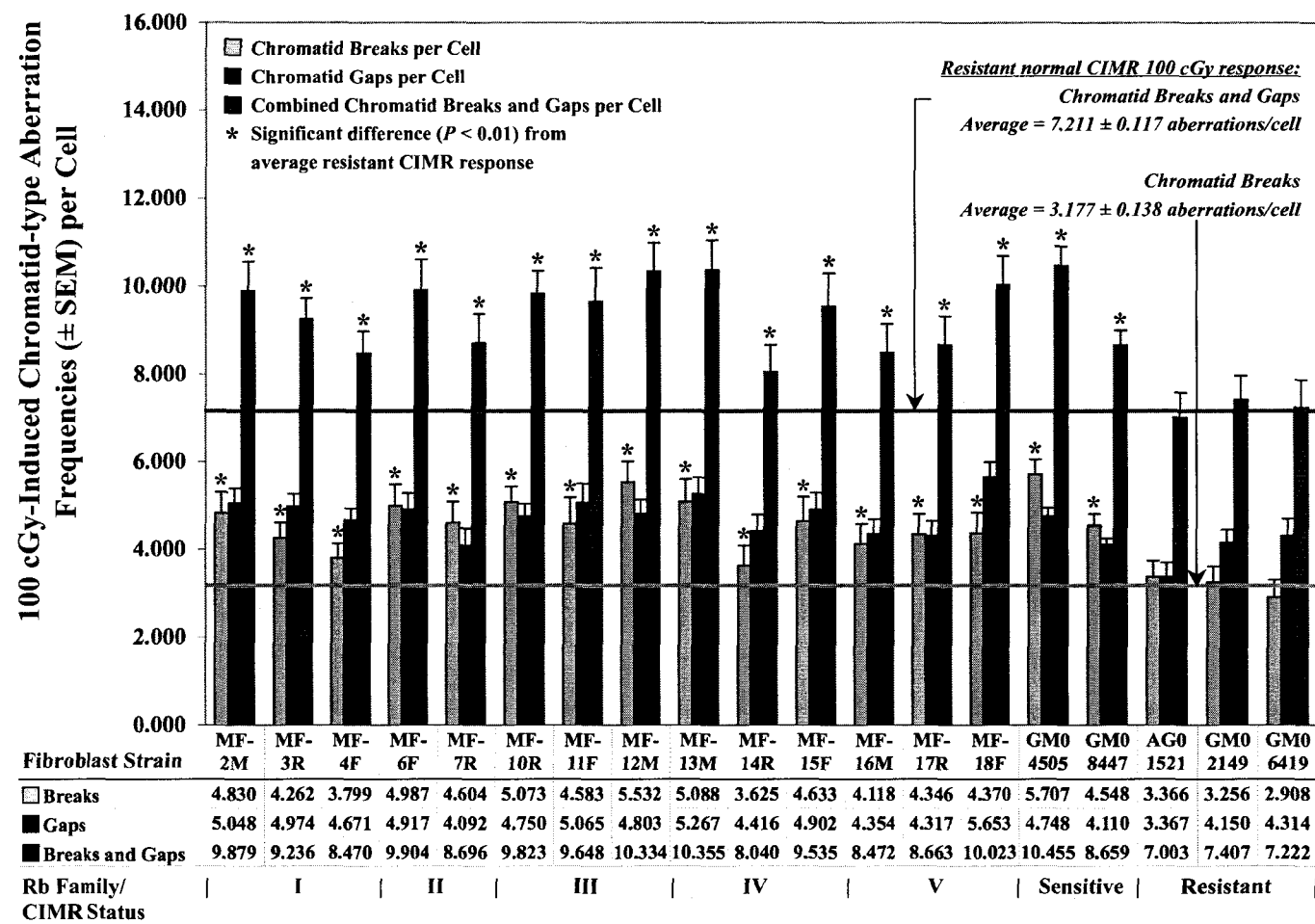


Figure 4.4. 100 cGy-induced chromatid gap and break frequencies ($\pm$ SEM) for the 14 Rb family member and 5 apparently normal and $Rb1^{+/-}$ CIMR fibroblast strains examined in the G2 chromosomal radiosensitivity assay in this chapter (see Table 4.3).

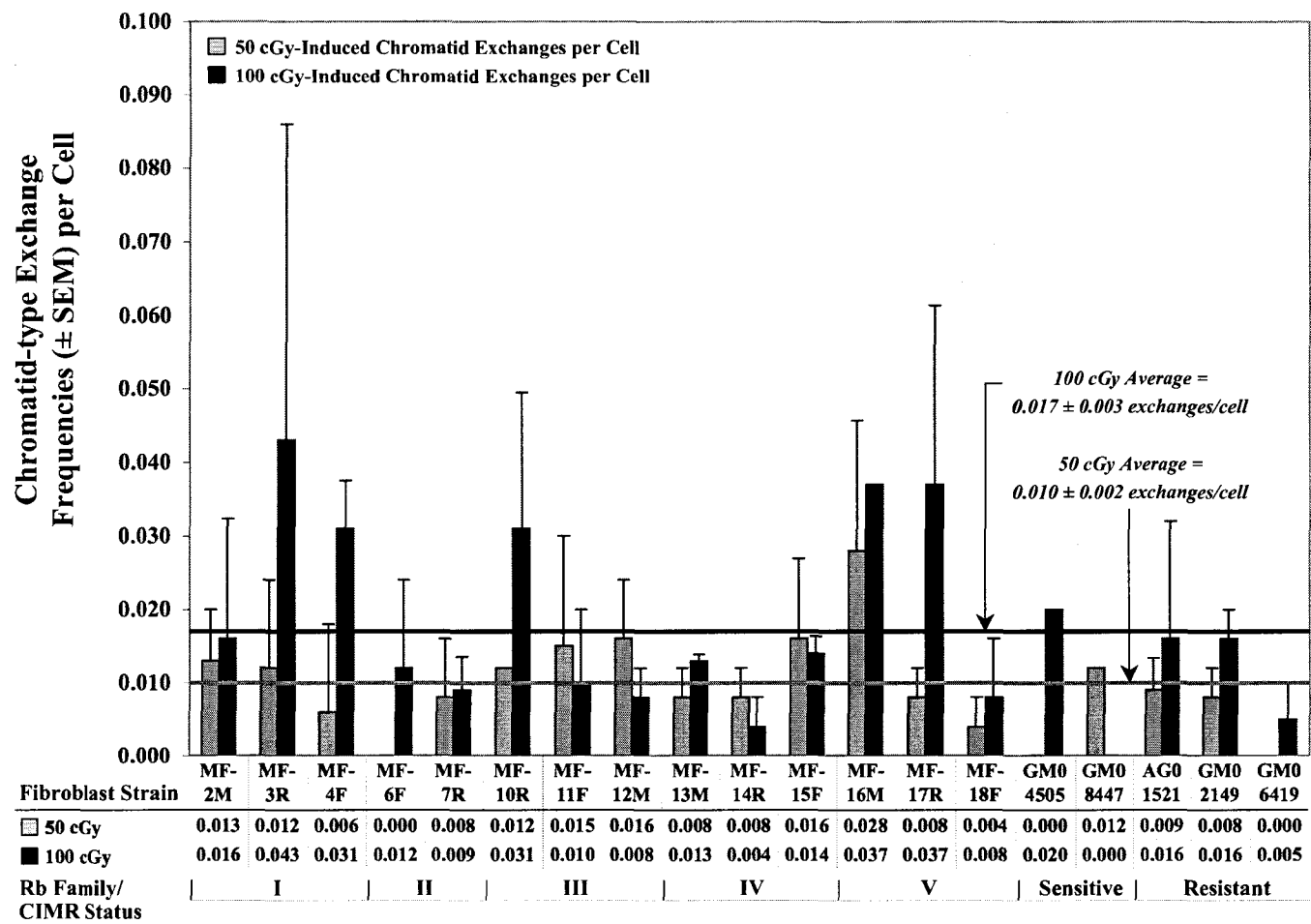


Figure 4.5. Chromatid-type exchange frequencies ($\pm$ SEM) for the 14 Rb family member and 5 apparently normal and *RbI*^{+/-} CIMR fibroblast strains examined in the G2 chromosomal radiosensitivity assay in this chapter (see Tables 4.2 and 4.3).

apparently normal CIMR strains, are denoted by asterisks for the particular strain and aberration type (chromatid gap data alone were not included in the statistical analyses). The average spontaneous (0 cGy) aberration frequencies in Figure 4.2 are derived from *all* 19 fibroblast strains (as no obvious trend in these spontaneous levels were observed for the different groups of fibroblasts); the average 50 cGy and 100 cGy aberration frequencies in Figures 4.3 and 4.4 are derived from only the three radioresistant CIMR control strains (AG01521, GM02149, and GM06419), as denoted in these figures. There were no significant differences in the 50 cGy and 100 cGy-induced chromatid-type exchange frequencies among the 19 fibroblast strains in Figure 4.5 (despite an ~2-fold increase in exchanges observed in 5 of the 14 Rb family member fibroblast strains after 100 cGy, as compared to the other fibroblast strains).

The average radiation-induced chromatid break and combined chromatid gap and break frequencies per cell per Gray ($\pm$ SD, averaged for both the 50 and 100 cGy data sets) for the 19 fibroblast strains and groups thereof (grouped by *Rb1* haplotype for the Rb family members and CIMR radiosensitivity status for the CIMR controls) are presented in Table 4.4. The chromatid break data are presented for inter-laboratory comparison purposes of this data to reports in the literature (the chromatid break is the typical aberration type scored by the Sanford NCI and Scott PICR groups in this assay). The combined chromatid gap and break data are presented since they more truly reflect the generation of acentric fragments in these cells that would be relegated to micronuclei and lost during further cell divisions. The 14 Rb family member fibroblast strains are ranked according to both these radiation-induced aberration frequencies from least (1) to most (14) radiosensitive (these rankings are identically listed in Tables 4.1–4.3).

Table 4.4. Average radiation-induced chromatid break and combined chromatid gap and break frequencies ($\pm$ SD) for the 14 Rb family member and 5 apparently normal and *Rb1*^{+/-} CIMR fibroblast strains, and groups thereof, in the G2 chromosomal radiosensitivity assay.

Fibroblast Strain/ Group	No. Expts. / Strains	Average Induced Chromatid Break Frequency per cell per Gray ($\pm$ SD, Range) ^a	Average Induced Chromatid Gap and Break Frequency per cell per Gray ($\pm$ SD, Range) ^a	Rb Family G2 Assay Rank ^b
<i>Retinoblastoma family members</i>				
MF-2M Rb Family I	3 expts.	4.84 $\pm$ 0.02*	10.37 $\pm$ 0.69*	11
MF-3R Rb Family I	2 expts.	4.06 $\pm$ 0.28*	9.06 $\pm$ 0.24*	5
MF-4F Rb Family I	2 expts.	3.56 $\pm$ 0.33	8.30 $\pm$ 0.24*	2
MF-6F Rb Family II	2 expts.	4.71 $\pm$ 0.39*	9.62 $\pm$ 0.40*	9
MF-7R Rb Family II	3 expts.	4.18 $\pm$ 0.60*	8.65 $\pm$ 0.07*	3
MF-10R Rb Family III	2 expts.	5.18 $\pm$ 0.15*	9.84 $\pm$ 0.03*	10
MF-11F Rb Family III	2 expts.	4.93 $\pm$ 0.49*	10.83 $\pm$ 1.67*	13
MF-12M Rb Family III	3 expts.	5.33 $\pm$ 0.29*	10.52 $\pm$ 0.26*	14
MF-13M Rb Family IV	3 expts.	5.06 $\pm$ 0.04*	10.35 $\pm$ 0.01*	12
MF-14R Rb Family IV	3 expts.	3.34 $\pm$ 0.41	7.74 $\pm$ 0.43	1
MF-15F Rb Family IV	3 expts.	4.37 $\pm$ 0.38*	9.41 $\pm$ 0.18*	7
MF-16M Rb Family V	3 expts.	4.15 $\pm$ 0.05*	8.95 $\pm$ 0.67*	4
MF-17R Rb Family V	3 expts.	4.26 $\pm$ 0.12*	8.93 $\pm$ 0.38*	6
MF-18F Rb Family V	3 expts.	4.30 $\pm$ 0.10*	9.97 $\pm$ 0.07*	8

Table 4.4. Continued.

Fibroblast Strain/ Group	No. Expts. / Strains	Average Induced Chromatid Break Frequency per cell per Gray ($\pm$ SD, Range) ^a	Average Induced Chromatid Gap and Break Frequency per cell per Gray ($\pm$ SD, Range) ^a
<i>Average retinoblastoma family member response per Rb1 haplotype</i>			
<i>Rb1</i> ^{+/+}	8 strains	4.54 $\pm$ 0.56* (3.56–5.33)	9.69 $\pm$ 0.78* (8.30–10.52)
<i>Rb1</i> ^{+/-}	6 strains	4.32 $\pm$ 0.66* (3.34–5.18)	9.18 $\pm$ 1.06* (7.74–10.83)
Average Rb family member response	14 strains	4.45 $\pm$ 0.59* (3.34–5.33)	9.47 $\pm$ 0.91* (7.74–10.83)
<i>Sensitive apparently normal CIMR controls</i>			
GM04505A	1 expt.	5.43 $\pm$ 0.40*	10.96 $\pm$ 0.72*
GM08447	1 expt.	4.34 $\pm$ 0.30*	9.26 $\pm$ 0.84*
Average sensitive CIMR response	2 strains	4.88 $\pm$ 0.77* (4.34–5.43)	10.11 $\pm$ 1.20* (9.26–10.96)
<i>Resistant apparently normal and Rb1^{+/-} CIMR controls</i>			
AG01521	3 expts.	3.25 $\pm$ 0.16	6.95 $\pm$ 0.07
GM02149	3 expts.	3.43 $\pm$ 0.24	8.12 $\pm$ 1.01
GM06419	3 expts.	2.77 $\pm$ 0.19	7.08 $\pm$ 0.20
Average resistant CIMR response	3 strains	3.15 $\pm$ 0.34 (2.77–3.43)	7.39 $\pm$ 0.64 (6.95–8.12)

^a Averaged for both the 50 and 100 cGy induced chromatid break and combined chromatid gap and break data sets (see Tables 4.2 and 4.3 and Figures 4.3 and 4.4).

^b The Rb family member fibroblast strains are ranked by their induced chromatid break and combined chromatid gap and break frequencies per cell per Gray from least (1) to most (14) radiosensitive.

* Significant difference ($P < 0.05$) from average resistant CIMR response.

From Table 4.4, the average radiation-induced chromatid break and combined chromatid gap and break frequencies $\pm$ SD for the three resistant CIMR strains was 3.15 ± 0.34 (range 2.77–3.43) and 7.39 ± 0.64 (range 6.95–8.12) aberrations per cell, respectively. The average radiation-induced chromatid break and combined chromatid gap and break frequencies $\pm$ SD for the two sensitive CIMR strains was 4.88 ± 0.77 (range 4.34–5.43) and 10.11 ± 1.20 (range 9.26–10.96) aberrations per cell, respectively. The average radiation-induced chromatid break and combined chromatid gap and break frequencies $\pm$ SD for the 14 Rb family members strains was 4.45 ± 0.59 (range 3.34–5.33) and 9.47 ± 0.91 (range 7.74–10.83) aberrations per cell, respectively.

Separating the Rb family members by *Rb1* genotype, the average radiation-induced chromatid break frequencies $\pm$ SD for *Rb1*^{+/+} and *Rb1*^{+/-} strains were 4.54 ± 0.56 (range 3.56–5.33) and 4.32 ± 0.66 (range 3.34–5.18) aberrations per cell, respectively. For combined radiation-induced chromatid gaps and breaks, the average frequencies $\pm$ SD for *Rb1*^{+/+} and *Rb1*^{+/-} strains were 9.69 ± 0.78 (range 8.30–10.52) and 9.18 ± 1.06 (range 7.74–10.83) aberrations per cell, respectively. Interestingly, the unaffected Rb family member fibroblast strains had slightly higher (though not at all significant) frequencies of these aberration types compared to the affected Rb family member strains. The Rb family member fibroblast strains (collectively and grouped by *Rb1* haplotype) and the radiosensitive apparently normal CIMR strains are all individually (except the affected proband MF-14R in Rb family IV) and per group significantly ($P < 0.05$) more radiosensitive than the resistant CIMR control strains when considering the average frequencies of radiation-induced chromatid breaks and combined chromatid gaps and breaks recorded in Table 4.4.

The average spontaneous endoreduplication and tetraploidy frequencies ($\pm$ SEM) for the 19 fibroblast strains are reported in Table 4.5 and Figure 4.6. These values have been averaged for the three dose points per experiment and the total number of experiments per strain. Since both of these phenotypes were generated spontaneously in culture prior to the G2-phase irradiation and mitotic collection procedures used in this assay (in a prior S-phase and mitosis, respectively), no radiation dose response would be expected. While a slight dose response may be evident in a few strains (*e.g.*, MF-6F, MF-7R, MF-15F), this is most likely due to radiation-induced mitotic delay of diploid cells at the higher doses. This would influence the total numbers of cells viewed per slide in order to achieve the desired number of diploid metaphase spreads (and thus reflect a higher probability of recording delay-resistant aneuploid cells in the total population); the remainder of cell strains either show no dose response or, paradoxically, decreases in the aneuploid frequencies as the dose is increased.

Three unaffected Rb parents (MF-15F in Rb family IV; MF-16M and MF-18F in Rb family V) had significantly ($P < 0.05$) higher levels of tetraploidy (~17%, 12%, and 8% respectively) compared to the average of the three resistant CIMR control strains (average of ~4%). Three unaffected Rb parents (MF-13M and MF-15F in Rb family IV; MF-16M in Rb family V) likewise had significantly high frequencies of endoreduplication (2–3% of the total population). Seven additional Rb family member strains were also observed to have endoreduplicated metaphase spreads (at a lower frequency of 0.1–0.4%). Only three of the ten Rb family member strains with endoreduplicated metaphase spreads were affected *Rb1*^{+/-} individuals: MF-3R, MF-7R, and MF-11F; and this phenotype was not observed in cultures of the five CIMR fibroblast

Table 4.5. Average endoreduplication and tetraploidy frequencies ($\pm$ SEM) for the 14 Rb family member and 5 apparently normal and *Rb1*^{+/-} CIMR fibroblast strains in the G2 chromosomal radiosensitivity assay.

Fibroblast Strain	No. Expts.	Endoreduplication Frequency ($\pm$ SEM)	Tetraploidy Frequency ($\pm$ SEM)
<i>Retinoblastoma Family Members</i>			
MF-2M Rb Family I	3	0.003 $\pm$ 0.002*	0.047 $\pm$ 0.012
MF-3R Rb Family I	2	0.004 $\pm$ 0.003*	0.024 $\pm$ 0.009
MF-4F Rb Family I	2	0.002 $\pm$ 0.002	0.027 $\pm$ 0.004
MF-6F Rb Family II	2	0.003 $\pm$ 0.003*	0.048 $\pm$ 0.010
MF-7R Rb Family II	3	0.001 $\pm$ 0.001	0.030 $\pm$ 0.008
MF-10R Rb Family III	2	0	0.012 $\pm$ 0.004
MF-11F Rb Family III	2	0.003 $\pm$ 0.003*	0.039 $\pm$ 0.013
MF-12M Rb Family III	3	0	0.023 $\pm$ 0.008
MF-13M Rb Family IV	3	0.026 $\pm$ 0.012*	0.021 $\pm$ 0.005
MF-14R Rb Family IV	3	0	0.018 $\pm$ 0.004
MF-15F Rb Family IV	3	0.017 $\pm$ 0.006*	0.171 $\pm$ 0.038*
MF-16M Rb Family V	3	0.021 $\pm$ 0.005*	0.117 $\pm$ 0.016*
MF-17R Rb Family V	3	0	0.065 $\pm$ 0.012
MF-18F Rb Family V	3	0.004 $\pm$ 0.003*	0.083 $\pm$ 0.011*
<i>Sensitive apparently normal CIMR controls</i>			
GM04505A	1	0	0.006 $\pm$ 0.006
GM08447	1	0	0.012 $\pm$ 0.007
<i>Resistant apparently normal and <i>Rb1</i>^{+/-} CIMR controls</i>			
AG01521	3	0	0.056 $\pm$ 0.009
GM02149	3	0	0.041 $\pm$ 0.006
GM06419	3	0	0.017 $\pm$ 0.005

* Significant difference ($P < 0.05$) from average resistant CIMR response.

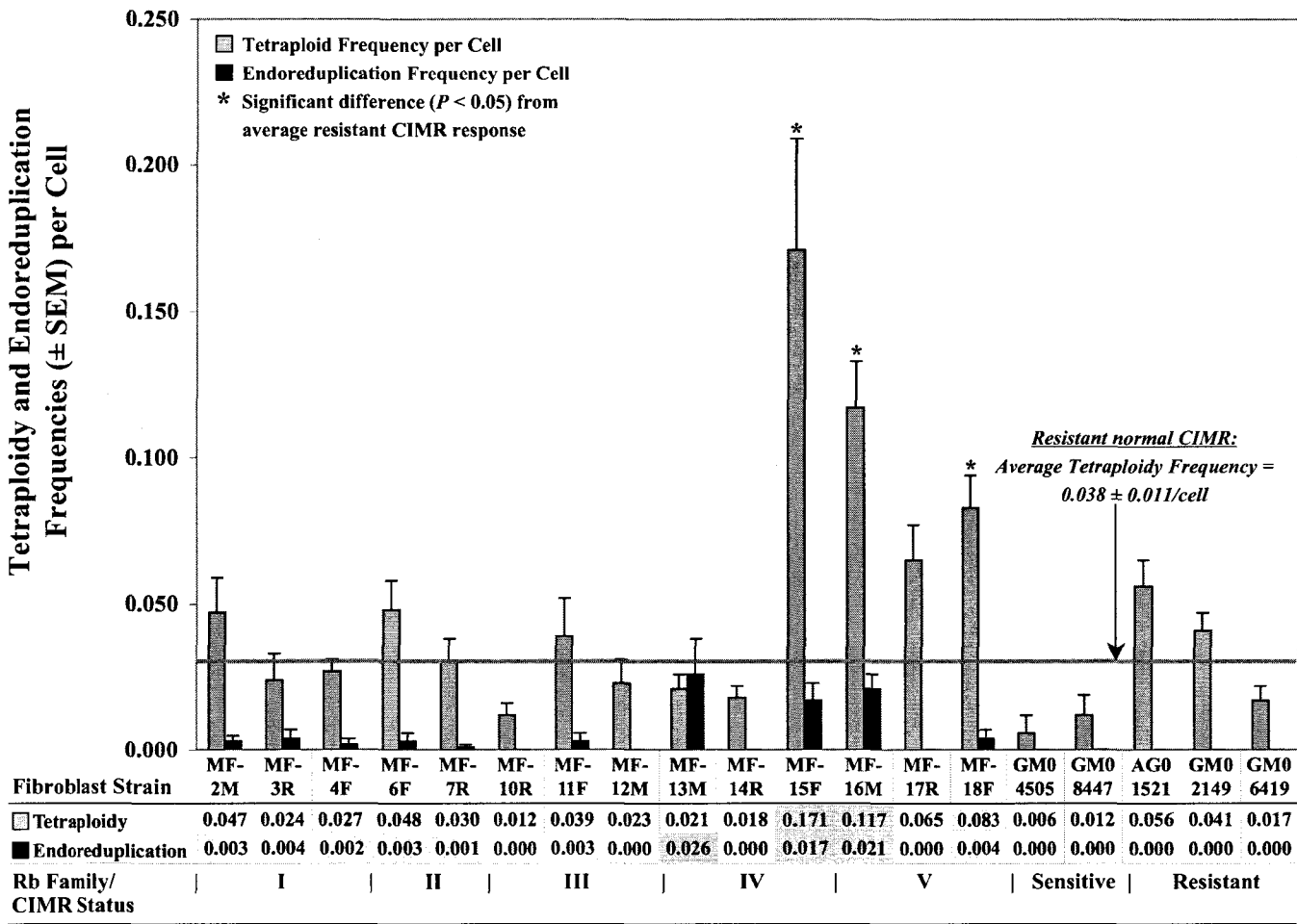


Figure 4.6. Spontaneous tetraploidy and endoreduplication frequencies ($\pm$ SEM) for the 14 Rb family member and 5 apparently normal and $Rb1^{+/-}$ CIMR fibroblast strains examined in the G2 chromosomal radiosensitivity assay in this chapter (see Table 4.5).

strains. There was no significant correlation between the frequencies of radiation-induced chromatid breaks and combined chromatid gaps and breaks and the frequencies of tetraploidy and endoreduplication ($P = 0.357\text{--}0.938$) for the 19 human fibroblast strains examined in this study.

The experimental data sets for the 19 fibroblast strains examined in this study (frequencies of all types of chromosome aberrations, tetraploidy, and endoreduplication per cell) are reported in Table 4.6. For the chromatid break data (grey lines) and combined chromatid gap and break data (black lines), experimental data histograms and Poisson distributions with the means of the total (solid lines) and the radiation-induced (0 cGy background-corrected, dashed lines) aberration frequencies are plotted in Figures 4.7–4.13. The average radiation-induced chromatid break (B) and combined chromatid gap and break (G+B) frequencies for the 50 and 100 cGy data sets are also reported for each fibroblast strain in the panels of Figures 4.7–4.13.

Histograms of the frequencies of radiation-induced chromatid breaks and combined chromatid gaps and breaks per cell per Gray for the 14 Rb family member, two radiosensitive apparently normal CIMR (GM04505A and GM08447), and three radioresistant CIMR control (AG01521, GM02149, and GM06419) fibroblast strains are shown in Figures 4.14 and 4.15, respectively (the “MF,” “AG,” and “GM” prefixes have been omitted). The distribution in Figure 4.14 for induced chromatid breaks appears bimodal, with all or most (in the case of MF-14R) of the Rb family member and radiosensitive CIMR fibroblast strains separated from the radioresistant CIMR fibroblast strains. The distribution in Figure 4.15 appears somewhat flatter, with more spread of the strains into a larger number of “bins” (9 bins in Figure 4.15, 6 bins in Figure 4.14). Both

Table 4.6. G2 chromosomal radiosensitivity assay experimental results for the 19 human fibroblast strains examined in this study. Chromatid break, gap, combined breaks and gaps, and exchange (including both symmetrical and asymmetrical types) data are reported as mean value per cell ($\pm$ SEM) and 95% confidence intervals. Chromosome-type aberration (dicentrics and rings), endoreduplication and tetraploidy frequency data are reported only as mean value per cell.

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
AG01521 – resistant apparently normal CIMR male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW063	0 cGy	71	0.324 $\pm$ 0.080 (0.165–0.483)	0.437 $\pm$ 0.069 (0.300–0.574)	0.761 $\pm$ 0.105 (0.551–0.970)	0	0	0	0.027
PFW070	0 cGy	82	0.098 $\pm$ 0.041 (0.016–0.179)	0.378 $\pm$ 0.064 (0.251–0.506)	0.476 $\pm$ 0.082 (0.313–0.638)	0	0	0	0.109
PFW090	0 cGy	82	0.061 $\pm$ 0.027 (0.008–0.114)	0.085 $\pm$ 0.031 (0.024–0.147)	0.146 $\pm$ 0.043 (0.061–0.232)	0	0	0	0.035
0 cGy Average		235	0.161 $\pm$ 0.094 (0–0.345)	0.300 $\pm$ 0.099 (0.105–0.495)	0.461 $\pm$ 0.140 (0.185–0.736)	0	0	0	0.057
PFW063	50 cGy	73	1.375 $\pm$ 0.174 (1.067–1.683)	1.714 $\pm$ 0.178 (1.387–2.041)	3.089 $\pm$ 0.266 (2.602–3.575)	0	0	0	0.039
PFW070	50 cGy	74	1.497 $\pm$ 0.150 (1.210–1.784)	1.798 $\pm$ 0.163 (1.500–2.096)	3.295 $\pm$ 0.212 (2.905–3.685)	0.014	0	0	0.026
PFW090	50 cGy	82	1.841 $\pm$ 0.161 (1.526–2.157)	1.476 $\pm$ 0.129 (1.226–1.725)	3.964 $\pm$ 0.218 (3.538–4.389)	0.012	0	0	0.068
50 cGy Average		229	1.571 $\pm$ 0.280 (1.019–2.123)	1.662 $\pm$ 0.273 (1.124–2.201)	3.449 $\pm$ 0.404 (2.653–4.245)	0.009 $\pm$ 0.004 (0.001–0.012)	0	0	0.044
PFW063	100 cGy	72	3.232 $\pm$ 0.247 (2.765–3.698)	3.716 $\pm$ 0.230 (3.279–4.153)	6.947 $\pm$ 0.356 (6.270–7.625)	0	0	0	0.069
PFW070	100 cGy	82	3.171 $\pm$ 0.211 (2.759–3.582)	3.232 $\pm$ 0.194 (2.867–3.597)	6.402 $\pm$ 0.331 (5.764–7.041)	0	0	0	0.079
PFW090	100 cGy	82	3.695 $\pm$ 0.201 (3.299–4.091)	3.963 $\pm$ 0.150 (3.671–4.256)	7.659 $\pm$ 0.286 (7.095–8.222)	0.049	0	0	0.057
100 cGy Average		236	3.366 $\pm$ 0.382 (2.614–4.118)	3.637 $\pm$ 0.336 (2.975–4.300)	7.003 $\pm$ 0.564 (5.891–8.115)	0.016 $\pm$ 0.016 (0–0.048)	0	0	0.068

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
GM02149 – resistant apparently normal CIMR female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW079	0 cGy	82	0.110 $\pm$ 0.039 (0.033–0.187)	0.427 $\pm$ 0.067 (0.293–0.561)	0.537 $\pm$ 0.084 (0.370–0.703)	0	0.012	0	0.024
PFW090A	0 cGy	82	0.061 $\pm$ 0.027 (0.008–0.114)	0.171 $\pm$ 0.042 (0.088–0.254)	0.232 $\pm$ 0.050 (0.132–0.331)	0	0	0	0.047
PFW090B	0 cGy	82	0.085 $\pm$ 0.036 (0.015–0.156)	0.195 $\pm$ 0.047 (0.101–0.289)	0.281 $\pm$ 0.066 (0.150–0.411)	0	0	0	0.047
0 cGy Average		246	0.085 $\pm$ 0.059 (0–0.202)	0.264 $\pm$ 0.092 (0.082–0.446)	0.350 $\pm$ 0.117 (0.118–0.581)	0	0.004	0	0.039
PFW079	50 cGy	82	1.744 $\pm$ 0.168 (1.418–2.070)	2.573 $\pm$ 0.155 (2.295–2.852)	4.317 $\pm$ 0.244 (3.862–4.773)	0	0	0	0.047
PFW090A	50 cGy	82	1.720 $\pm$ 0.135 (1.457–1.983)	2.573 $\pm$ 0.143 (2.302–2.845)	4.293 $\pm$ 0.234 (3.838–4.747)	0.012	0	0	0.012
PFW090B	50 cGy	82	1.939 $\pm$ 0.143 (1.663–2.215)	2.707 $\pm$ 0.132 (2.462–2.953)	4.646 $\pm$ 0.230 (4.207–5.086)	0.012	0	0	0.047
50 cGy Average		246	1.801 $\pm$ 0.259 (1.291–2.311)	2.618 $\pm$ 0.249 (2.128–3.108)	4.419 $\pm$ 0.409 (3.613–5.224)	0.008 $\pm$ 0.004 (0–0.016)	0	0	0.035
PFW079	100 cGy	82	3.561 $\pm$ 0.241 (3.089–4.033)	3.939 $\pm$ 0.186 (3.595–4.283)	7.500 $\pm$ 0.342 (6.840–8.160)	0.012	0	0	0.068
PFW090A	100 cGy	82	2.951 $\pm$ 0.176 (2.605–3.297)	4.329 $\pm$ 0.170 (4.001–4.657)	7.281 $\pm$ 0.296 (6.701–7.861)	0.012	0	0	0.024
PFW090B	100 cGy	82	3.256 $\pm$ 0.209 (2.846–3.666)	4.183 $\pm$ 0.157 (3.885–4.481)	7.439 $\pm$ 0.308 (6.841–8.037)	0.024	0	0	0.057
100 cGy Average		246	3.256 $\pm$ 0.364 (2.539–3.973)	4.150 $\pm$ 0.297 (3.566–4.735)	7.407 $\pm$ 0.547 (6.329–8.484)	0.016 $\pm$ 0.004 (0.008–0.024)	0	0	0.050

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
GM06419 – resistant *Rb1*^{+/–} CIMR male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell (± SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW063	0 cGy	67	0.119 ± 0.045 (0.029–0.210)	0.313 ± 0.061 (0.192–0.435)	0.433 ± 0.077 (0.278–0.587)	0	0	0	0.043
PFW122A	0 cGy	82	0.061 ± 0.032 (0–0.124)	0.085 ± 0.036 (0.015–0.156)	0.146 ± 0.046 (0.054–0.238)	0	0	0	0.012
PFW122B	0 cGy	82	0.061 ± 0.027 (0.008–0.114)	0.183 ± 0.043 (0.097–0.268)	0.244 ± 0.051 (0.143–0.345)	0	0	0	0.012
0 cGy Average		231	0.080 ± 0.061 (0–0.201)	0.194 ± 0.083 (0.031–0.357)	0.274 ± 0.103 (0.071–0.478)	0	0	0	0.022
PFW063	50 cGy	54	1.029 ± 0.170 (0.700–1.357)	1.927 ± 0.197 (1.553–2.302)	2.956 ± 0.271 (2.436–3.477)	0	0	0	0.036
PFW122A	50 cGy	82	1.500 ± 0.188 (1.131–1.869)	2.305 ± 0.144 (2.027–2.583)	3.805 ± 0.259 (3.299–4.311)	0	0	0	0
PFW122B	50 cGy	82	1.427 ± 0.152 (1.129–1.724)	2.232 ± 0.141 (1.964–2.499)	3.659 ± 0.244 (3.184–4.133)	0	0	0	0
50 cGy Average		218	1.319 ± 0.295 (0.736–1.901)	2.155 ± 0.282 (1.600–2.709)	3.473 ± 0.447 (2.593–4.353)	0	0	0	0.012
PFW063	100 cGy	68	2.748 ± 0.257 (2.243–3.253)	4.745 ± 0.249 (4.263–5.228)	7.494 ± 0.387 (6.736–8.252)	0.015	0	0	0.029
PFW122A	100 cGy	82	2.927 ± 0.207 (2.521–3.333)	4.524 ± 0.220 (4.092–4.957)	7.451 ± 0.359 (6.744–8.159)	0	0	0	0.024
PFW122B	100 cGy	82	3.049 ± 0.220 (2.615–3.483)	3.671 ± 0.187 (3.309–4.033)	6.720 ± 0.343 (6.044–7.395)	0	0	0	0
100 cGy Average		232	2.908 ± 0.396 (2.127–3.689)	4.314 ± 0.382 (3.562–5.065)	7.222 ± 0.630 (5.981–8.462)	0.005 ± 0.005 (0–0.015)	0	0	0.018

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
GM04505A – sensitive apparently normal CIMR female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW126	0 cGy	82	0.073 $\pm$ 0.029 (0.016–0.131)	0.232 $\pm$ 0.053 (0.126–0.337)	0.305 $\pm$ 0.057 (0.192–0.418)	0	0	0	0
PFW126	50 cGy	51	2.574 $\pm$ 0.226 (2.125–3.023)	3.160 $\pm$ 0.147 (2.884–3.436)	5.734 $\pm$ 0.309 (5.123–6.345)	0	0	0	0.019
PFW126	100 cGy	50	5.707 $\pm$ 0.344 (5.017–6.397)	4.748 $\pm$ 0.194 (4.374–5.122)	10.455 $\pm$ 0.447 (9.554–11.346)	0.020	0	0	0

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
GM08447 – sensitive apparently normal CIMR female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW126	0 cGy	82	0.098 $\pm$ 0.045 (0.009–0.186)	0.244 $\pm$ 0.051 (0.143–0.345)	0.341 $\pm$ 0.074 (0.194–0.489)	0	0	0	0
PFW126	50 cGy	82	2.061 $\pm$ 0.155 (1.765–2.357)	2.866 $\pm$ 0.143 (2.601–3.131)	4.927 $\pm$ 0.248 (4.456–5.398)	0.012	0.012	0	0.024
PFW126	100 cGy	82	4.548 $\pm$ 0.259 (4.041–5.055)	4.110 $\pm$ 0.139 (3.853–4.367)	8.659 $\pm$ 0.331 (8.018–9.300)	0	0	0	0.012

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-2M – apparently normal Rb family I female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW080	0 cGy	82	0.098 $\pm$ 0.033 (0.032–0.163)	0.354 $\pm$ 0.066 (0.223–0.485)	0.451 $\pm$ 0.078 (0.296–0.606)	0	0	0	0.035
PFW087A	0 cGy	82	0.098 $\pm$ 0.037 (0.023–0.172)	0.110 $\pm$ 0.035 (0.041–0.179)	0.207 $\pm$ 0.048 (0.111–0.303)	0	0	0	0.012
PFW087B	0 cGy	82	0.073 $\pm$ 0.029 (0.016–0.131)	0.195 $\pm$ 0.044 (0.108–0.283)	0.268 $\pm$ 0.055 (0.159–0.378)	0	0	0	0.047
0 cGy Average		246	0.090 $\pm$ 0.057 (0–0.203)	0.220 $\pm$ 0.087 (0.048–0.392)	0.309 $\pm$ 0.107 (0.097–0.521)	0	0	0	0.031
PFW080	50 cGy	70	2.588 $\pm$ 0.187 (2.222–2.955)	3.646 $\pm$ 0.254 (3.158–4.135)	6.235 $\pm$ 0.337 (5.581–6.889)	0.014	0	0	0.125
PFW087A	50 cGy	82	2.212 $\pm$ 0.152 (1.919–2.505)	2.475 $\pm$ 0.111 (2.265–2.685)	4.720 $\pm$ 0.215 (4.303–5.137)	0.024	0	0	0.024
PFW087B	50 cGy	82	2.488 $\pm$ 0.180 (2.134–2.842)	2.842 $\pm$ 0.156 (2.544–3.140)	5.330 $\pm$ 0.278 (4.788–5.872)	0	0	0	0.012
50 cGy Average		234	2.429 $\pm$ 0.301 (1.833–3.025)	2.988 $\pm$ 0.318 (2.358–3.618)	5.428 $\pm$ 0.487 (4.464–6.392)	0.013 $\pm$ 0.007 (0–0.027)	0	0	0.054
PFW080	100 cGy	70	5.260 $\pm$ 0.297 (4.670–5.849)	6.132 $\pm$ 0.244 (5.664–6.560)	11.392 $\pm$ 0.423 (10.563–12.111)	0	0	0	0.067
PFW087A	100 cGy	82	4.841 $\pm$ 0.282 (4.285–5.397)	4.817 $\pm$ 0.160 (4.507–5.127)	9.659 $\pm$ 0.382 (8.905–10.413)	0	0	0.012	0.057
PFW087B	100 cGy	82	4.390 $\pm$ 0.240 (3.915–4.865)	4.195 $\pm$ 0.186 (3.835–4.555)	8.586 $\pm$ 0.360 (7.877–9.295)	0.049	0	0.012	0.047
100 cGy Average		234	4.830 $\pm$ 0.475 (3.900–5.771)	5.048 $\pm$ 0.346 (4.363–5.733)	9.879 $\pm$ 0.674 (8.544–11.214)	0.016 $\pm$ 0.016 (0–0.048)	0	0.008	0.057

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-3R – *Rb1*^{+/-} Rb family I female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW080	0 cGy	82	0.159 $\pm$ 0.047 (0.064–0.253)	0.366 $\pm$ 0.056 (0.254–0.478)	0.524 $\pm$ 0.084 (0.358–0.691)	0	0.012	0.012	0.012
PFW087	0 cGy	82	0.061 $\pm$ 0.032 (0–0.124)	0.159 $\pm$ 0.041 (0.078–0.239)	0.220 $\pm$ 0.058 (0.105–0.334)	0	0	0	0.012
0 cGy Average		164	0.110 $\pm$ 0.057 (0–0.223)	0.263 $\pm$ 0.069 (0.126–0.373)	0.372 $\pm$ 0.102 (0.170–0.574)	0	0.006	0.006	0.012
PFW080	50 cGy	82	2.098 $\pm$ 0.180 (1.753–2.443)	2.049 $\pm$ 0.135 (1.805–2.293)	4.146 $\pm$ 0.255 (3.647–4.645)	0.024	0	0	0.068
PFW087	50 cGy	82	1.768 $\pm$ 0.132 (1.512–2.024)	2.976 $\pm$ 0.106 (2.781–3.171)	4.744 $\pm$ 0.201 (4.360–5.128)	0	0	0	0.012
50 cGy Average		164	1.933 $\pm$ 0.223 (1.491–2.375)	2.513 $\pm$ 0.172 (2.172–2.854)	4.445 $\pm$ 0.325 (3.802–5.089)	0.012 $\pm$ 0.012 (0–0.036)	0	0	0.040
PFW080	100 cGy	70	4.599 $\pm$ 0.284 (4.040–5.157)	5.177 $\pm$ 0.241 (4.709–5.645)	9.776 $\pm$ 0.382 (9.032–10.519)	0	0	0.014	0.028
PFW087	100 cGy	70	3.925 $\pm$ 0.208 (3.514–4.336)	4.770 $\pm$ 0.157 (4.468–5.072)	8.695 $\pm$ 0.301 (8.105–9.284)	0.086	0	0	0.014
100 cGy Average		140	4.262 $\pm$ 0.352 (3.565–4.959)	4.974 $\pm$ 0.288 (4.404–5.544)	9.236 $\pm$ 0.486 (8.274–10.198)	0.043 $\pm$ 0.043 (0–0.128)	0	0.007	0.021

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF- 4F – apparently normal Rb family I male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW080	0 cGy	82	0.073 $\pm$ 0.038 (0.006–0.140)	0.268 $\pm$ 0.055 (0.159–0.378)	0.342 $\pm$ 0.072 (0.198–0.485)	0	0	0	0.012
PFW087	0 cGy	82	0.085 $\pm$ 0.036 (0.015–0.156)	0.207 $\pm$ 0.048 (0.111–0.303)	0.293 $\pm$ 0.066 (0.161–0.424)	0	0	0	0.024
0 cGy Average		164	0.079 $\pm$ 0.052 (0–0.182)	0.238 $\pm$ 0.073 (0.093–0.383)	0.318 $\pm$ 0.098 (0.124–0.512)	0	0	0	0.018
PFW080	50 cGy	58	1.910 $\pm$ 0.199 (1.517–2.302)	2.646 $\pm$ 0.158 (2.349–2.942)	4.555 $\pm$ 0.289 (3.994–5.116)	0	0	0	0.033
PFW087	50 cGy	82	1.415 $\pm$ 0.125 (1.177–1.652)	2.159 $\pm$ 0.110 (1.962–2.356)	3.573 $\pm$ 0.186 (3.227–3.920)	0.012	0	0	0.035
50 cGy Average		140	1.663 $\pm$ 0.235 (1.198–2.128)	2.403 $\pm$ 0.193 (2.021–2.785)	4.064 $\pm$ 0.344 (3.383–4.745)	0.006 $\pm$ 0.012 (0–0.030)	0	0	0.034
PFW080	100 cGy	82	3.854 $\pm$ 0.255 (3.351–4.356)	4.305 $\pm$ 0.213 (3.896–4.714)	8.159 $\pm$ 0.391 (7.395–8.922)	0.037	0	0.012	0.035
PFW087	100 cGy	82	3.744 $\pm$ 0.211 (3.330–4.158)	5.037 $\pm$ 0.152 (4.751–5.323)	8.781 $\pm$ 0.311 (8.177–9.384)	0.024	0	0	0.024
100 cGy Average		164	3.799 $\pm$ 0.331 (3.144–4.454)	4.671 $\pm$ 0.262 (4.152–5.190)	8.470 $\pm$ 0.500 (7.480–9.460)	0.031 $\pm$ 0.007 (0.018–0.044)	0	0.006	0.030

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-6F – apparently normal Rb family II male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW063	0 cGy	50	0.120 ± 0.055 (0.011–0.230)	0.520 ± 0.087 (0.346–0.695)	0.640 ± 0.102 (0.435–0.845)	0	0	0	0.020
PFW117	0 cGy	50	0.120 ± 0.046 (0.027–0.213)	0.380 ± 0.080 (0.219–0.541)	0.500 ± 0.100 (0.299–0.701)	0	0	0.020	0.020
0 cGy Average		100	0.120 ± 0.072 (0–0.263)	0.450 ± 0.118 (0.216–0.684)	0.570 ± 0.143 (0.287–0.853)	0	0	0.010	0.020
PFW063	50 cGy	71	2.176 ± 0.199 (1.793–2.558)	2.381 ± 0.207 (2.006–2.757)	4.557 ± 0.278 (4.041–5.074)	0	0	0	0.066
PFW117	50 cGy	72	2.253 ± 0.194 (1.869–2.637)	2.525 ± 0.145 (2.238–2.812)	4.778 ± 0.255 (4.273–5.283)	0	0	0	0.040
50 cGy Average		143	2.215 ± 0.278 (1.665–2.765)	2.453 ± 0.253 (1.952–2.954)	4.668 ± 0.377 (3.922–5.414)	0	0	0	0.053
PFW063	100 cGy	70	5.094 ± 0.285 (4.536–5.652)	4.509 ± 0.240 (4.062–4.955)	9.603 ± 0.382 (8.869–10.336)	0	0	0	0.079
PFW117	100 cGy	44	4.880 ± 0.396 (4.088–5.672)	5.325 ± 0.264 (4.818–5.831)	10.205 ± 0.589 (9.034–11.375)	0.023	0	0	0.064
100 cGy Average		114	4.987 ± 0.488 (4.021–5.953)	4.917 ± 0.357 (4.210–5.624)	9.904 ± 0.702 (8.514–11.294)	0.012 ± 0.012 (0–0.036)	0	0	0.072

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-7R – *RbI*^{+/-} Rb family II male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW063	0 cGy	50	0.080 $\pm$ 0.048 (0–0.177)	0.420 $\pm$ 0.071 (0.278–0.562)	0.500 $\pm$ 0.077 (0.345–0.655)	0	0	0	0.038
PFW070	0 cGy	82	0.159 $\pm$ 0.053 (0.052–0.265)	0.268 $\pm$ 0.060 (0.149–0.388)	0.427 $\pm$ 0.080 (0.269–0.585)	0	0.024	0	0.012
PFW117	0 cGy	62	0.113 $\pm$ 0.047 (0.020–0.206)	0.371 $\pm$ 0.074 (0.224–0.518)	0.484 $\pm$ 0.094 (0.296–0.672)	0	0	0	0
0 cGy Average		194	0.117 $\pm$ 0.086 (0–0.286)	0.353 $\pm$ 0.118 (0.120–0.586)	0.470 $\pm$ 0.145 (0.184–0.756)	0	0.008	0	0.017
PFW063	50 cGy	70	1.863 $\pm$ 0.193 (1.491–2.235)	2.880 $\pm$ 0.203 (2.501–3.259)	4.743 $\pm$ 0.277 (4.211–5.274)	0	0	0	0.041
PFW070	50 cGy	82	1.488 $\pm$ 0.145 (1.219–1.756)	2.122 $\pm$ 0.151 (1.846–2.398)	3.610 $\pm$ 0.242 (3.155–4.065)	0.024	0.024	0	0.024
PFW117	50 cGy	82	2.277 $\pm$ 0.179 (1.934–2.620)	2.275 $\pm$ 0.151 (2.014–2.537)	4.553 $\pm$ 0.270 (4.050–5.056)	0	0	0	0.012
50 cGy Average		234	1.876 $\pm$ 0.300 (1.285–2.467)	2.426 $\pm$ 0.294 (1.846–3.006)	4.302 $\pm$ 0.456 (3.403–5.201)	0.008 $\pm$ 0.008 (0–0.024)	0.008	0	0.026
PFW063	100 cGy	66	4.632 $\pm$ 0.265 (4.112–5.153)	4.550 $\pm$ 0.283 (4.003–5.097)	9.182 $\pm$ 0.402 (8.395–9.969)	0.015	0	0	0.057
PFW070	100 cGy	82	4.488 $\pm$ 0.311 (3.879–5.097)	3.744 $\pm$ 0.219 (3.326–4.162)	8.232 $\pm$ 0.437 (7.376–9.087)	0	0	0	0.068
PFW117	100 cGy	82	4.692 $\pm$ 0.256 (4.191–5.193)	3.983 $\pm$ 0.144 (3.738–4.228)	8.675 $\pm$ 0.318 (8.071–9.278)	0.012	0	0.012	0.012
100 cGy Average		230	4.604 $\pm$ 0.482 (3.654–5.554)	4.092 $\pm$ 0.385 (3.333–4.851)	8.696 $\pm$ 0.673 (7.370–10.023)	0.009 $\pm$ 0.005 (0–0.018)	0	0.004	0.046

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-10R – *Rbl*^{+/-} Rb family III male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW080	0 cGy	82	0.061 $\pm$ 0.032 (0–0.124)	0.183 $\pm$ 0.046 (0.091–0.275)	0.244 $\pm$ 0.062 (0.122–0.366)	0	0	0	0
PFW117	0 cGy	82	0.098 $\pm$ 0.037 (0.024–0.172)	0.439 $\pm$ 0.074 (0.292–0.586)	0.537 $\pm$ 0.094 (0.350–0.723)	0	0	0	0
0 cGy Average		164	0.080 $\pm$ 0.049 (0–0.177)	0.311 $\pm$ 0.087 (0.139–0.483)	0.391 $\pm$ 0.113 (0.167–0.615)	0	0	0	0
PFW080	50 cGy	82	2.220 $\pm$ 0.174 (1.879–2.560)	2.244 $\pm$ 0.134 (1.995–2.493)	4.463 $\pm$ 0.252 (3.978–4.949)	0.012	0	0	0.012
PFW117	50 cGy	82	3.061 $\pm$ 0.192 (2.687–3.435)	2.342 $\pm$ 0.138 (2.110–2.573)	5.402 $\pm$ 0.281 (4.875–5.930)	0.012	0	0	0.024
50 cGy Average		164	2.641 $\pm$ 0.259 (2.128–3.154)	2.293 $\pm$ 0.192 (1.913–2.673)	4.933 $\pm$ 0.377 (4.187–5.679)	0.012	0	0	0.018
PFW080	100 cGy	82	4.268 $\pm$ 0.292 (3.692–4.845)	4.732 $\pm$ 0.230 (4.284–5.180)	9.000 $\pm$ 0.428 (8.158–9.842)	0.049	0	0	0.012
PFW117	100 cGy	82	5.878 $\pm$ 0.226 (5.435–6.322)	4.768 $\pm$ 0.187 (4.427–5.110)	10.646 $\pm$ 0.299 (10.081–11.212)	0.012	0	0	0.024
100 cGy Average		164	5.073 $\pm$ 0.369 (4.342–5.804)	4.750 $\pm$ 0.296 (4.164–5.336)	9.823 $\pm$ 0.522 (8.789–10.857)	0.031 $\pm$ 0.019 (0–0.068)	0	0	0.018

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-11F – *Rb1*^{+/-} Rb family III male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW080	0 cGy	70	0.143 $\pm$ 0.042 (0.059–0.227)	0.314 $\pm$ 0.066 (0.183–0.446)	0.457 $\pm$ 0.090 (0.277–0.637)	0	0	0	0.091
PFW117	0 cGy	40	0.150 $\pm$ 0.067 (0.014–0.286)	0.225 $\pm$ 0.067 (0.090–0.360)	0.375 $\pm$ 0.106 (0.162–0.588)	0	0	0	0.024
0 cGy Average		110	0.147 $\pm$ 0.079 (0–0.303)	0.270 $\pm$ 0.094 (0.084–0.456)	0.416 $\pm$ 0.139 (0.141–0.691)	0	0	0	0.058
PFW080	50 cGy	35	2.686 $\pm$ 0.264 (2.156–3.216)	2.829 $\pm$ 0.227 (2.387–3.271)	5.514 $\pm$ 0.363 (4.800–6.229)	0.029	0	0	0.054
PFW117	50 cGy	30	2.583 $\pm$ 0.214 (2.168–2.998)	3.908 $\pm$ 0.190 (3.532–4.284)	6.492 $\pm$ 0.298 (5.923–7.062)	0	0	0	0.032
50 cGy Average		65	2.635 $\pm$ 0.340 (1.962–3.308)	3.369 $\pm$ 0.296 (2.783–3.955)	6.003 $\pm$ 0.470 (5.072–6.934)	0.015 $\pm$ 0.015 (0–0.045)	0	0	0.043
PFW080	100 cGy	53	4.065 $\pm$ 0.302 (3.464–4.665)	4.176 $\pm$ 0.249 (3.694–4.659)	8.241 $\pm$ 0.423 (7.411–9.071)	0.019	0.019	0.019	0
PFW117	100 cGy	28	5.100 $\pm$ 0.510 (4.063–6.137)	5.954 $\pm$ 0.363 (5.222–6.686)	11.054 $\pm$ 0.642 (9.783–12.325)	0	0	0	0.034
100 cGy Average		81	4.583 $\pm$ 0.593 (3.409–5.757)	5.065 $\pm$ 0.440 (4.194–5.936)	9.648 $\pm$ 0.769 (8.125–11.171)	0.010 $\pm$ 0.010 (0–0.030)	0.010	0.010	0.017

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-12M – apparently normal Rb family III female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW080	0 cGy	82	0.098 ± 0.045 (0.009–0.186)	0.378 ± 0.062 (0.255–0.501)	0.476 ± 0.084 (0.309–0.642)	0	0	0	0.047
PFW117A	0 cGy	80	0.088 ± 0.036 (0.015–0.160)	0.300 ± 0.052 (0.197–0.403)	0.388 ± 0.058 (0.273–0.502)	0	0	0	0.012
PFW117B	0 cGy	82	0.122 ± 0.040 (0.042–0.202)	0.366 ± 0.061 (0.244–0.488)	0.488 ± 0.085 (0.318–0.658)	0	0	0	0.024
0 cGy Average		244	0.103 ± 0.070 (0–0.242)	0.348 ± 0.101 (0.148–0.548)	0.451 ± 0.133 (0.188–0.714)	0	0	0	0.028
PFW080	50 cGy	82	2.122 ± 0.146 (1.846–2.398)	2.329 ± 0.133 (2.094–2.565)	4.451 ± 0.216 (4.055–4.848)	0	0	0	0.079
PFW117A	50 cGy	82	3.144 ± 0.212 (2.728–3.560)	2.834 ± 0.136 (2.583–3.085)	5.978 ± 0.276 (5.441–6.516)	0.024	0	0	0.012
PFW117B	50 cGy	82	2.415 ± 0.178 (2.069–2.761)	3.219 ± 0.148 (2.952–3.486)	5.634 ± 0.279 (5.105–6.163)	0.024	0	0	0
50 cGy Average		246	2.560 ± 0.313 (1.940–3.180)	2.794 ± 0.241 (2.317–3.271)	5.354 ± 0.448 (4.467–6.241)	0.016 ± 0.008 (0–0.032)	0	0	0.030
PFW080	100 cGy	82	5.756 ± 0.325 (5.116–6.397)	4.378 ± 0.223 (3.952–4.805)	10.134 ± 0.432 (9.291–10.978)	0.012	0	0	0.024
PFW117A	100 cGy	82	6.242 ± 0.251 (5.594–6.736)	5.042 ± 0.177 (4.705–5.378)	11.283 ± 0.331 (10.635–11.931)	0	0	0	0
PFW117B	100 cGy	82	4.598 ± 0.241 (4.124–5.072)	4.988 ± 0.175 (4.662–5.335)	9.585 ± 0.356 (8.896–10.274)	0.012	0	0	0.012
100 cGy Average		246	5.532 ± 0.476 (4.590–6.474)	4.803 ± 0.334 (4.142–5.464)	10.334 ± 0.650 (9.047–11.621)	0.008 ± 0.004 (0–0.016)	0	0	0.012

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-13M – apparently normal Rb family IV female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW063	0 cGy	50	0.140 $\pm$ 0.070 (0–0.281)	0.500 $\pm$ 0.092 (0.316–0.684)	0.640 $\pm$ 0.127 (0.385–0.895)	0	0	0.020	0.038
PFW123A	0 cGy	82	0.110 $\pm$ 0.035 (0.041–0.179)	0.268 $\pm$ 0.055 (0.159–0.378)	0.378 $\pm$ 0.066 (0.246–0.510)	0	0	0.035	0
PFW123B	0 cGy	82	0.244 $\pm$ 0.059 (0.127–0.361)	0.232 $\pm$ 0.053 (0.126–0.337)	0.476 $\pm$ 0.094 (0.289–0.662)	0	0	0	0.012
0 cGy Average		214	0.165 $\pm$ 0.098 (0–0.357)	0.333 $\pm$ 0.119 (0.099–0.568)	0.498 $\pm$ 0.171 (0.160–0.835)	0	0	0.018	0.016
PFW063	50 cGy	73	1.970 $\pm$ 0.206 (1.584–2.356)	2.719 $\pm$ 0.180 (2.410–3.029)	4.689 $\pm$ 0.284 (4.183–5.194)	0	0	0	0.014
PFW123A	50 cGy	82	2.756 $\pm$ 0.210 (2.345–3.167)	3.037 $\pm$ 0.164 (2.728–3.345)	5.793 $\pm$ 0.317 (5.176–6.409)	0.012	0	0	0.024
PFW123B	50 cGy	82	2.329 $\pm$ 0.188 (1.974–2.685)	2.707 $\pm$ 0.134 (2.462–2.952)	5.037 $\pm$ 0.270 (4.532–5.541)	0.012	0	0.068	0.012
50 cGy Average		237	2.516 $\pm$ 0.349 (1.829–3.204)	2.821 $\pm$ 0.278 (2.273–3.369)	5.173 $\pm$ 0.504 (4.180–6.165)	0.008 $\pm$ 0.004 (0–0.016)	0	0.023	0.016
PFW063	100 cGy	66	3.830 $\pm$ 0.261 (3.327–4.333)	4.742 $\pm$ 0.236 (4.309–5.176)	8.572 $\pm$ 0.402 (7.810–9.334)	0.015	0	0.015	0.043
PFW123A	100 cGy	80	5.640 $\pm$ 0.318 (5.011–6.269)	5.669 $\pm$ 0.191 (5.306–6.033)	11.310 $\pm$ 0.403 (10.519–12.100)	0.013	0	0	0.036
PFW123B	100 cGy	82	5.793 $\pm$ 0.304 (5.199–6.386)	5.390 $\pm$ 0.211 (4.984–5.797)	11.183 $\pm$ 0.398 (10.414–11.952)	0.012	0	0.099	0.012
100 cGy Average		228	5.088 $\pm$ 0.512 (4.080–6.096)	5.267 $\pm$ 0.369 (4.540–5.995)	10.355 $\pm$ 0.694 (8.987–11.723)	0.013 $\pm$ 0.001 (0.011–0.015)	0	0.038	0.030

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-14R – *Rb1*^{+/-} Rb family IV female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW063	0 cGy	50	0.220 $\pm$ 0.077 (0.065–0.375)	0.400 $\pm$ 0.076 (0.248–0.552)	0.620 $\pm$ 0.107 (0.406–0.834)	0	0	0	0.038
PFW070	0 cGy	82	0.134 $\pm$ 0.045 (0.044–0.224)	0.256 $\pm$ 0.052 (0.154–0.359)	0.390 $\pm$ 0.077 (0.237–0.544)	0	0	0	0.024
PFW123	0 cGy	82	0.183 $\pm$ 0.050 (0.085–0.281)	0.293 $\pm$ 0.061 (0.171–0.415)	0.476 $\pm$ 0.080 (0.317–0.635)	0	0	0	0.024
0 cGy Average		214	0.179 $\pm$ 0.169 (0–0.512)	0.316 $\pm$ 0.110 (0.099–0.533)	0.495 $\pm$ 0.154 (0.192–0.798)	0	0	0	0.029
PFW063	50 cGy	69	1.186 $\pm$ 0.168 (0.888–1.484)	2.441 $\pm$ 0.193 (2.087–2.794)	3.626 $\pm$ 0.271 (3.130–4.123)	0	0	0	0.014
PFW070	50 cGy	82	1.561 $\pm$ 0.165 (1.245–1.877)	2.024 $\pm$ 0.149 (1.746–2.303)	3.585 $\pm$ 0.248 (3.116–4.055)	0.012	0	0	0.024
PFW123	50 cGy	82	1.829 $\pm$ 0.173 (1.499–2.160)	2.110 $\pm$ 0.135 (1.871–2.349)	3.939 $\pm$ 0.260 (3.447–4.431)	0.012	0	0	0
50 cGy Average		233	1.525 $\pm$ 0.292 (0.950–2.101)	2.192 $\pm$ 0.279 (1.643–2.740)	3.717 $\pm$ 0.450 (2.830–4.603)	0.008 $\pm$ 0.004 (0–0.016)	0	0	0.013
PFW063	100 cGy	69	2.910 $\pm$ 0.283 (2.367–3.454)	5.296 $\pm$ 0.263 (4.794–5.797)	8.206 $\pm$ 0.382 (7.475–8.937)	0	0	0	0.014
PFW070	100 cGy	82	3.561 $\pm$ 0.258 (3.056–4.066)	3.573 $\pm$ 0.183 (3.225–3.922)	7.134 $\pm$ 0.311 (6.534–7.734)	0.012	0	0	0.012
PFW123	100 cGy	82	4.403 $\pm$ 0.268 (3.879–4.926)	4.378 $\pm$ 0.202 (3.995–4.761)	8.781 $\pm$ 0.365 (8.072–9.489)	0	0	0	0.012
100 cGy Average		233	3.625 $\pm$ 0.467 (2.704–4.545)	4.416 $\pm$ 0.378 (3.671–5.161)	8.040 $\pm$ 0.613 (6.833–9.248)	0.004 $\pm$ 0.004 (0–0.012)	0	0	0.013

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-15F – apparently normal Rb family IV male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW063	0 cGy	50	0.180 ± 0.074 (0.032–0.329)	0.360 ± 0.074 (0.211–0.509)	0.540 ± 0.100 (0.339–0.741)	0	0	0.020	0.020
PFW070	0 cGy	82	0.159 ± 0.051 (0.058–0.259)	0.402 ± 0.067 (0.269–0.536)	0.561 ± 0.095 (0.372–0.750)	0	0	0	0.248
PFW123	0 cGy	82	0.122 ± 0.040 (0.042–0.202)	0.329 ± 0.063 (0.205–0.454)	0.451 ± 0.074 (0.304–0.598)	0	0	0	0.226
0 cGy Average		214	0.154 ± 0.098 (0–0.347)	0.364 ± 0.118 (0.131–0.596)	0.517 ± 0.156 (0.209–0.826)	0	0	0.007	0.165
PFW063	50 cGy	68	1.732 ± 0.211 (1.338–2.126)	2.949 ± 0.247 (2.479–3.419)	4.681 ± 0.310 (4.095–5.266)	0	0	0.014	0.014
PFW070	50 cGy	82	1.707 ± 0.186 (1.351–2.064)	2.085 ± 0.152 (1.813–2.358)	3.793 ± 0.278 (3.273–4.312)	0.037	0	0	0.204
PFW123	50 cGy	82	2.707 ± 0.203 (2.312–3.102)	2.744 ± 0.151 (2.470–3.018)	5.451 ± 0.289 (4.895–6.008)	0.012	0	0.057	0.261
50 cGy Average		232	2.049 ± 0.347 (1.366–2.732)	2.593 ± 0.327 (1.948–3.237)	4.642 ± 0.507 (3.643–5.640)	0.016 ± 0.011 (0–0.038)	0	0.024	0.160
PFW063	100 cGy	86	3.750 ± 0.282 (3.209–4.291)	5.187 ± 0.230 (4.754–5.619)	8.937 ± 0.418 (8.130–9.743)	0.012	0	0.011	0.044
PFW070	100 cGy	54	4.527 ± 0.367 (3.798–5.255)	4.116 ± 0.231 (3.673–4.559)	8.643 ± 0.478 (7.703–9.583)	0.019	0	0.036	0.308
PFW123	100 cGy	82	5.622 ± 0.313 (5.005–6.239)	5.402 ± 0.207 (5.010–5.795)	11.024 ± 0.413 (10.215–11.834)	0.012	0	0.012	0.212
100 cGy Average		222	4.633 ± 0.559 (3.532–5.733)	4.902 ± 0.386 (4.142–5.661)	9.535 ± 0.758 (8.042–11.027)	0.014 ± 0.002 (0.009–0.019)	0	0.020	0.188

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-16M – apparently normal Rb family V female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW093A	0 cGy	82	0.171 $\pm$ 0.052 (0.068–0.273)	0.305 $\pm$ 0.059 (0.187–0.423)	0.476 $\pm$ 0.078 (0.320–0.631)	0	0.012	0	0.068
PFW093B	0 cGy	82	0.220 $\pm$ 0.070 (0.081–0.358)	0.305 $\pm$ 0.064 (0.177–0.433)	0.524 $\pm$ 0.102 (0.322–0.726)	0	0	0.012	0.035
PFW125	0 cGy	82	0.085 $\pm$ 0.031 (0.024–0.147)	0.366 $\pm$ 0.064 (0.239–0.493)	0.451 $\pm$ 0.076 (0.300–0.602)	0	0	0.035	0.089
0 cGy Average		246	0.159 $\pm$ 0.092 (0–0.340)	0.325 $\pm$ 0.108 (0.112–0.538)	0.484 $\pm$ 0.149 (0.191–0.777)	0	0.004	0.016	0.064
PFW093A	50 cGy	82	2.085 $\pm$ 0.175 (1.753–2.418)	2.415 $\pm$ 0.139 (2.165–2.664)	4.500 $\pm$ 0.236 (4.057–4.944)	0	0	0	0.172
PFW093B	50 cGy	82	2.049 $\pm$ 0.186 (1.705–2.392)	2.293 $\pm$ 0.142 (2.042–2.544)	4.342 $\pm$ 0.272 (3.839–4.844)	0.061	0	0.024	0.180
PFW125	50 cGy	82	2.146 $\pm$ 0.146 (1.863–2.430)	3.146 $\pm$ 0.151 (2.874–3.419)	5.293 $\pm$ 0.241 (4.838–5.748)	0.024	0	0.024	0.128
50 cGy Average		246	2.094 $\pm$ 0.294 (1.514–2.673)	2.618 $\pm$ 0.249 (2.127–3.109)	4.711 $\pm$ 0.434 (3.857–5.565)	0.028 $\pm$ 0.018 (0–0.063)	0	0.016	0.160
PFW093A	100 cGy	82	4.024 $\pm$ 0.264 (3.510–4.539)	4.415 $\pm$ 0.187 (4.062–4.767)	8.439 $\pm$ 0.364 (7.731–9.147)	0.037	0	0.047	0.099
PFW093B	100 cGy	82	4.537 $\pm$ 0.286 (3.985–5.089)	4.183 $\pm$ 0.179 (3.851–4.515)	8.720 $\pm$ 0.386 (7.979–9.461)	0.037	0	0.024	0.118
PFW125	100 cGy	82	3.793 $\pm$ 0.266 (3.267–4.318)	4.463 $\pm$ 0.216 (4.054–4.873)	8.256 $\pm$ 0.398 (7.478–9.034)	0.037	0	0.024	0.163
100 cGy Average		246	4.118 $\pm$ 0.462 (3.207–5.028)	4.354 $\pm$ 0.337 (3.690–5.017)	8.472 $\pm$ 0.664 (7.164–9.779)	0.037	0	0.032	0.127

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-17R – *Rbl*^{+/-} Rb family V female fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW093A	0 cGy	82	0.110 $\pm$ 0.043 (0.025–0.194)	0.232 $\pm$ 0.053 (0.126–0.337)	0.342 $\pm$ 0.072 (0.198–0.485)	0	0	0	0.047
PFW093B	0 cGy	82	0.207 $\pm$ 0.062 (0.084–0.331)	0.244 $\pm$ 0.054 (0.137–0.351)	0.451 $\pm$ 0.092 (0.268–0.634)	0	0	0	0.024
PFW125	0 cGy	82	0.024 $\pm$ 0.017 (0–0.059)	0.159 $\pm$ 0.044 (0.071–0.246)	0.183 $\pm$ 0.046 (0.091–0.275)	0	0	0	0.109
0 cGy Average		246	0.114 $\pm$ 0.077 (0–0.266)	0.211 $\pm$ 0.087 (0.039–0.383)	0.325 $\pm$ 0.126 (0.077–0.573)	0	0	0	0.060
PFW093A	50 cGy	82	2.963 $\pm$ 0.205 (2.564–3.363)	2.707 $\pm$ 0.175 (2.376–2.882)	5.671 $\pm$ 0.302 (5.087–6.254)	0.012	0	0	0.089
PFW093B	50 cGy	82	1.659 $\pm$ 0.159 (1.368–1.949)	2.220 $\pm$ 0.139 (1.965–2.474)	3.878 $\pm$ 0.239 (3.439–4.318)	0.012	0	0	0.047
PFW125	50 cGy	82	1.646 $\pm$ 0.158 (1.334–1.959)	2.598 $\pm$ 0.142 (2.329–2.866)	4.244 $\pm$ 0.260 (3.734–4.754)	0	0	0	0.118
50 cGy Average		246	2.089 $\pm$ 0.304 (1.491–2.688)	2.508 $\pm$ 0.265 (1.987–3.029)	4.598 $\pm$ 0.465 (3.682–5.514)	0.008 $\pm$ 0.004 (0–0.016)	0	0	0.085
PFW093A	100 cGy	82	4.451 $\pm$ 0.263 (3.934–4.968)	4.317 $\pm$ 0.186 (3.963–4.671)	8.768 $\pm$ 0.382 (8.021–9.515)	0.085	0	0	0.035
PFW093B	100 cGy	82	4.342 $\pm$ 0.269 (3.820–4.863)	3.842 $\pm$ 0.162 (3.538–4.145)	8.183 $\pm$ 0.339 (7.533–8.833)	0.012	0	0	0.035
PFW125	100 cGy	82	4.244 $\pm$ 0.262 (3.724–4.764)	4.793 $\pm$ 0.212 (4.380–5.205)	9.037 $\pm$ 0.395 (8.257–9.816)	0.012	0	0	0.079
100 cGy Average		246	4.346 $\pm$ 0.459 (3.442–5.249)	4.317 $\pm$ 0.325 (3.677–4.957)	8.663 $\pm$ 0.646 (7.390–9.935)	0.037 $\pm$ 0.024 (0–0.085)	0	0	0.050

Table 4.6. G2 chromosomal radiosensitivity assay experimental results:
MF-18F – apparently normal Rb family V male fibroblast strain.

Experiment	Dose (cGy)	n	Induced Chromatid-Type Aberrations per Cell ($\pm$ SEM)				Chromosome-Type Aberrations per Cell	Endoredup. Frequency	Tetraploidy Frequency
			Breaks	Gaps	Breaks and Gaps	Exchanges			
PFW093A	0 cGy	82	0.061 $\pm$ 0.027 (0.008–0.114)	0.171 $\pm$ 0.042 (0.088–0.254)	0.232 $\pm$ 0.053 (0.126–0.337)	0	0	0	0.057
PFW093B	0 cGy	82	0.110 $\pm$ 0.043 (0.025–0.194)	0.183 $\pm$ 0.046 (0.091–0.275)	0.293 $\pm$ 0.061 (0.171–0.415)	0	0	0.012	0.035
PFW125	0 cGy	82	0.049 $\pm$ 0.030 (0–0.108)	0.232 $\pm$ 0.050 (0.132–0.331)	0.281 $\pm$ 0.066 (0.150–0.411)	0	0	0	0.137
0 cGy Average		246	0.073 $\pm$ 0.058 (0–0.188)	0.195 $\pm$ 0.080 (0.038–0.353)	0.268 $\pm$ 0.104 (0.063–0.474)	0	0	0.004	0.076
PFW093A	50 cGy	82	2.268 $\pm$ 0.208 (1.858–2.678)	3.037 $\pm$ 0.175 (2.699–3.374)	5.305 $\pm$ 0.294 (4.729–5.881)	0.012	0	0	0.079
PFW093B	50 cGy	82	2.329 $\pm$ 0.183 (1.975–2.683)	2.793 $\pm$ 0.157 (2.494–3.092)	5.122 $\pm$ 0.252 (4.636–5.608)	0	0	0	0.109
PFW125	50 cGy	82	1.744 $\pm$ 0.182 (1.387–2.101)	2.720 $\pm$ 0.139 (2.461–2.978)	4.463 $\pm$ 0.260 (3.962–4.965)	0	0	0.024	0.099
50 cGy Average		246	2.114 $\pm$ 0.331 (1.461–2.766)	2.850 $\pm$ 0.273 (2.311–3.388)	4.963 $\pm$ 0.467 (4.044–5.882)	0.004 $\pm$ 0.004 (0–0.012)	0	0.008	0.096
PFW093A	100 cGy	80	5.002 $\pm$ 0.287 (4.434–5.570)	6.167 $\pm$ 0.201 (5.776–6.558)	11.168 $\pm$ 0.394 (10.391–11.945)	0.025	0	0	0.101
PFW093B	100 cGy	80	4.853 $\pm$ 0.306 (4.250–5.455)	5.805 $\pm$ 0.198 (5.421–6.188)	10.657 $\pm$ 0.413 (9.845–11.469)	0	0	0	0.059
PFW125	100 cGy	82	3.256 $\pm$ 0.203 (2.857–3.655)	4.988 $\pm$ 0.198 (4.606–5.369)	8.244 $\pm$ 0.332 (7.597–8.891)	0	0	0	0.068
100 cGy Average		242	4.370 $\pm$ 0.466 (3.453–5.287)	5.653 $\pm$ 0.345 (4.974–6.332)	10.023 $\pm$ 0.660 (8.723–11.323)	0.008 $\pm$ 0.008 (0–0.024)	0	0	0.076

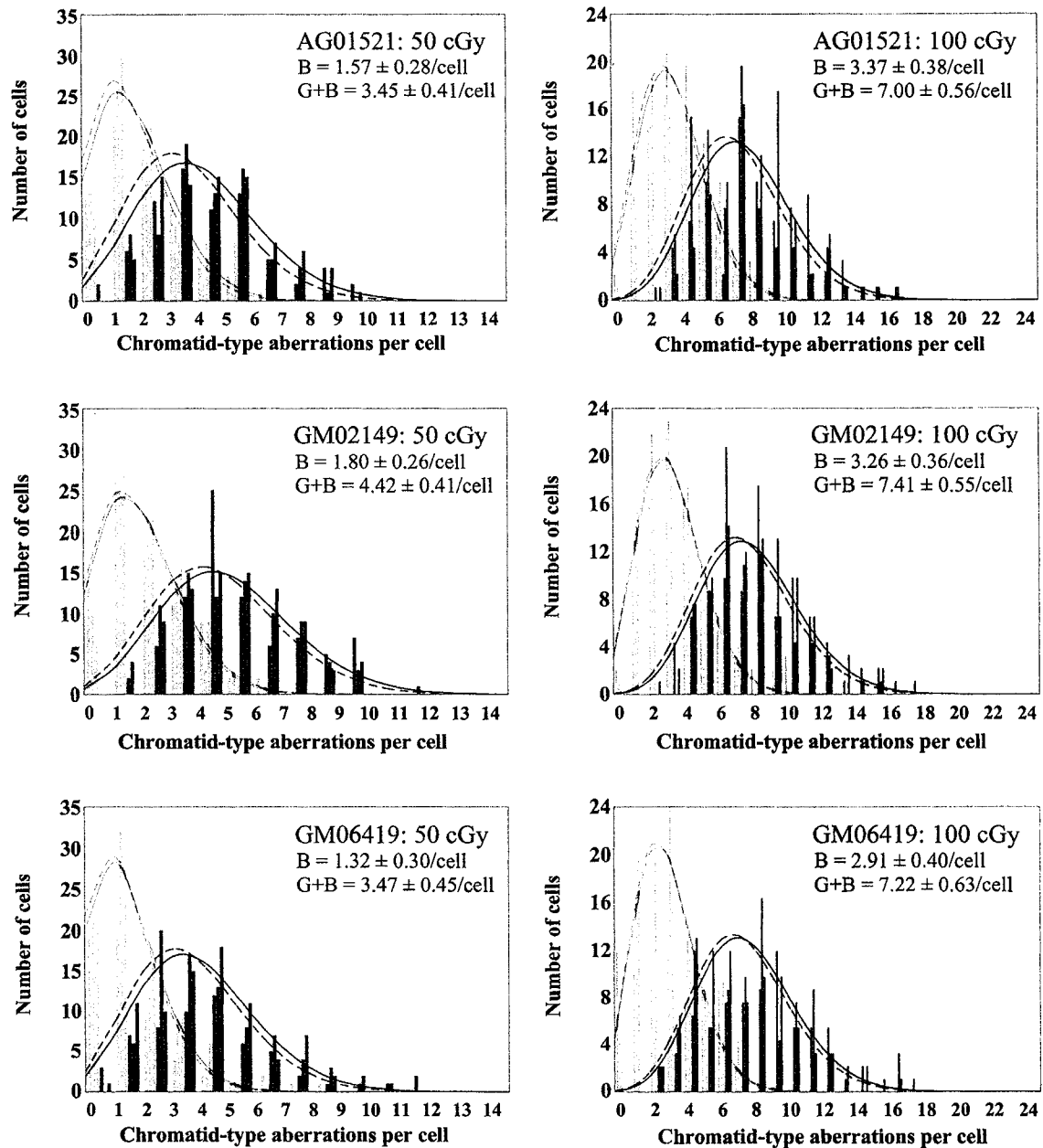


Figure 4.7. G2 chromosomal radiosensitivity assay data histograms and calculated Poisson distributions for the resistant CIMR fibroblast controls: apparently normal strains AG01521 and GM02149 and *RbI*^{+/−} strain GM06419. Light gray bars represent total chromatin breaks and dark gray bars represent total combined chromatin gaps and breaks for three independent experiments (see Table 4.6). Light and dark gray curves represent Poisson distributions with means of the total (solid lines) and induced (0 cGy background-corrected, dashed lines) chromatin break and combined chromatin gap and break data, respectively.

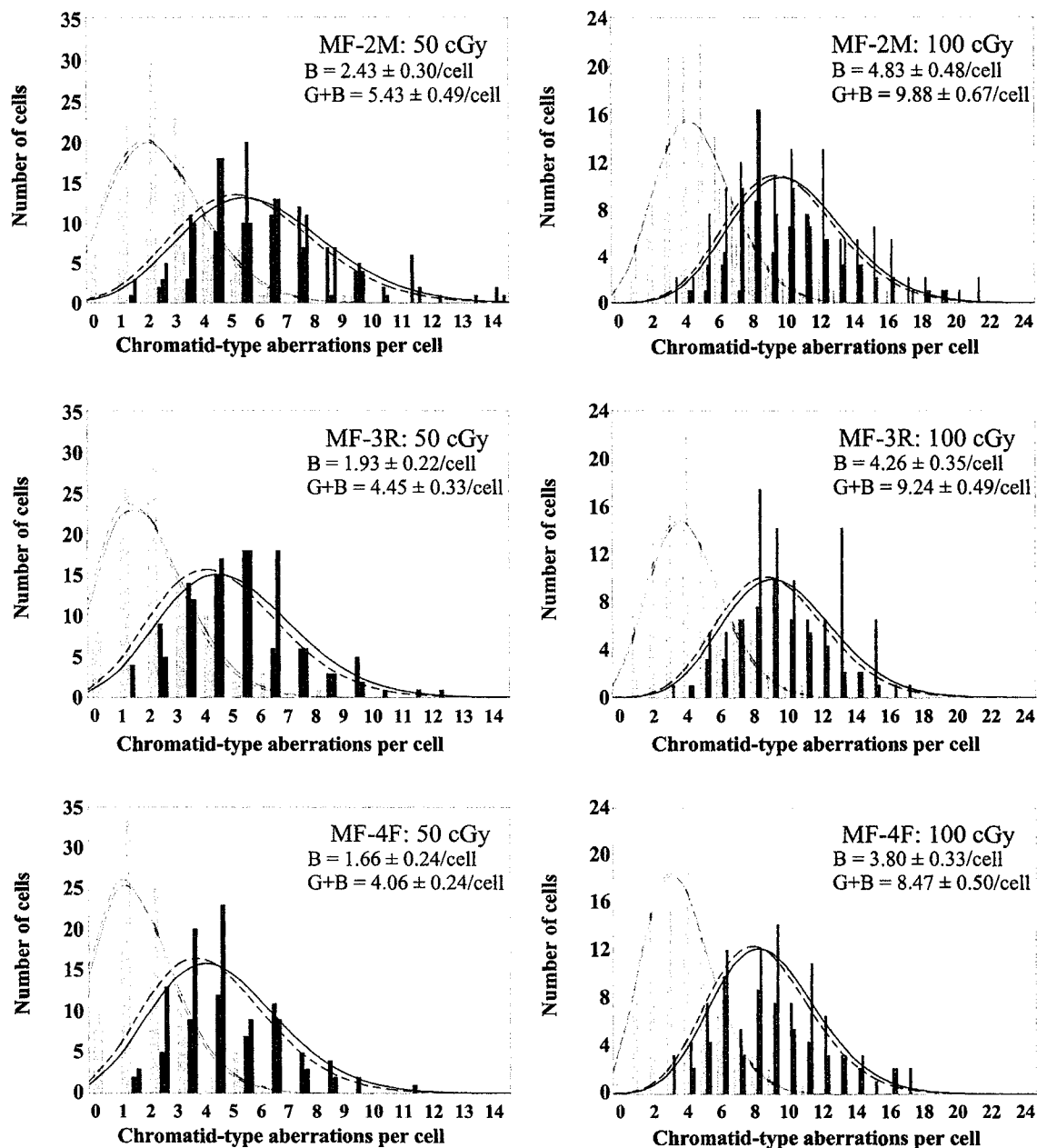


Figure 4.8. G2 chromosomal radiosensitivity assay data histograms and calculated Poisson distributions for members of Rb Family I: apparently normal strains MF-2M and MF-4F and *Rbl*^{+/−} strain MF-3R. Light gray bars represent total chromatin breaks and dark gray bars represent total combined chromatin gaps and breaks for two to three independent experiments (see Table 4.6). Light and dark gray curves represent Poisson distributions with means of the total (solid lines) and induced (0 cGy background-corrected, dashed lines) chromatin break and combined chromatin gap and break data, respectively.

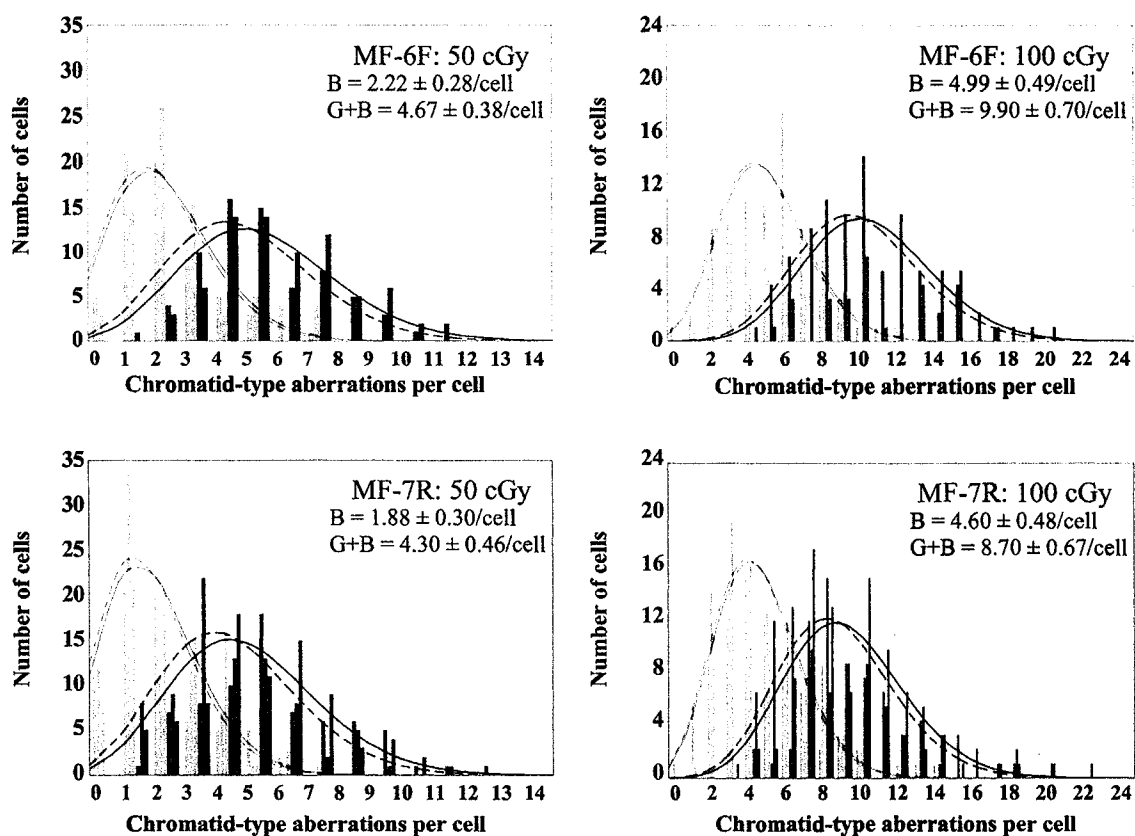


Figure 4.9. G2 chromosomal radiosensitivity assay data histograms and calculated Poisson distributions for members of Rb Family II: apparently normal strain MF-6F and *Rb1*^{+/-} strain MF-7R. Light gray bars represent total chromatid breaks and dark gray bars represent total combined chromatid gaps and breaks for two to three independent experiments (see Table 4.6). Light and dark gray curves represent Poisson distributions with means of the total (solid lines) and induced (0 cGy background-corrected, dashed lines) chromatid break and combined chromatid gap and break data, respectively.

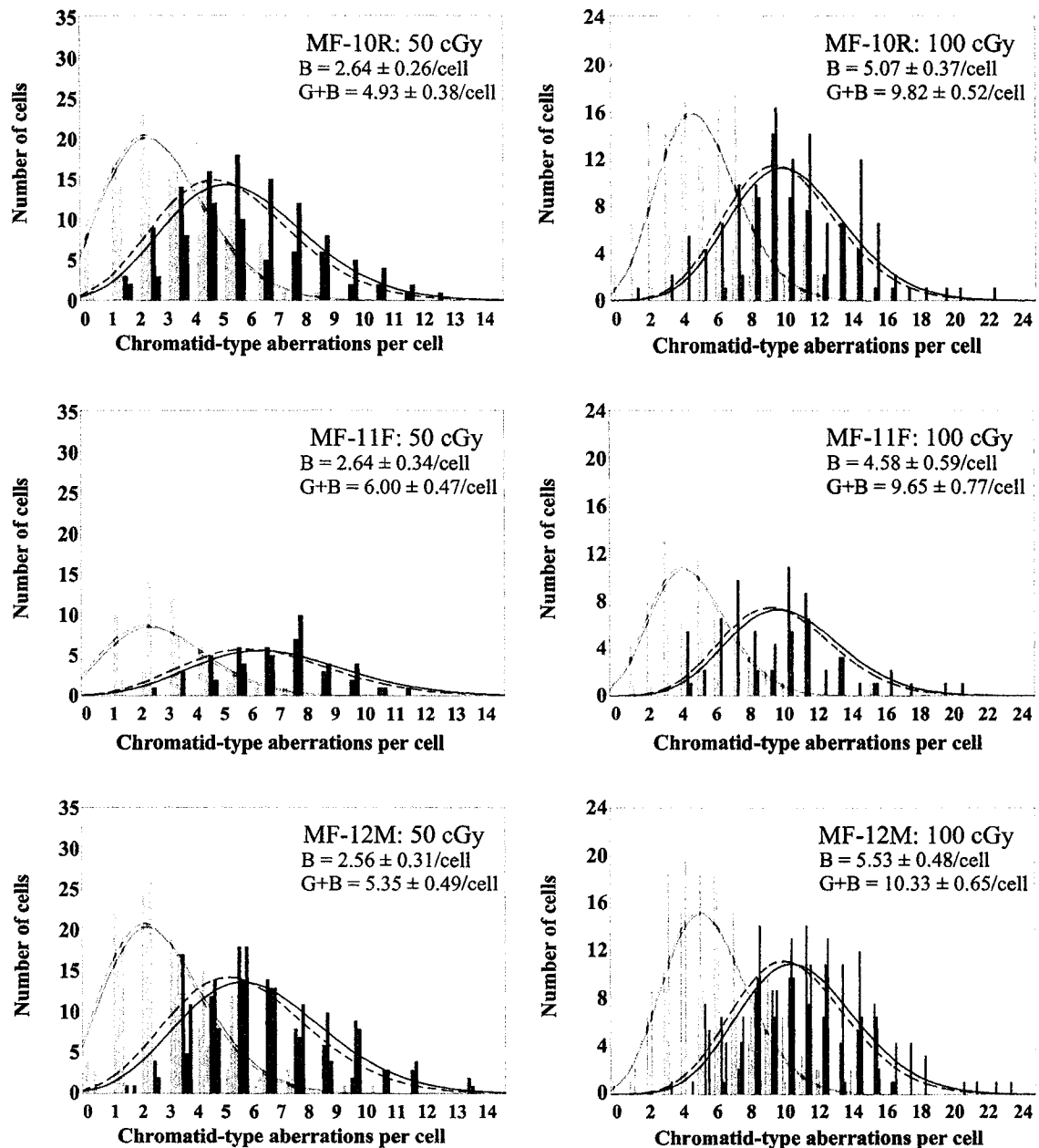


Figure 4.10. G2 chromosomal radiosensitivity assay data histograms and calculated Poisson distributions for members of Rb Family III: apparently normal strain MF-12M and *Rb1*^{+/−} strains MF-10R and MF-11F. Light gray bars represent total chromatin breaks and dark gray bars represent total combined chromatin gaps and breaks for two to three independent experiments (see Table 4.6). Light and dark gray curves represent Poisson distributions with means of the total (solid lines) and induced (0 cGy background-corrected, dashed lines) chromatin break and combined chromatin gap and break data, respectively.

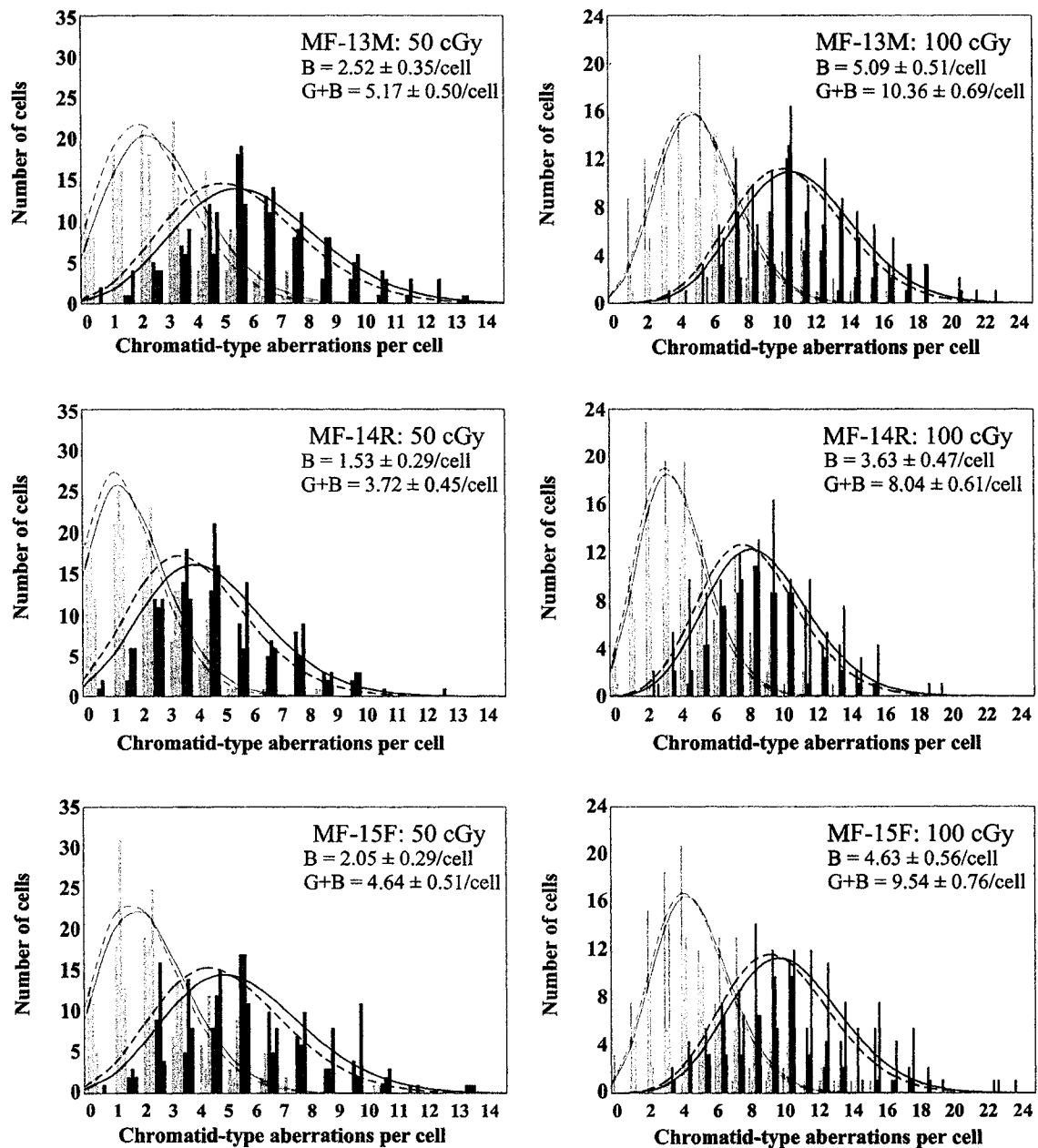


Figure 4.11. G2 chromosomal radiosensitivity assay data histograms and calculated Poisson distributions for members of Rb Family IV: apparently normal strains MF-13M and MF-15F and *Rb1*^{+/+} strain MF-14R. Light gray bars represent total chromatid breaks and dark gray bars represent total combined chromatid gaps and breaks for three independent experiments (see Table 4.6). Light and dark gray curves represent Poisson distributions with means of the total (solid lines) and induced (0 cGy background-corrected, dashed lines) chromatid break and combined chromatid gap and break data, respectively.

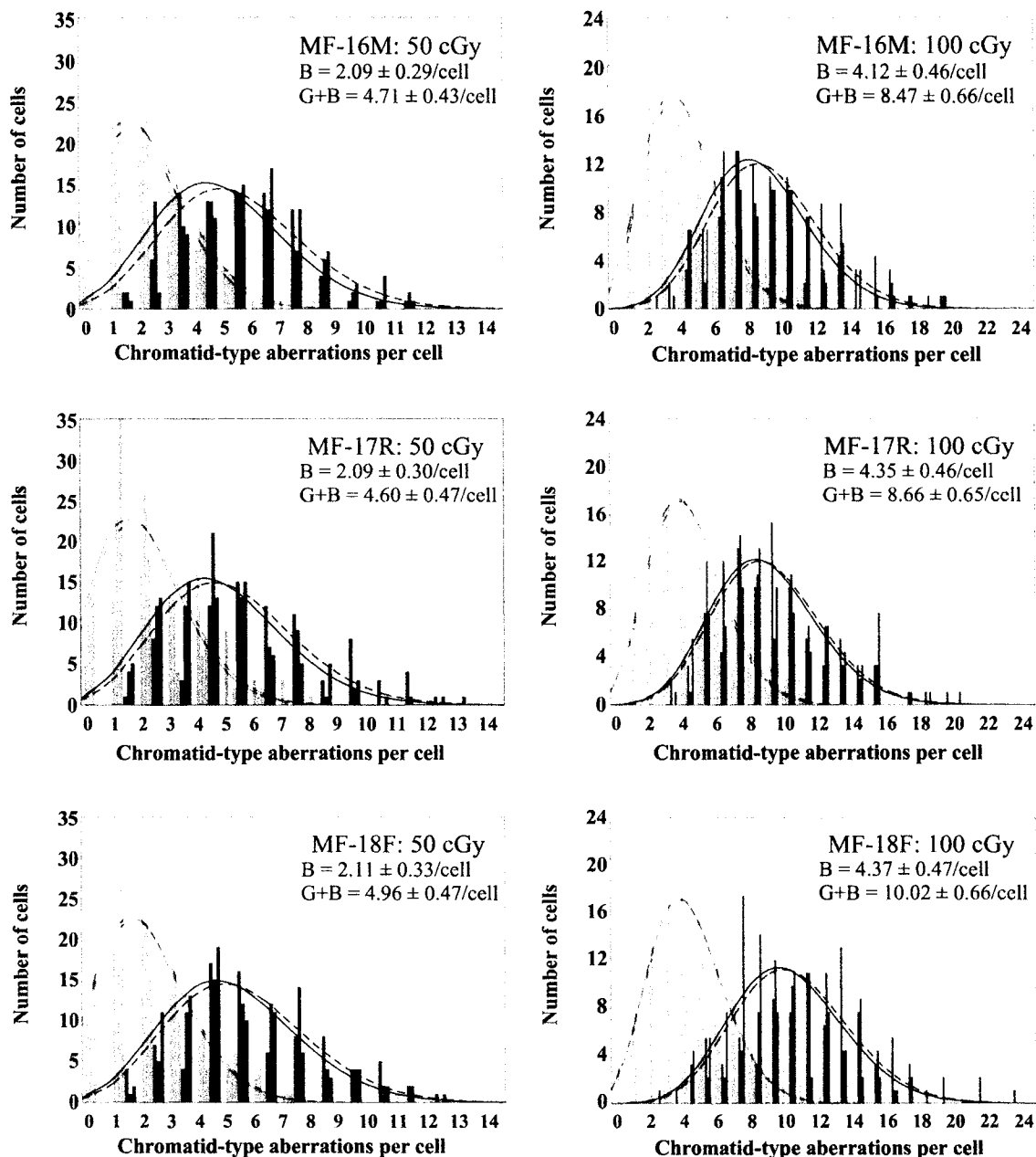


Figure 4.12. G2 chromosomal radiosensitivity assay data histograms and calculated Poisson distributions for members of Rb Family V: apparently normal strains MF-16M and MF-18F and *Rbl*^{+/-} strain MF-17R. Light gray bars represent total chromatid breaks and dark gray bars represent total combined chromatid gaps and breaks for three independent experiments (see Table 4.6). Light and dark gray curves represent Poisson distributions with means of the total (solid lines) and induced (0 cGy background-corrected, dashed lines) chromatid break and combined chromatid gap and break data, respectively.

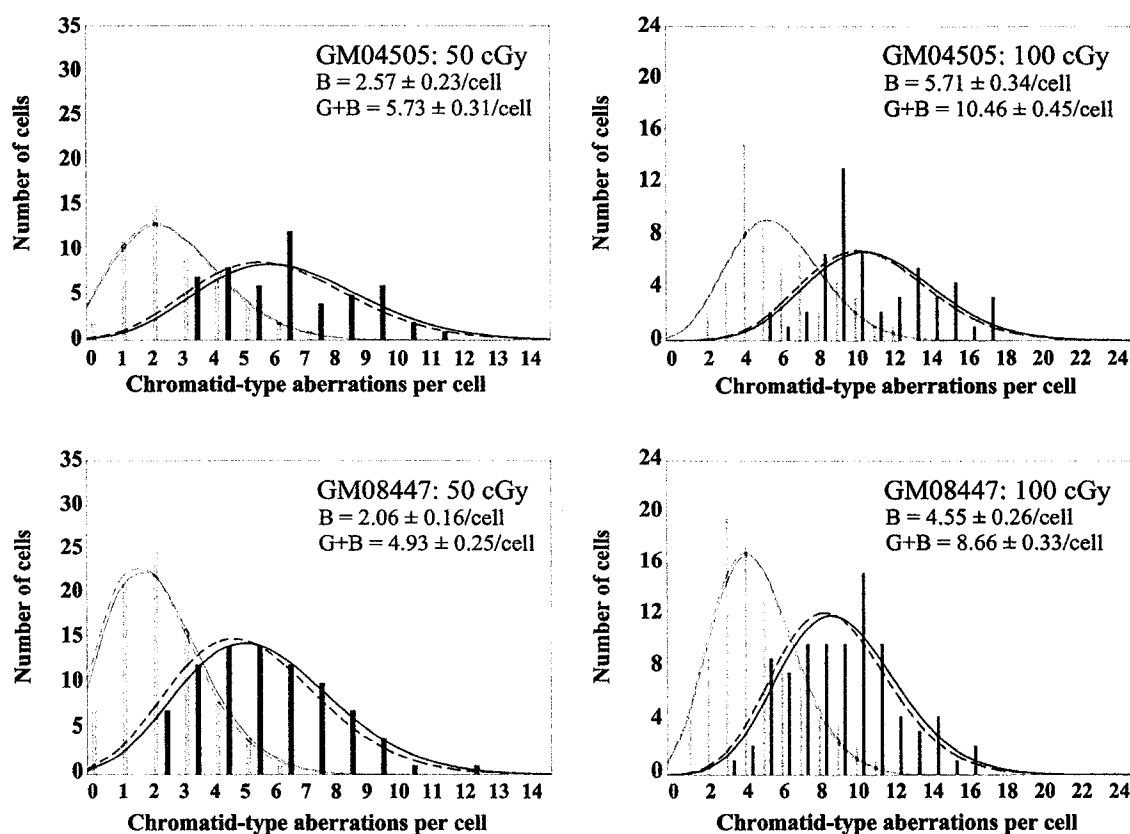


Figure 4.13. G2 chromosomal radiosensitivity assay data histograms and calculated Poisson distributions for the sensitive CIMR fibroblast controls: apparently normal strains GM04505 and GM08447. Light gray bars represent total chromatid breaks and dark gray bars represent total combined chromatid gaps and breaks for one experiment only (see Table 4.6). Light and dark gray curves represent Poisson distributions with means of the total (solid lines) and induced (0 cGy background-corrected, dashed lines) chromatid break and combined chromatid gap and break data, respectively.

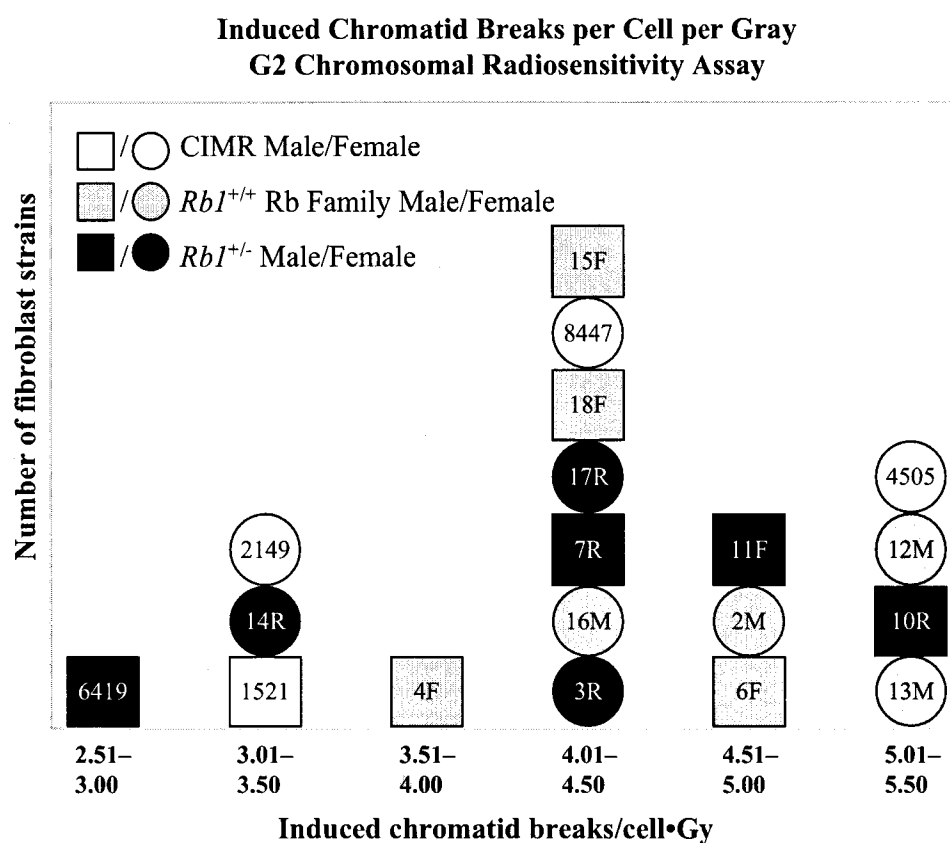


Figure 4.14. Distribution of radiation-induced chromatid breaks per cell per Gray for the 14 Rb family member and 5 apparently normal and *RbI*^{+/-} CIMR control fibroblast strains examined in the G2 chromosomal radiosensitivity assay (see Table 4.4).

**Induced Total Chromatid Gaps and Breaks per Cell per Gray
G2 Chromosomal Radiosensitivity Assay**

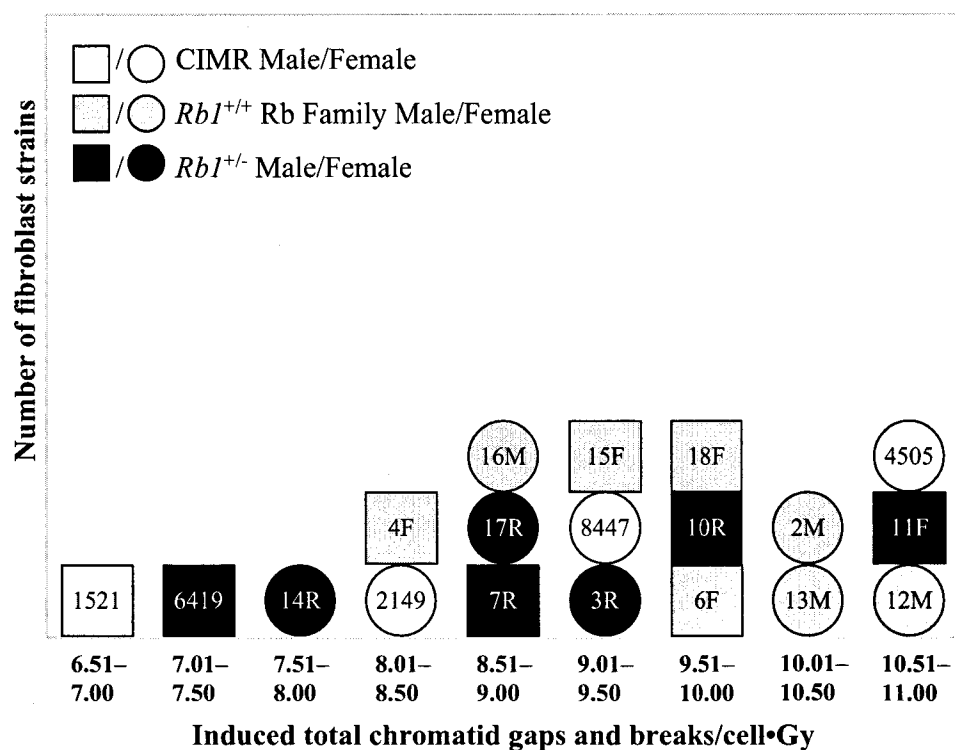


Figure 4.15. Distribution of radiation-induced total chromatid gaps and breaks per cell per Gray for the 14 Rb family member and 5 apparently normal and *RbI*^{+/-} CIMR control fibroblast strains examined in the G2 chromosomal radiosensitivity assay (see Table 4.4).

figures suggest a possible *trimodal* distribution, with separation of the Rb family members into moderately radiosensitive (4–5 induced breaks per cell per Gray in Figure 4.14 and ~8.5–10 induced gaps and breaks per cell per Gray in Figure 4.15) and hyper-radiosensitive (>5 and >10 aberrations per cell per Gray, respectively) categories.

The ratios of chromatid gaps (chromatid fragments displaced at a distance *less* than the chromatid arm width) to chromatid breaks (chromatid fragments displaced at a distance *greater* than the chromatid arm width) per cell for 0, 50 and 100 cGy doses are plotted in Figure 4.16 for the 19 fibroblast strains. Interestingly, the mean value of this ratio is 2.7 for the spontaneous (0 cGy) aberrations, and is significantly ($P < 0.0001$) lower for the 50 and 100 cGy-irradiated groups (ratios of ~1.3 and ~1.1, respectively). The 50 and 100 cGy ratios are also significantly different from each other ($P = 0.0015$). The numbers of chromatid gaps and breaks per cell for the three dose points are compared in Figure 4.17. As expected, the two aberration types are significantly correlated with one another for all three doses ($P = 0.028$, 0.0006 , and 0.023 , respectively).

The induction of radiation-induced chromatid exchanges is plotted in Figure 4.18 for the 19 human fibroblast strains. This includes both symmetrical and asymmetrical forms of chromatid exchanges (equivalent to chromatid translocations and dicentrics, respectively). The induction of these aberrations appears to follow a linear dose response of ~0.019 chromatid exchanges per Gray for the G2 chromosomal radiosensitivity assay protocol employed in this study.

In Figure 4.19, the frequencies of radiation-induced chromatid breaks and combined chromatid gaps and breaks (Table 4.4) are compared to the two acute

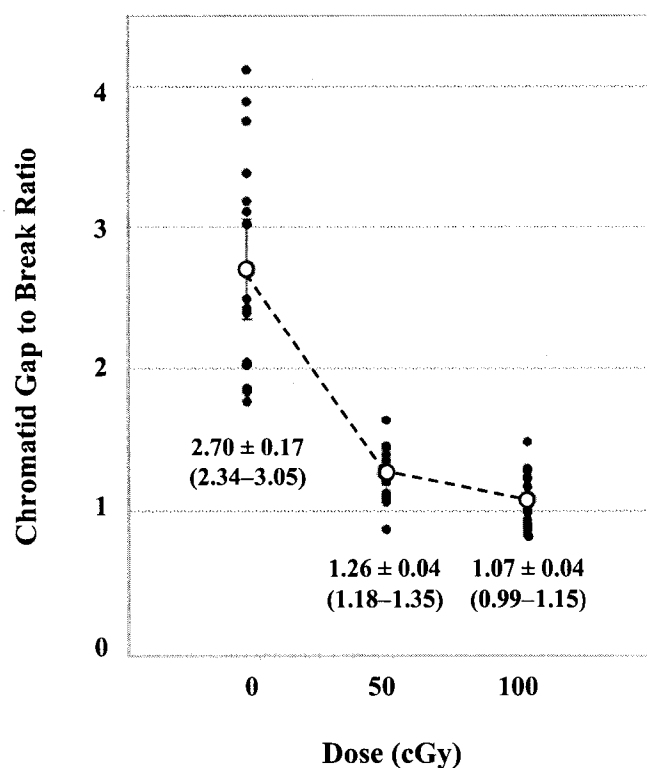


Figure 4.16. Plot of the ratio of chromatid gaps (fragments displaced at a distance *smaller* than the chromatid arm width) to chromatid breaks (fragments displaced at a distance *larger* than the chromatid arm width) per cell and the mean value $\pm$ SEM (95% C.I., open circles) for 0, 50, and 100 cGy doses of cesium-137 gamma rays for the 19 human fibroblast strains examined in the G2 chromosomal radiosensitivity assay (see Tables 4.1–4.3 and Figures 4.2–4.4).

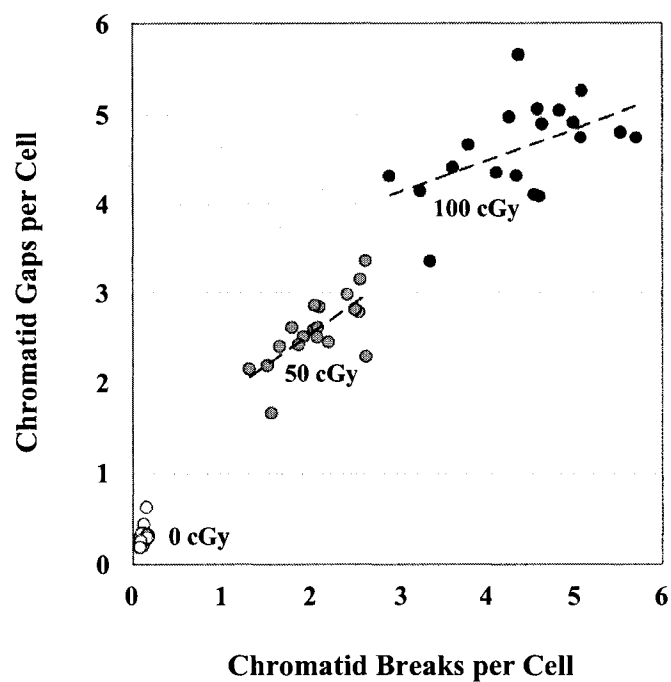


Figure 4.17. Plot of chromatid gaps to chromatid breaks per cell for 0 cGy (open circles), 50 cGy (grey-filled circles), and 100 cGy (black-filled circles) doses of cesium-137 gamma rays for the 19 human fibroblast strains examined in the G2 chromosomal radiosensitivity assay (see Tables 4.1–4.3 and Figures 4.2–4.4).

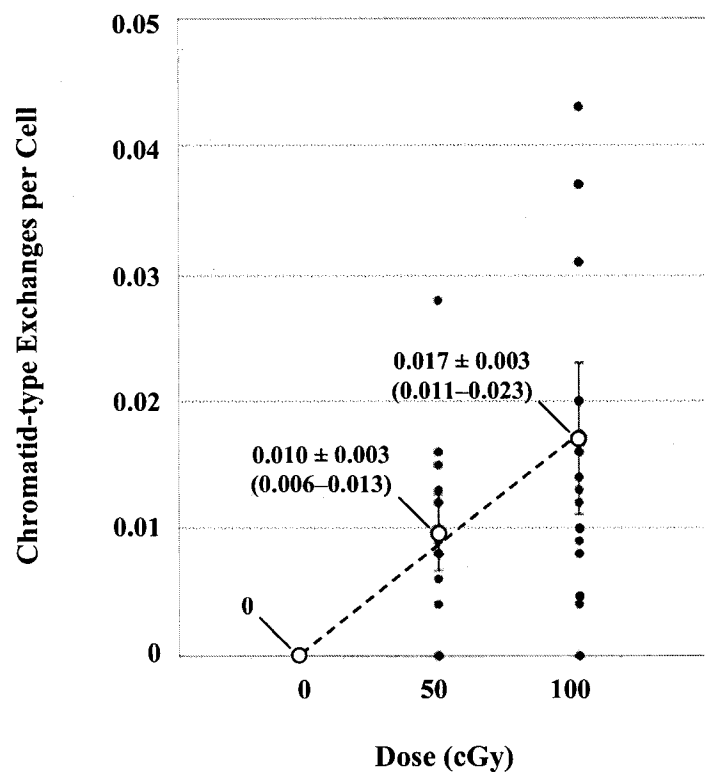


Figure 4.18. Plot of combined symmetrical and asymmetrical chromatid-type exchange frequencies per cell and the mean value $\pm$ SEM (95% C.I., open circles) for 0, 50, and 100 cGy doses of cesium-137 gamma rays for the 19 human fibroblast strains examined in the G2 chromosomal radiosensitivity assay (see Tables 4.1–4.3 and Figure 4.5). The induction of chromatid-type exchanges appears to follow a simple linear dose response.

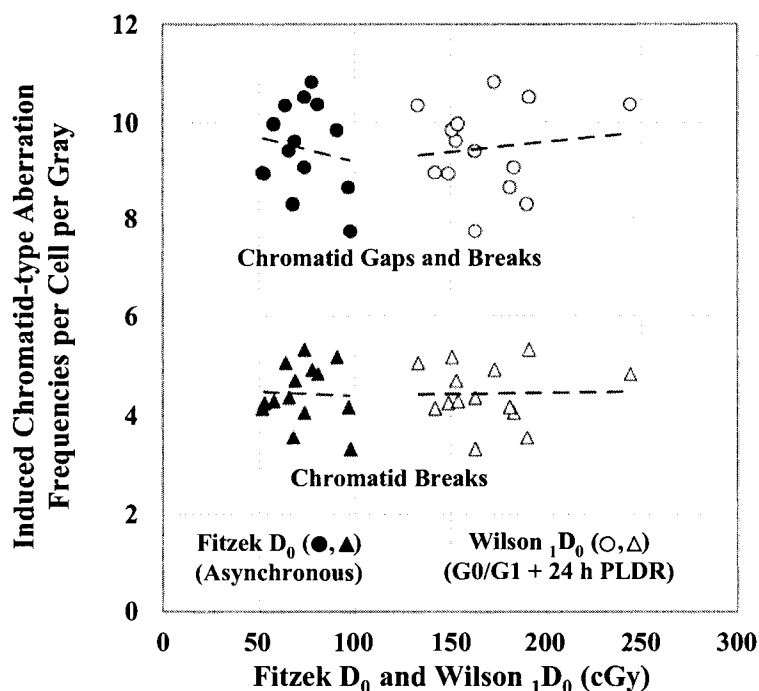


Figure 4.19. Plot of the frequencies of radiation-induced chromatid breaks (triangles) and combined chromatid gaps and breaks (circles) per cell per Gray in the G2 chromosomal radiosensitivity assay (see Table 4.4) compared with two acute irradiation survival experiment radiosensitivity parameters recorded for the 14 Rb family member fibroblast strains: D_0 values reported in Fitzek *et al.* (2002) and Table 1.1 (filled symbols) and ${}_1D_0$ values reported in Table 3.1 (open symbols). The frequencies of both groups of radiation-induced aberrations in the G2 chromosomal radiosensitivity assay and the two acute irradiation survival parameters are not significantly correlated ($P = 0.562$ – 0.931).

irradiation survival parameters, D_0 and ${}_1D_0$, available for the 14 Rb family member fibroblast strains (Fitzek, *et al.*, 2002; see Tables 1.1 and 3.1). The frequencies of both groups of radiation-induced chromatid aberrations in the G2 chromosomal radiosensitivity assay were not significantly correlated with either of the two acute irradiation survival parameters ($P = 0.562\text{--}0.931$).

4.5. Discussion

This large series of cytogenetic measurements of G2-phase chromosomal radiosensitivity clearly supports the assignment of moderate radiosensitivity in both the unaffected and affected Rb family member and the radiosensitive CIMR controls fibroblast strains, GM04505A and GM08447 (originally identified in the LDR studies in Chapter 5). The three radioresistant CIMR reference strains, AG01521, GM02149, and GM06419, have combined average values of ~ 3.2 chromatid breaks and ~ 7.4 total combined chromatid gaps and breaks induced per cell per Gray, while the Rb family and radiosensitive CIMR strains have ~ 1.5 -fold higher aberration frequencies (averages of 4.5–4.9 breaks and 9.5–10.1 gaps and breaks induced per cell per Gray, respectively). Interestingly, GM06419, a bilateral Rb fibroblast strain, demonstrated the lowest aberration frequencies of all the cell strains examined (~ 2.8 breaks and 7.1 gaps and breaks induced per cell per Gray). A similar radioresistant response for GM06419 was observed in Sanford, *et al.* (1989, 1996) and was the only Rb strain examined in common by the Sanford NCI group and myself (and provides further evidence that *Rb1* mutation alone is not strongly correlated with radiosensitivity).

There is some discrepancy in the range of G2 assay indices recorded for the apparently normal fibroblast strains in this study and those by other research groups (particularly the Sanford NCI group), as would be expected from the discussions above. The chromatid-type aberration frequencies in the three resistant, apparently normal CIMR controls (AG01521, GM02419, and GM06419) measured in these experiments are roughly four times higher than those observed in the 1989 and 1996 Sanford reports (average of ~ 3.2 breaks/cell $\cdot$ Gy versus ~ 0.8 breaks/cell $\cdot$ Gy), including the one cell strain examined in common (the radioresistant GM06419). Reevaluation of the Sanford strains by Scott, *et al.* (1996) raised the average number of breaks to ~ 2.2 /cell $\cdot$ Gy and broadened the much more limited Sanford normal range to 1.1–3.3 for a total of 53 normal fibroblast strains, which is much more comparable to the data in this chapter and Nagasawa and Little (1988). Despite these differences observed for the normal cell strains, the radiation-induced chromatid break frequencies measured for the affected Rb patients (average ~ 4.3 breaks/cell $\cdot$ Gy, range 3.3–5.2; see Table 4.4) in this study are quite consistent with the average range of 4.4–6.4 breaks/cell $\cdot$ Gy reported by Sanford, *et al.* (1989, 1996) and Scott, *et al.*, (1996).

The significantly high levels of tetraploidy and endoreduplication observed in the cultures of the *unaffected* parents in the Rb family member fibroblast strains is somewhat surprising, since these phenotype are usually associated with *Rb1* mutation (Di Leonardo, *et al.*, 1997; Niculescu, *et al.*, 1998; Flatt, *et al.*, 2000; Borel, *et al.*, 2002; Margolis, *et al.*, 2003; Hernando, *et al.*, 2004). As mentioned earlier, endoreduplication is generated in a previous S-phase by a second round of DNA replication occurring in the same cell cycle without mitotic division (Avni, *et al.*, 2003). Tetraploidy is generated in a previous

mitosis due to spindle apparatus disruption and the failure of karyokinesis and cytokinesis (Margolis, *et al.*, 2003; Storchova and Pellman, 2004; Kops, *et al.*, 2005). These two phenotypes occur spontaneously and could not have been induced by G2-phase irradiation, as they must have been generated in the previous cell cycle(s) prior to any irradiation. The significantly higher frequencies of these aneuploidy phenotypes in the parents of the Rb families, compared to the apparently normal CIMR strains, suggests that low-penetrance or hypomorphic mutations affecting the integrity of DNA replication, repair, proper mitotic function, or their associated cell cycle checkpoint pathways may underlie their radiosensitivity phenotype.

Interestingly, as evidenced by Figures 4.16 and 4.17, chromatid fragments spontaneously displaced from the chromosome seem to preferentially associate more closely to their original, intact positions (perhaps reflecting the integrity of sister chromatid cohesin-based binding) than those induced by irradiation. Spontaneous fragments may result from a single DNA DSB and more limited chromatin reorganization, while radiation-induced fragments may be generated from both “dirtier” radiation-induced DNA DSBs plus additional localized chromatin structure damage which sever more of the protein-based connections of the fragment to the parent chromosome [see Bryant, *et al.* (2004), Savage (2004), and Chapter 2 for further discussions of this topic]. There is a long-standing debate regarding the scoring of chromatid “gaps” and “breaks,” depending on the laboratory and individual researcher, which contributes to a high degree of variability in the comparison of results of this assay (Scott, *et al.*, 1996; Vral, *et al.*, 2002, 2004). To avoid this issue, the number of breaks (displaced at a distance larger than the chromatid arm width) and the total number of

fully displaced chromatid fragments (gaps and breaks together, regardless of distance from the original chromatid arm) were reported in this study.

The variation in *absolute* aberration yields among various research groups does not change the more important fact that the *relative* aberration yields measured in this study clearly identify the Rb family member fibroblast strains, both affected patients and unaffected first-degree relatives, as a moderately radiosensitive group. This study also supports the utility of the G2 chromosomal radiosensitivity assay as a means to provide a fast and accurate measure of an individual fibroblast strain's relative radiosensitivity compared to a group of *validated* reference normal strains. The high aberration yields observed in the unaffected first-degree relatives of the Rb families and the breast cancer families examined by Burrill, *et al.* (2000) clearly implies the existence of low-penetrance and variably expressive genetic deficiencies associated with familial cancer. The results presented in this chapter provide an additional reference data set, along with acute irradiation survival data sets of Fitzek, *et al.* (2002) and Chapter 3, with which to test the radiation response of a potentially corrected Rb family member fibroblast strain generated from more promising genetic complementation and correction strategies employing continuous LDR irradiation colony formation (see Chapters 5 and 7).

CHAPTER 5

Effects of Continuous Low Dose-Rate Irradiation on Rb Family Member and Apparently Normal and *RbI*^{+/-} CIMR Fibroblast Strains

5.1. Abstract

Expanding upon the continuous low dose-rate (LDR) irradiation selection strategy originally employed by Stackhouse and Bedford (1993) that resulted in the successful isolation of the hyper-radiosensitive CHO mutant *irs*-20, measurements of the continuous LDR (0.5–8.4 cGy/h) colony formation ability of fibroblast strains derived from the 14 Rb family members and 14 age- and sex-matched apparently normal and *RbI*^{+/-} CIMR controls were made. Fibroblasts derived from the 14 Rb family members and from 7 of the 14 (50%) apparently normal CIMR controls were significantly more radiosensitive (1.5–2.3-fold, $P < 0.05$) than the remaining radioresistant CIMR controls, using the endpoint of dose rates required to reduce relative survival to 10% and 1%. The average dose rates required to reduce relative survival to 10% and 1% were ~2.6 and 4.6 cGy/h, respectively, for the 7 radioresistant CIMR fibroblast strains; ~1.7 and 3.1 cGy/h, respectively, for the 10 moderately radiosensitive Rb family member and apparently normal CIMR strains; and, ~1.1 and 2.0 cGy/h, respectively for the 11 hyper-radiosensitive Rb family member and apparently normal CIMR strains. The continuous LDR irradiation responses were significantly correlated ($P < 0.001$) with plating efficiency (PE) and the frequencies of radiation-induced chromatid aberrations measured

in G2 chromosomal radiosensitivity assay measurements made for these strains in Chapter 4.

At the dose rates required to reduce relative survival to 1% for the moderately and hyper-radiosensitive fibroblast strains (~2–3 cGy/h), the survival of the resistant apparently normal strains is consistently greater by 5–100+-fold (dose rates ≥ 4 cGy/h are required to reduce relative survival to 1% in this group). The large degree of differential survival provides the basis for a complete cell cycle-based genetic screen for human cells harboring mutations rendering them hypersensitive to LDR radiation-induced cell killing or perturbations in cell cycle regulatory networks. With the ability to possibly recover a significant fraction (5–30+%) of correcting sequence-complemented cells of apparently normal radioresistance (though the use of a cDNA library transfection scheme discussed further in Chapter 7), this strategy could help identify the genetic basis underlying the hyper-radiosensitivity of the unaffected parents in the Rb families, and other familial cancer types associated with radiation sensitivity and chromosomal instability. The similar degree of hyper-radiosensitivity observed in some of the CIMR control strains implies that this strategy could be extended to “apparently normal” individuals from families not typically associated with familial cancer predisposition.

5.2. Introduction

The CHO *irs-20* series of low dose-rate experiments reported by Stackhouse, Bedford and associates from 1993–1998 provide the rationale for the series of experiments involving the Rb family member and age- and sex-matched apparently normal CIMR control fibroblast strains reported in the following sections. This strategy

was designed “to select for cells hypersensitive to inhibition of cell population growth during continuous low dose-rate irradiation” (Stackhouse and Bedford, 1993). This would “include in the selection system any mutants that were either hypersensitive to cell cycle perturbations or hypersensitive during only a portion of the cell cycle relative to wild-type cells, or cells that were hypersensitive to cell killing,” in general.

Briefly, microcarrier beads and replica plating techniques were used to identify ethyl methane sulfonate (EMS)-mutagenized CHO cells (Harvey and Bedford, 1988) incapable of proliferation during 4 days of continuous low dose-rate irradiation at 6 cGy/h, a dose rate that had little influence on the survival of the wild-type cells (see Chapter 6). Nineteen putative radiosensitive mutants were isolated and re-tested for low dose-rate hypersensitivity; only one, (curiously named) *irs-20*, was deemed hypersensitive by the criteria of colony formation, proliferative capacity, and the formation of giant cells in irradiated colonies (Stackhouse and Bedford, 1993a; discussion of this latter phenotype continues in Chapter 6). The *irs-20* line was hypersensitive to the radiomimetic drug bleomycin but wild type in resistance to the alkylating agent EMS, the cross-linking agent mitomycin C (MMC), and 254-nm UV-C radiation (which forms pyrimidine dimers and 6’–4’-photoproducts). The *irs-20* cells maintained stable chromosome complements and the radiosensitivity phenotype, including an almost complete lack of a dose-rate effect (low dose-rate recovery), in continued culture (Stackhouse and Bedford, 1993a, 1993b).

The concept of introducing (and identifying) a human gene that could restore wild-type radiosensitivity in the *irs-20* line by continuous low dose-rate irradiation selection was presented in the second Stackhouse and Bedford report (1993b). Lin, *et al.*

(1997) demonstrated concordance between restoration of wild-type radiosensitivity during 6 cGy/h continuous irradiation and the presence of human chromosome 8 in *irs-20* x human fibroblast (HSF42) somatic cell hybrids, as confirmed by G-banding and fluorescence *in situ* hybridization (FISH) analyses with human chromosome 8-specific FISH painting probes and chromosome-8 PCR primers (which helped narrow the region to 8q). Complementation attempted by cell fusion of *irs-20* and similarly hyper-radiosensitive *scid* mouse cells (Fulop and Phillip, 1990; Biedermann, *et al.*, 1991; Hendrickson, *et al.*, 1991) was unsuccessful in restoring normal radiosensitivity in any hybrid clones, suggesting they were both members of the same genetic complementation group. Also similar to *scid* mouse cells, transient RAG1/RAG2-mediated V(D)J recombination assays performed with *irs-20* cells demonstrated ~10% wild-type capacity for signal joint formation and ~3% wild-type capacity for coding joint formation in the reporter plasmid substrates.

The mutation responsible for the hyper-radiosensitivity of *irs-20* was determined in Priestly, *et al.* (1998). A single G→A base change in the *prkdc* gene (encoding the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs) results in substitution of a lysine residue for glutamic acid in a conserved C-terminal domain outside the PI-3 kinase domain, a region shared by other PIKK kinases (*e.g.*, ATM and ATR). Compared to mouse *scid* cells and V-3 cells, another *prkdc*-deficient CHO cell line (derived from wild-type strain AA8; Peterson, *et al.*, 1995), *irs-20* cells retain higher levels of DNA-PKcs protein (undetectable in V-3 cells). Similar to the murine *scid* DNA-PKcs molecule (which is prematurely truncated at residue 4045), the *irs-20* DNA-PKcs molecule can bind DNA and Ku70/80 heterodimers but is unable to function as a

protein kinase (*i.e.*, a “kinase-dead” mutation; Priestly, *et al.*, 1998). Complementation was achieved by transfecting *irs-20* cells with yeast artificial chromosomes (YACs) containing mouse and human *prkdc* genes, and confirmed by the restoration of wild-type V(D)J recombination capability and near-wild-type acute dose and continuous low dose-rate radiation response.

The purpose of this present study was to determine if exposing fibroblast strains derived from the Rb family members to continuous LDR irradiation could result in a greater degree of differential survival when compared to the survival of validated apparently normal CIMR strains at the same dose rates, as was observed for *irs-20* cells compared to wild-type CHO 10B2 cells. Generation of a sufficient degree of differential radiation survival would provide a basis for a genetic complementation and correction screening strategy, similar to that employed for *irs-20* but using a genome-wide cDNA library, to identify potential candidate sequences that underlie the observed radiosensitivity of the Rb family members (Fitzek, *et al.*, 2002, this volume).

5.3. Materials and Methods

5.3.1. Dosimetry of the CSU Low Dose-Rate Irradiation Facilities MRB008 and MRB012

The Colorado State University (CSU) MRB008 and MRB012 low dose-rate (LDR) ^{137}Cs irradiation facilities are located in the basement of the Molecular and Radiological Biosciences (MRB) Building in Fort Collins, Colorado. The MRB012 facility consists of a custom-built 79 cm-diameter 12-source array housed in a 37°C warm room without CO₂ supply (see Figure 5.1; Stackhouse and Bedford, 1993). The

activity of each of the twelve ^{137}Cs sources is approximately 2.2 mCi (as of September 2004; Amersham). Exposure rate measurements taken directly above each independently exposed source revealed the source strengths are not uniform [the relative strength of each source compared to the strongest source (number 3) are shown under the source labels in Figures 5.3–5.5]. The sources are delivered into the MRB012 warm room under a 107.5 x 96.5 cm shelving unit by a series of copper tubes down which the sources (connected at the end of steel cables) are pushed into various positions to achieve the desired dose-rate levels. A locked cabinet with the source cable controls and a large cell culture preparation area (lab bench, sink, *etc.*) are located in the anteroom MRB010. A total culturable cell surface area of $\sim 4.6 \text{ m}^2$ (630 T-75 flasks) was available for each experimental setup depicted in Figure 5.1.

The MRB008 facility (see Figures 5.6 and 6.4) is equipped with a J. L. Sheperd and Associates Model 81-14 beam irradiator containing a single $\sim 21 \text{ Ci } ^{137}\text{Cs}$ source placed $\sim 100 \text{ cm}$ above a large, water-jacketed copper incubator that is maintained at 37°C without CO_2 supply. Six shelves are available in the MRB008 incubator with sufficient space to hold up to 16 T-75 or 50 T-25 tissue culture flasks per level (see Figure 6.4), and flasks may be stacked onto these shelves (setup depicted in Figure 6.4) or the shelves completely removed such that columns made of 34 stacked T-75s may be constructed (setup depicted in Figure 5.6).

Dosimetry of the MRB008 and MRB012 irradiation facilities was performed using a Radcal Corporation Model 2025 ion chamber equipped with a 12-cm diameter pancake probe (courtesy of Dr. James Durham). The ion chamber was initially calibrated to NIST-traceable standards, and annually calibrated by C. Sampier in this department

who has reported the accuracy of the detector to $\leq 3\%$. The ion chamber probe was placed into the 37°C irradiation chambers (without the sources exposed) for a minimum of one hour to provide temperature equilibration before taking any measurements. Exposure rate measurements (in R/h) were taken with the ion chamber pancake probe enclosed in a modified T-150 cell culture flask (Greiner Bio-One) to ensure appropriate buildup at the cell culture surface. Additional T-75 flasks containing the correct volumes of water or air (empty) were then arranged above, below, and to the side of the probe-containing T-150 flask to mimic the experimental exposure conditions depicted in Figures 5.1 and 5.6. Repeated measurements during my years of operation (2002–2005) of both the MRB008 and MRB012 facilities yielded very consistent exposure rate measurements (safely measured in the anteroom MRB010 through the use of a long cable connecting the pancake probe and the readout meter).

For the human fibroblast continuous LDR irradiation setup illustrated in Figure 5.1, four of the MRB012 facility's twelve ~ 2.2 mCi ^{137}Cs sources (numbered 1, 4, 7, and 10) were engaged and exposure rates were measured at seventeen evenly-spaced locations across the experimental plane and for every odd-numbered flask position (1–35) where flasks containing cells would be placed. Odd-numbered flask levels that were not used for cell culture were filled with 25 ml of water to ensure consistent attenuation; this also allowed the experimenter to achieve a specific number and variety of dose rates.

The dose rates delivered to cells within these flasks ($\dot{D}$, in cGy/h) was calculated by correcting the measured ion chamber exposure rates ($\dot{X}$, in R/h; the Roentgen (R) is defined only for photons in air) for the temperature ($T=23\text{--}37^\circ\text{C}$ or $296\text{--}310$ K) and pressure (~ 85 kPa) of the irradiation chambers at the time of measurement and the mass

absorption coefficients of muscle (0.0323 cm²/g) and air (0.0293 cm²/g) for 662-keV gamma ray photons (Johns and Cunningham, 1983), using the following formula:

$$\dot{D} = \dot{X} \cdot \left(\frac{\mu_{abMUSCLE}}{\mu_{abAIR}} \right) \cdot (0.873cGy / R)$$

The dose-rate field isocurves depicted in Figures 5.3–5.5 were generated by SigmaPlot using the software's three-dimensional smoothing and interpolation functions for dose rate and position values measured within the experimental area.

5.3.2. Cell Culture and Irradiations

Low-passage human fibroblast cell strains identified in Tables 5.1 and 5.2 were obtained from the Coriell Cell Repositories (CCR) at the Coriell Institute for Medical Research (CIMR, Camden, NJ), and from members of five retinoblastoma families obtained at the Massachusetts Eye and Ear Infirmary and the Harvard School of Public Health (Boston, MA) as reported in Fitzek *et al.* (2002) and Chapter 1, respectively. All fibroblast strains were passaged regularly at a 1:4 dilution and maintained in α MEM medium (GIBCO/Invitrogen) supplemented with 15% FBS, 50 U/ml penicillin-G, and 50 μ g/ml streptomycin sulfate (all Sigma) in a 37°C humidified incubator supplied with 95% air/5% CO₂ gas mixture prior to each experiment. A single lot of serum (Sigma lot number 32K8401) was used for the entire set of experiments detailed in this report to reduce experimental variability.

Cultures were detached with 0.25% trypsin (GIBCO/Invitrogen)/1 mM EDTA in Ca²⁺/Mg²⁺-free phosphate-buffered saline (Dulbecco's "PBS-A" formulation), counted with a Coulter Z-1 particle data counter (Beckman Coulter, Inc.), and plated as single cell suspensions of 150, 500, and 1500 cells per flask into replicate T-75 flasks with 25 ml of

pH-equilibrated α MEM medium provided by the addition of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (GIBCO/Invitrogen) to ensure pH equilibration ($\sim$ 7.2–7.3) without supplied CO₂. Seeded flasks were then capped tightly and placed in a 37°C warm room (also without 5% CO₂ supply) overnight to allow for plating.

The plating efficiencies of all the fibroblast strains used in these experiments (see Tables 5.1 and 5.2) were sufficiently high to obviate the use of feeder cell layers. Initial cellular multiplicities of 1 were obtained by carefully plating single cell suspensions and initiating irradiations <16–18 hours later (strains have $\sim$ 24–28 hour doubling times). An experiment where exposures were initiated three days after plating did *not* significantly affect measured survival levels in fibroblast strains AG01521, AG01522, MF-4F, MF-6F, MF-7R, MF-11F, MF-14R, MF-15F, and MF-16M, *i.e.*, strains encompassing the observed range of LDR responses in these studies (data not shown).

Following overnight (<16–18 hours) incubation in the 37°C warm room, the flasks were transferred to the CSU MRB012 low dose-rate irradiation facility, which is maintained at 37°C without CO₂ supply. Flasks were stacked into 34 columns with 35 flasks per column (for more than 4 m² of culturable surface area) onto the shelf located 56 cm above the 79-cm diameter source array (see Figure 5.1). Odd-numbered flasks (1–35) contained either 25 ml of culture medium with cells or 25 ml of water without cells to ensure consistent attenuation, while even-numbered flasks (2–34) were empty. Once arranged, four of the facility's twelve $\sim$ 2.2-mCi ¹³⁷Cs sources (numbered 1, 4, 7, and 10) were exposed and the cells were continuously irradiated for 7–9 days with dose-rates exponentially attenuating from 8.4 cGy/h at the bottom of the columns to 0.5 cGy/h at the

top of the columns. After 7–9 days, the flasks were removed from the irradiation facility; the cell medium exchanged with fresh α MEM medium with 10% FBS, antibiotics, and 25 mM HEPES buffer; and the flasks were returned to the irradiation facility for an additional 7–9 days, for a total exposure time of approximately 14–18 days.

After irradiations were completed, the flasks were removed from the LDR irradiation facility and the culture medium aspirated. The flasks were rinsed twice with 0.9% w/v sodium chloride, fixed with 95% ethanol, and stained with a 0.1% w/v crystal violet/methanol solution. Colonies with >50–100 viable cells were scored as survivors. Due to the number of cells plated per flask (150, 500, or 1500) and the plating efficiencies of the various fibroblast strains examined, the detection limit for survivors in this assay is approximately 1 in 1000 (10^{-3}).

5.3.3. Data and Statistical Analyses

Estimates for absolute survival were calculated as the average number of colonies counted per number of cells plated per dose rate. The logarithm of these survival estimates were averaged for 2–9 independent experiments per fibroblast strain, and fitted with a linear ($y=ax+c$) least-squares-weighted regression function for survival data measured for dose rates of ≤ 3 cGy/h only, where y is the average absolute survival for a dose rate x (SigmaPlot). If the calculated intercept (c) was >5% different than the measured zero-dose rate plating efficiency (PE) value for a particular strain, the calculated intercept value c was used as the zero-dose plating efficiency to calculate the average relative surviving fraction (*i.e.*, the absolute surviving fractions corrected for the zero-dose plating efficiencies), otherwise the average measured PE value was used to

normalize the absolute survival data. Standard errors of the mean (SEM) were calculated by combining the standard deviations of the absolute surviving fractions by the square root of the sum-of-the-squares method (Bevington and Robinson, 1992) and dividing this value by the square root of the number of experiments, and constitute the error bars for Figures 5.8, 5.9, and 5.11–5.15. The average survival response for the specific groups (radioresistant, moderately and hyper-radiosensitive, and Fitzek D_0 class ranges) are reported in Table 5.3 and Figures 5.10 and 5.16 as arithmetic means and standard deviations (SD) of relative continuous LDR colony formation ability for the fibroblast strains of the group.

Statistical differences were tested by Student t -tests for two independent sample distributions (SigmaPlot; Tarone, *et al.*, 1983). Correlations between the different radiosensitivity parameters (PE, D_0 , ${}_1D_0$, G2 assay aberration frequencies, continuous irradiation dose rates for 10% and 1% relative survival) were assessed using the Pearson product-moment correlation coefficient, fit by the least-squares method to the respective data sets being compared (SigmaPlot).

As no parameter-based radiobiological model exists for these types of continuous LDR exposures coupled with variable cell growth and differential *dose rate-dependent* cell cycle-phase sensitivities (such studies have classically only used density-inhibited, G0/G1-phase cell populations), the best-fit lines for the data sets in Figures 5.8–5.16 have been drawn for illustrative purposes only. The lines were drawn using the least-squares fit linear-exponential regression curve for the ≤ 3 cGy/h survival data and best fit by eye to the data points for dose rates > 3 cGy/h (assuming an asymptotic decrease in survival as the dose rate approaches the proliferation-limiting LDR). This asymptotic decrease in

survival is similar to the shape of dose response curves seen for many chemical agents and is best typified for LDR exposures by the rodent cell responses in the continuous LDR colony formation assay described in Priestly, *et al.* (1998) and Chapter 6.

The only radiosensitivity parameter reported for the individual fibroblast strains and groups thereof in Tables 5.1–5.3 is the continuous irradiation dose rate required to reduce relative survival to 10% and 1%. Classification of fibroblast strains as radioresistant, moderately radiosensitive, or hyper-radiosensitive in this assay were based on arbitrary cutoff values for the dose-rates required to reduce relative survival to 1%. Strains requiring ≥ 4 cGy/h to reduce relative survival to 1% were considered radioresistant, strains requiring dose rates ≥ 2.5 cGy/h but < 4 cGy/h to reduce relative survival to 1% were considered moderately radiosensitive, and strains requiring dose rates < 2.5 cGy/h to reduce relative survival to 1% were considered hyper-radiosensitive.

5.4. Results

The MRB012 LDR facility experimental setup used for these series of experiments is shown in Figure 5.1. The setup consists of 34 columns of 35 T-75 flasks each (for a total of 1190 flasks) housed in a 37°C warm room without CO₂ supply. Flasks containing 25 ml of cell culture medium with cells or 25 ml of water (for consistent attenuation) were arranged on the 18 odd-numbered flask levels (1–35) for a total of 630 available T-75 culture flasks for these experiments (the even-numbered flasks 2–34 were empty). The flask columns were arranged on a large shelf located 56 cm above the twelve ~ 2.2 -mCi ¹³⁷Cs source array, only four of which (sources 1, 4, 7, and

Human Fibroblast LDR Irradiation Setup: MRB012

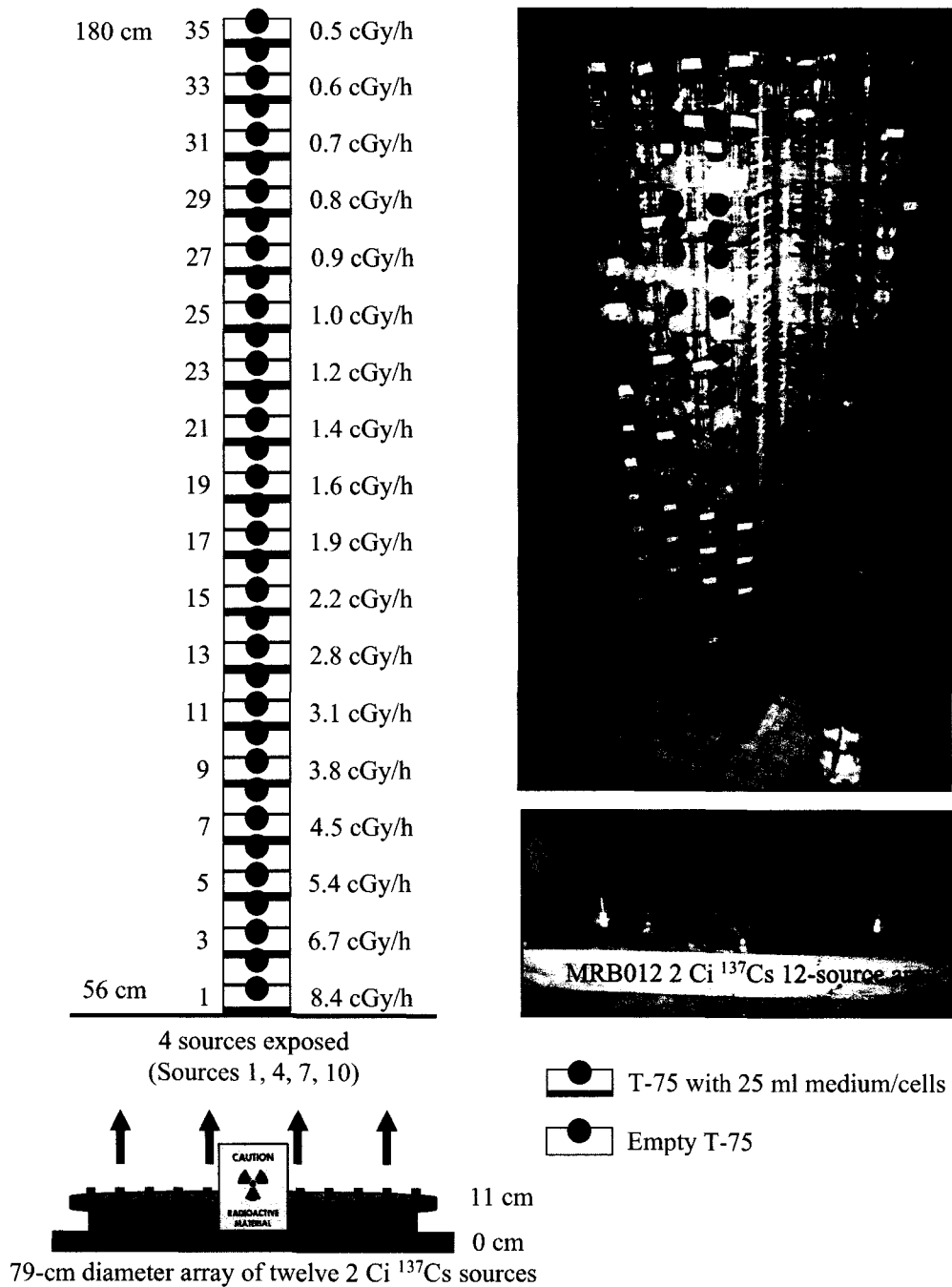


Figure 5.1. Experimental setup for the MRB012 low dose-rate irradiation facility discussed in this chapter.

10) were exposed for this series of experiments to achieve dose rates in the range of 0.5–8.4 cGy/h in the flask columns from top to bottom.

Ion chamber dosimetry measurements of the MRB012 LDR facility, with four of the twelve cesium sources engaged demonstrate a smooth, exponential decrease in the dose-rate as the flask level increases, as plotted in Figure 5.2. Exposure measurements taken across the plane of the experimental area demonstrate no more than $\pm 6.25\%$ relative variation in dose-rate for any given flask level; this percent variation constitutes the error bars for Figure 5.2. Figures 5.3–5.5 are isodose-rate contour plots of the experimental plane for flask row 1 (8.4 ± 0.5 cGy/h), flask row 5 (5.4 ± 0.3 cGy/h), and flask row 15 (2.2 ± 0.1 cGy/h), with each gray level representing a 3–5% relative change in dose-rate. The pattern of the dose-rate field is closely maintained at every flask level within the experimental setup. To gauge the relative contribution from each of the twelve MRB012 sources independently, exposure rates were measured directly above each and are reported as a ratio relative to the strongest source (source 3) below the source labels in Figures 5.3–5.5. This variation in the source strengths accounts for the slight skewing of the dose-rate field toward sources 1 and 4 when the four sources are exposed.

The MRB008 LDR facility experimental setup, used for a smaller series of very low dose-rate (VLDR) experiments reported in Figure 5.18, is shown in Figure 5.6. Shielding the 21 Ci ^{137}Cs MRB008 source with ~ 1.3 cm of lead sheet (equivalent to ~ 2 half-value layers for cesium-137 photons) reduced the dose-rates delivered to the flask columns in the incubator to 0.06–0.6 cGy/h, slightly less than an order of magnitude lower than the unshielded setup described in Figures 6.4 and 6.5 (used in the MEF LDR studies discussed in the next chapter).

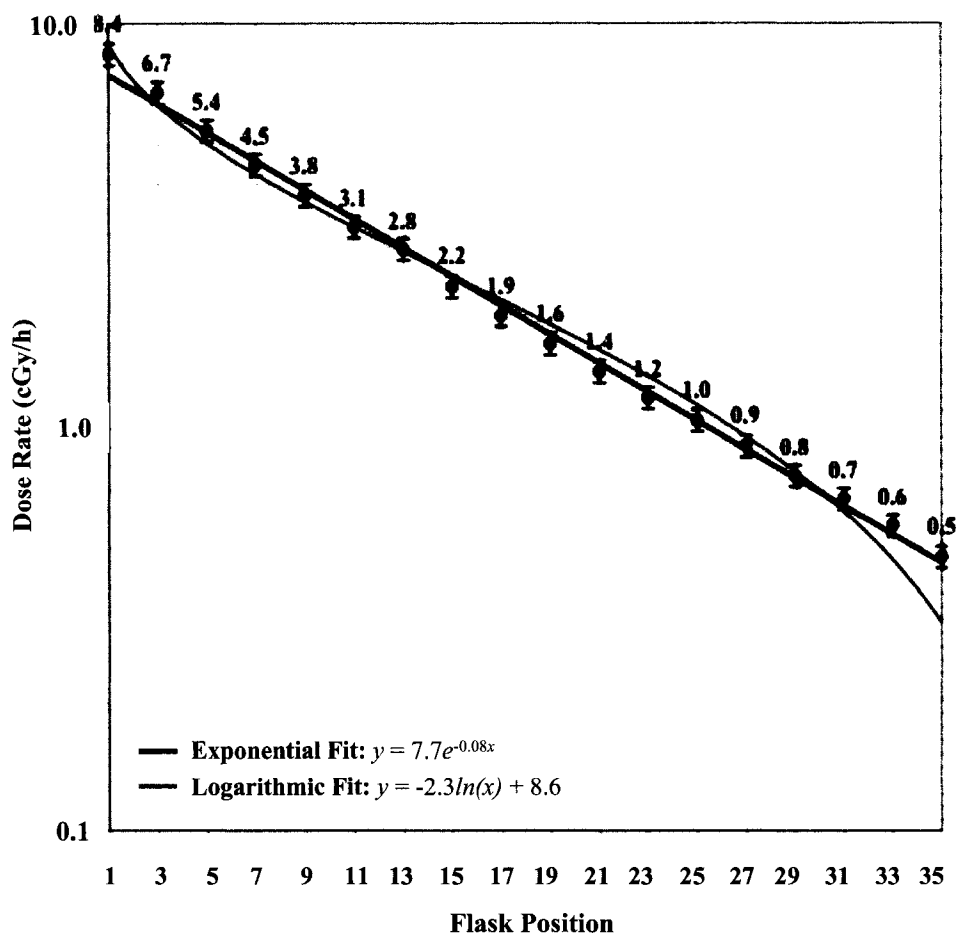


Figure 5.2. Semi-logarithmic plot of MRB012 dose rates (in cGy/h) with four sources exposed (numbers 1, 4, 7, 10) versus experimental flask position (see Figure 5.1). Dose rates (cGy/h) are calculated from Radcal Corporation Model 2025 ion chamber exposure measurements (R/h) corrected for facility temperature (37°C) and pressure (85 kPa), and the mass attenuation coefficients for tissue (0.0293 cm²/g) and air (0.0323 cm²/g).

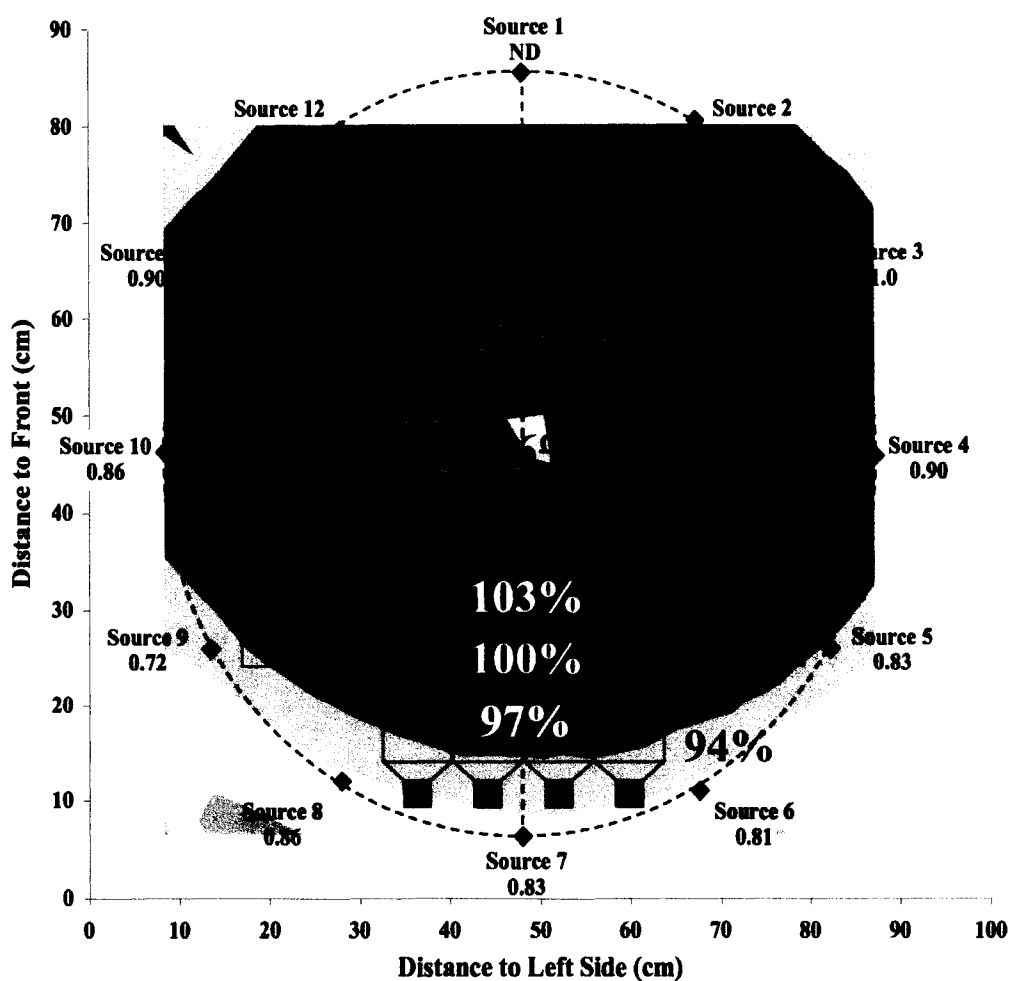


Figure 5.3. Isodose-rate contour plot of the flask position 1 (8.4 ± 0.5 cGy/h) field plane with MRB012 sources 1, 4, 7, and 10 exposed in the experimental setup detailed in Figure 5.1. Individual source strengths (relative to the strongest source, source 3) are denoted below each source label. ND = not determined.

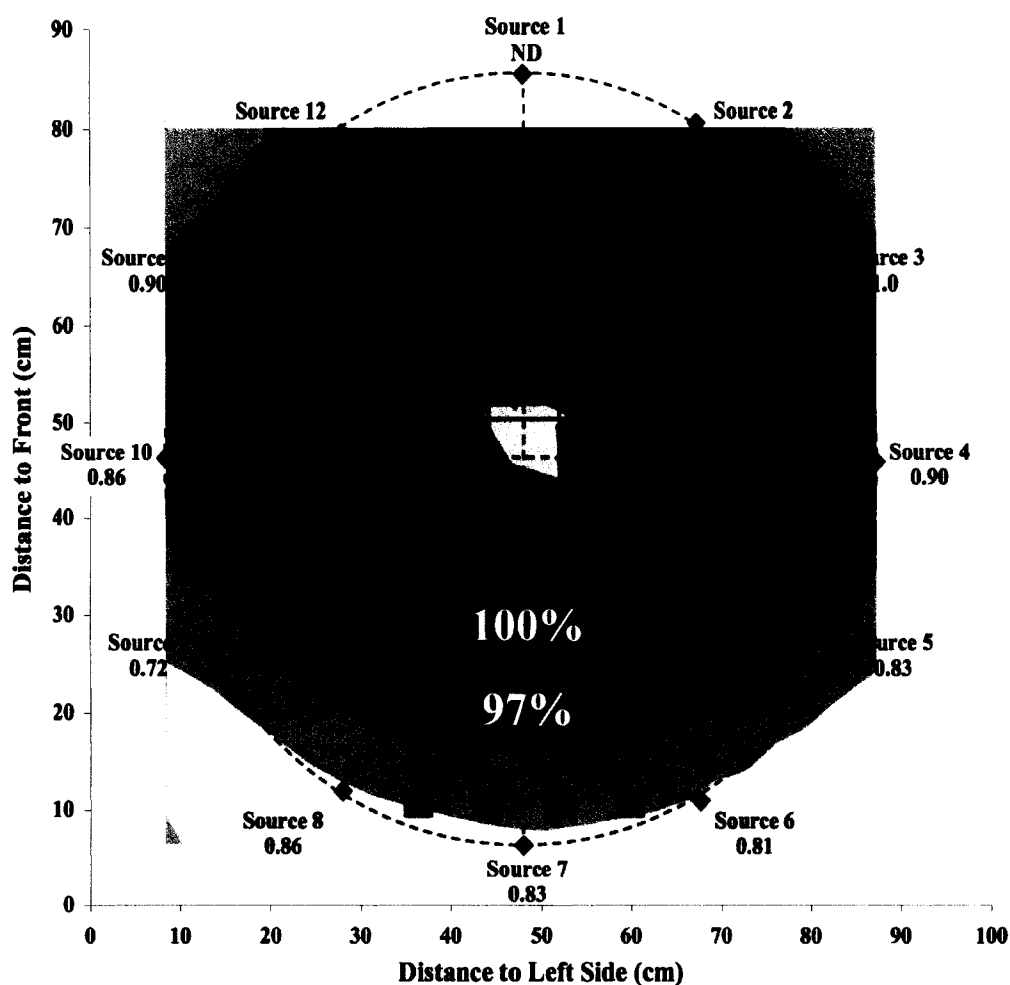


Figure 5.4. Isodose-rate contour plot of the flask position 5 (5.4 ± 0.3 cGy/h) field plane with MRB012 sources 1, 4, 7, and 10 exposed in the experimental setup detailed in Figure 5.1. Individual source strengths (relative to the strongest source, source 3) are denoted below each source label. ND = not determined.

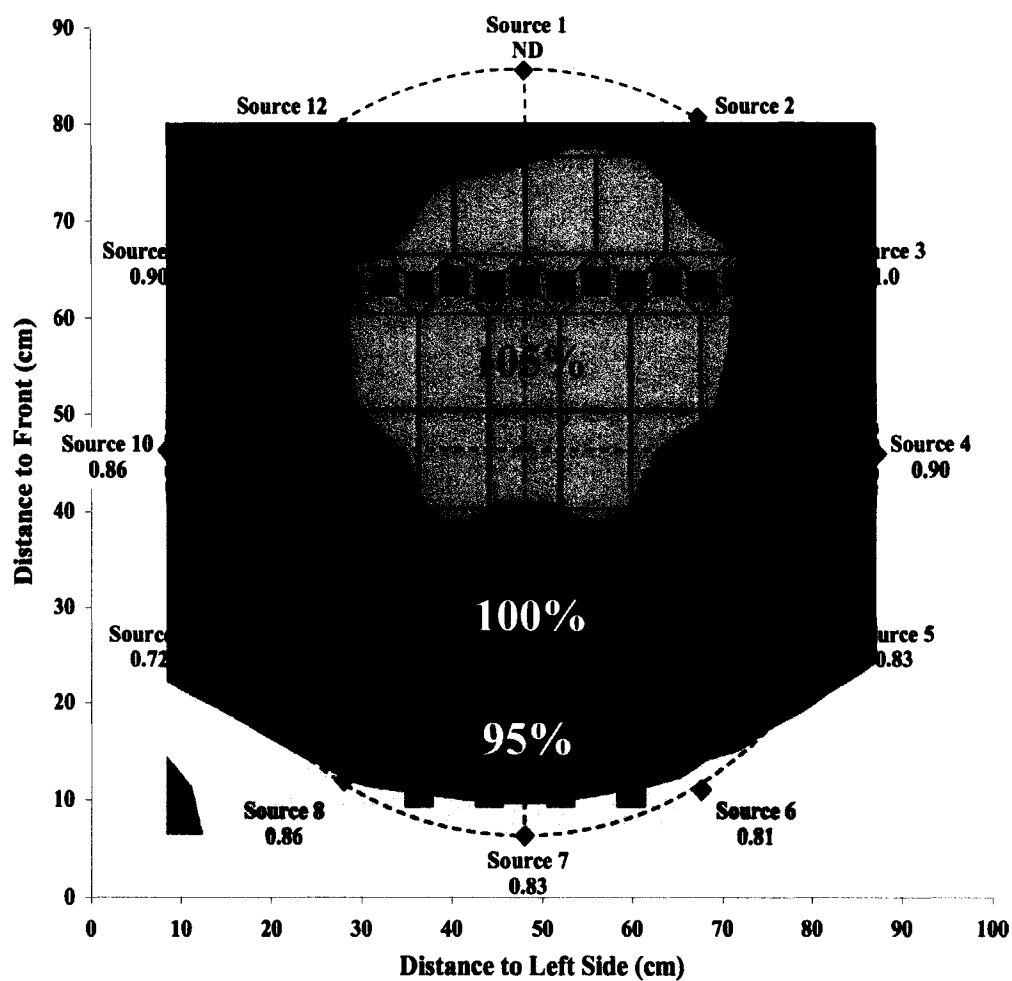
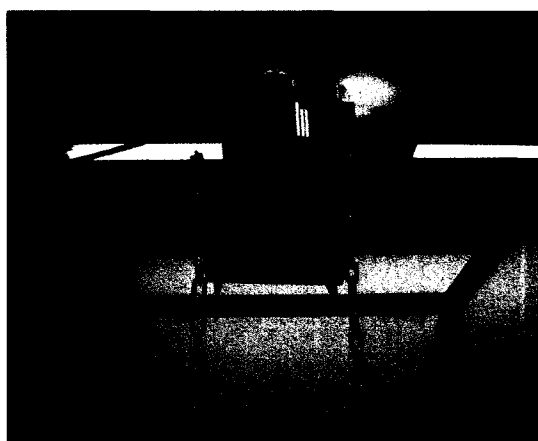
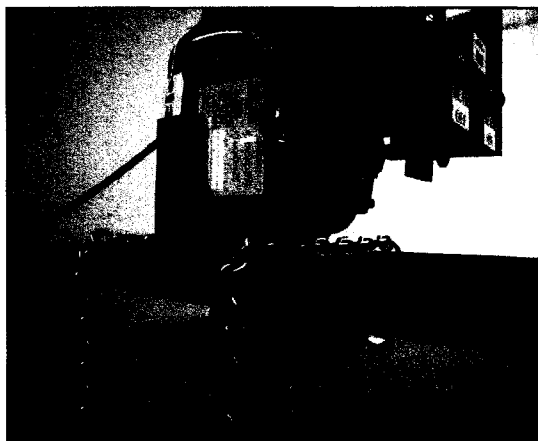
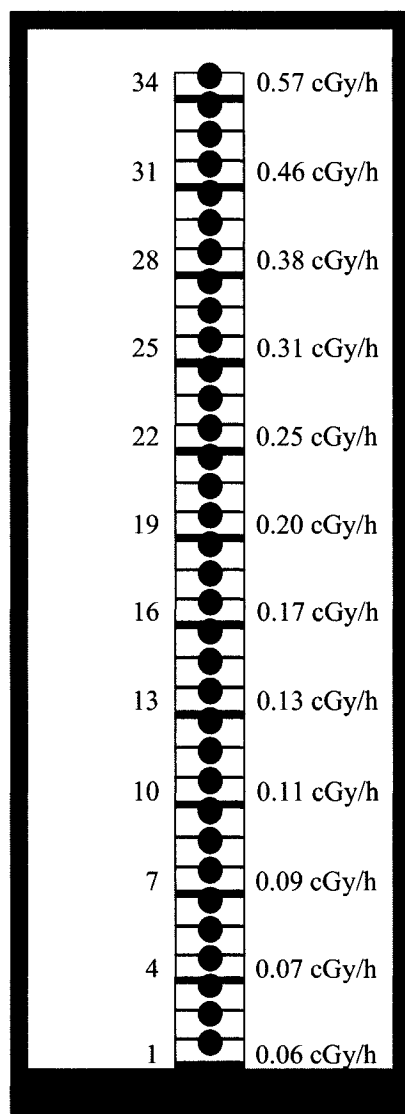
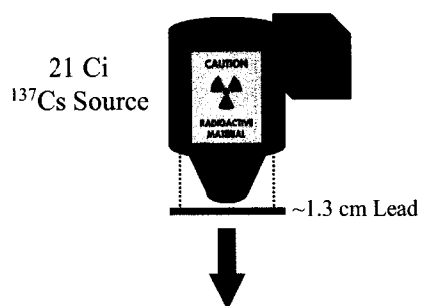


Figure 5.5. Isodose-rate contour plot of the flask position 15 (2.2 ± 0.1 cGy/h) field plane with MRB012 sources 1, 4, 7, and 10 exposed in the experimental setup detailed in Figure 5.1. Individual source strengths (relative to the strongest source, source 3) are denoted below each source label. ND = not determined.

Human Fibroblast VLDR Irradiation Setup: MRB008 with shielding



- T-75 with 25 ml medium/cells
- Empty T-75

Figure 5.6. Experimental setup for the lead-shielded MRB008 low dose-rate irradiation facility discussed in this chapter.

The growth rates (the slope of the growth curve in exponential growth phase) of several radioresistant (GM06419), moderately radiosensitive (MF-7R, MF-10R, and MF-11F), and hyper-radiosensitive (MF-6F, MF-12M, MF-15F, and GM08447) *RbI*^{+/+} and *RbI*^{+/-} fibroblast strains exposed to LDR irradiation are shown in Figure 5.7. These measurements yield population doubling times of approximately 25–30 hours for unirradiated cultures, ~25–50 hours at 1 cGy/h, ~55–90 hours at 3 cGy/h, and >200 hours at 8.4 cGy/h, which did not appear to tightly correlate with the strain radiosensitivity. These curves are qualitatively similar to LDR experiments performed with cells of other genetic origins and species in this laboratory (Mitchell, *et al.*, 1979a; Wells and Bedford, 1983; Stackhouse and Bedford, 1993). Overall, human cells seem to be the species most sensitive to continuous LDR irradiation growth inhibition.

For dose rates ≤ 3 cGy/h (including the unirradiated controls), the flasks in the LDR growth experiments became nearly confluent (density-inhibited) prior to the end of the exposure period due to the higher numbers of cell initially plated ($\geq 3 \times 10^4$ per flask, necessary for flow cytometry analysis). The long population doubling times (>200 hours) at 8.4 cGy/h indicate that a cell, on average, may be able to undergo only one or two divisions at most in a two to three week LDR exposure period. This is evidenced by the complete lack of colony formation at this dose rate for any human fibroblast strain examined (*i.e.*, the proliferation-limiting LDR is <8.4 cGy/h for human fibroblasts). The continuous irradiation flow cytometry profiles for these strains consistently indicated that the cultures became increasingly heterogeneous, with evidence of many cells distributed through the S-phase and G2/M-phases as the dose-rate approaches the proliferation-limiting LDR (data not shown).

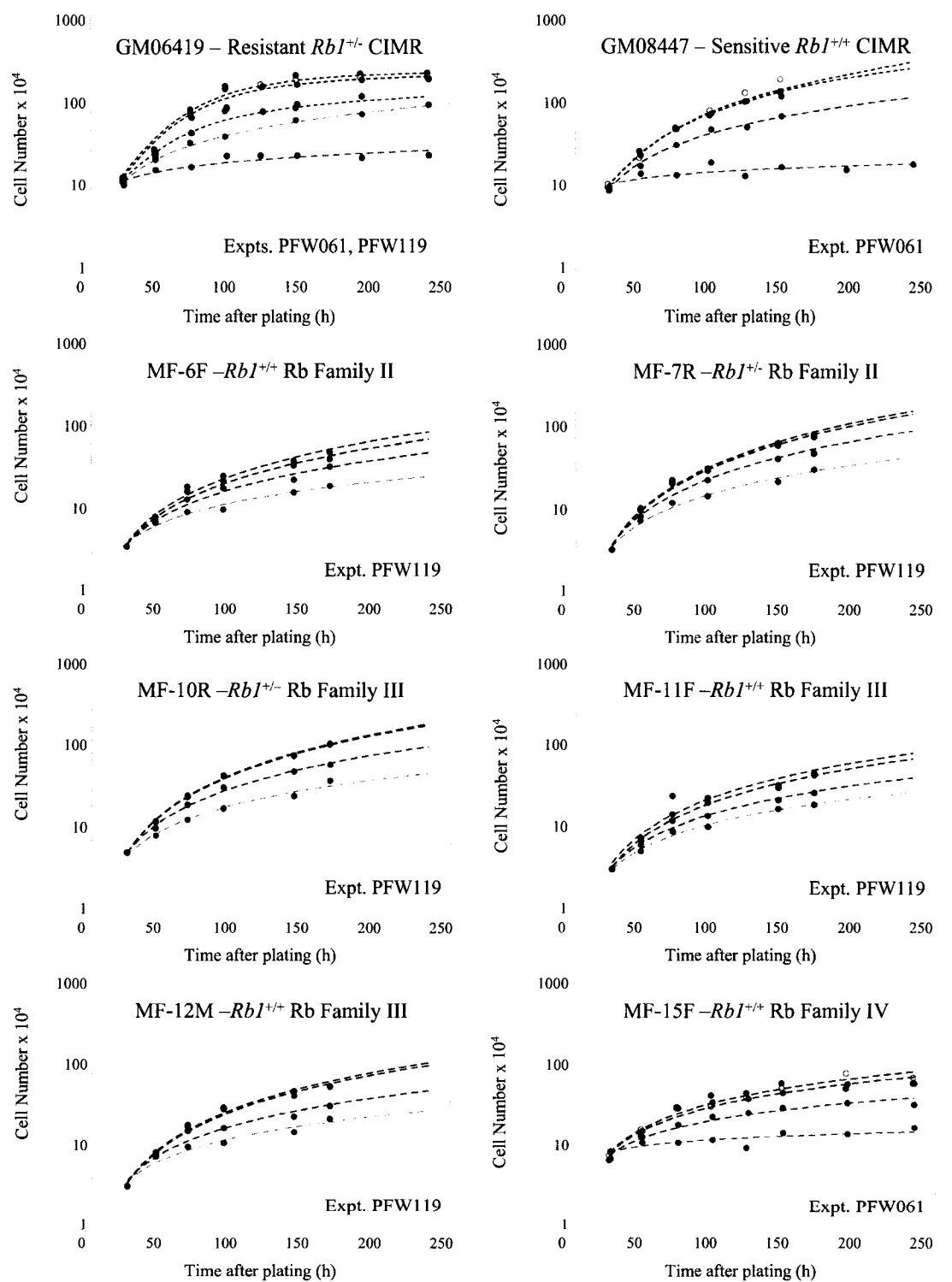


Figure 5.7. Growth curves for various human fibroblast strains grown at low dose-rates of 1, 3, 6, and 8 cGy/h.

● Incubator ●/○ 1 cGy/h ● 6 cGy/h
○ Warm Room ●/○ 3 cGy/h ● 8 cGy/h

Alternatively, these cells may have undergone endoreduplication becoming G1 cells with 4N DNA content (see Chapter 4). Cultures of Rb family IV strain MF-15F fibroblasts, observed to have significantly high frequencies of endoreduplicated metaphase spreads (~17%) in the G2 chromosomal radiosensitivity assay (see Table 4.5 and Figure 4.6 in Chapter 4), also contained cells with 4N to 8N (*i.e.*, G2-phase or mitotic tetraploid cells) DNA content in its LDR flow cytometry profiles. Interestingly, *Rb1* heterozygote cells, especially GM06419, routinely achieved significantly higher confluent densities ($\sim 3\text{--}3.5 \times 10^6$ cells/T-25) than the wild-type strains ($\sim 1.5\text{--}2 \times 10^6$ cells/T-25). This phenotype was also observed for *rb1*-deficient mouse 3T3 (Classon, *et al.*, 2000) and mouse embryo fibroblast (MEF) cells (Herrera, *et al.*, 1996; Johnson, *et al.*, 2004). Density inhibition was not a concern for the continuous LDR survival experiments described next, as only 150–1500 cells were plated per T-75 flask for the colony formation.

The effect of continuous LDR irradiation on the retention of proliferative capacity for the 14 apparently normal and *Rb1*^{+/-} CIMR fibroblast strains identified in Table 5.1 are shown in Figures 5.8–5.10. In these figures, the top panels are plots of absolute survival (*i.e.*, not corrected for PE) versus dose-rate for each group and the lower panels are plots of the same data corrected for the PE or *y*-intercept (*c*) of the log-transformed absolute survival (if *c* is >5% different than the zero-dose PE) for each cell strain and averaged for the number of experiments per strain. The CIMR control fibroblast strain LDR responses are summarized in Table 5.1, with the strains ranked from least (1) to most (14) radiosensitive in this assay.

Table 5.1. Results of the continuous LDR (0.5–8.4 cGy/h) irradiation colony formation assays for the 14 apparently normal and *Rb1*^{+/-} CIMR control fibroblast strains. Strains are ranked from least (1) to most (14) radiosensitive.

Table 5.1.

Fibroblast Strain	Sex	<i>Rb1</i> Genotype	No. Expts.	PE $\pm$ SEM _{PE} (Range)	Dose rate (cGy/h) for relative survival level of:		CIMR Continuous LDR Rank	Continuous LDR Classification
					0.10	0.01		
AG01521	M	+/+	2	0.447 $\pm$ 0.153 (0.231–0.663)	2.32 cGy/h	4.50 cGy/h	3	Normal (Radioresistant)
AG01522	M	+/+	2	0.380 $\pm$ 0.079 (0.269–0.492)	3.24 cGy/h	5.24 cGy/h	1	Normal (Radioresistant)
GM02673	M	+/+	3	0.160 $\pm$ 0.049 (0.010–0.301)	2.50 cGy/h	4.18 cGy/h	4	Normal (Radioresistant)
GM05757	M	+/+	1	0.315	2.38 cGy/h	4.25 cGy/h	6	Normal (Radioresistant)
GM06419	M	+/-	5	0.240 $\pm$ 0.026 (0.099–0.409)	2.82 cGy/h	5.28 cGy/h	2	Normal (Radioresistant)
GM08333B	M	+/+	3	0.292 $\pm$ 0.031 (0.237–0.400)	2.43 cGy/h	4.25 cGy/h	5	Normal (Radioresistant)
GM08429	M	+/+	9	0.347 $\pm$ 0.022 (0.094–0.645)	2.18 cGy/h	4.25 cGy/h	7	Normal (Radioresistant)

Table 5.1. Continued.

Fibroblast Strain	Sex	<i>Rb1</i> Genotype	No. Expts.	PE $\pm$ SEM _{PE} (Range)	Dose rate (cGy/h) for relative survival level of:		CIMR Continuous LDR Rank	Continuous LDR Classification
					0.10	0.01		
GM00041C	F	+/+	2	0.075 $\pm$ 0.006 (0.066–0.084)	1.02 cGy/h	1.98 cGy/h	14	Hyper-Radiosensitive
GM00969D	F	+/+	3	0.064 $\pm$ 0.013 (0.035–0.108)	1.90 cGy/h	2.88 cGy/h	10	Moderately Radiosensitive
GM03440C	M	+/+	3	0.178 $\pm$ 0.037 (0.049–0.249)	2.14 cGy/h	3.72 cGy/h	8	Moderately Radiosensitive
GM04503B	F	+/+	2	0.085 $\pm$ 0.017 (0.061–0.110)	1.16 cGy/h	2.28 cGy/h	11	Hyper-Radiosensitive
GM04505A	F	+/+	2	0.077 $\pm$ 0.029 (0.036–0.117)	1.08 cGy/h	2.14 cGy/h	12	Hyper-Radiosensitive
GM05756A	M	+/+	3	0.151 $\pm$ 0.039 (0.057–0.284)	2.14 cGy/h	3.24 cGy/h	9	Moderately Radiosensitive
GM08447	F	+/+	1	0.090	1.02 cGy/h	2.04 cGy/h	13	Hyper-Radiosensitive

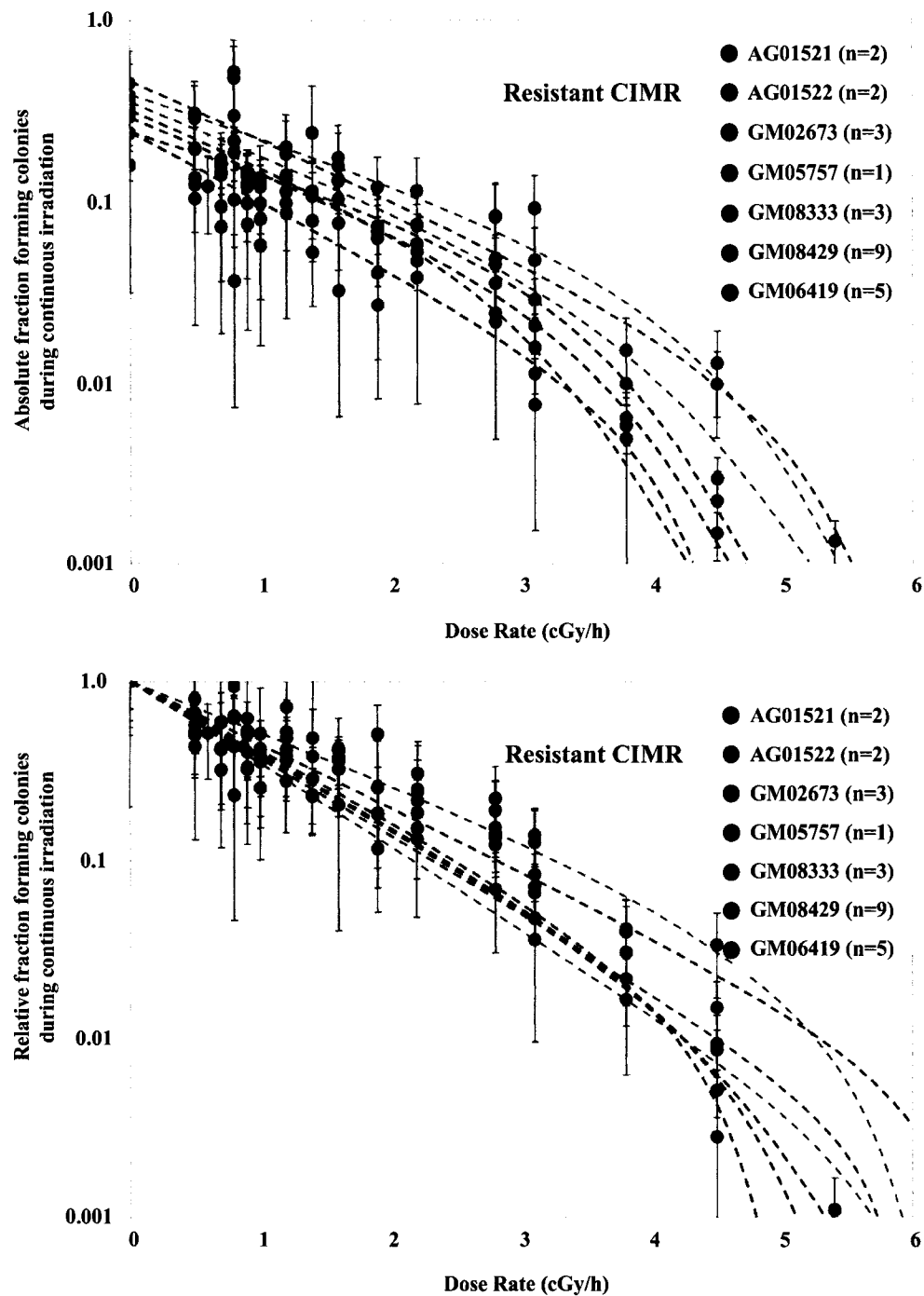


Figure 5.8. Results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from the radioresistant apparently normal (AG01521, AG01522, GM02673, GM05757, GM08333, and GM08429) and *Rb1*^{+/-} (GM06419) CIMR controls (see Table 5.1). Individual strain error bars represent standard errors of the mean, n = number of experiments.

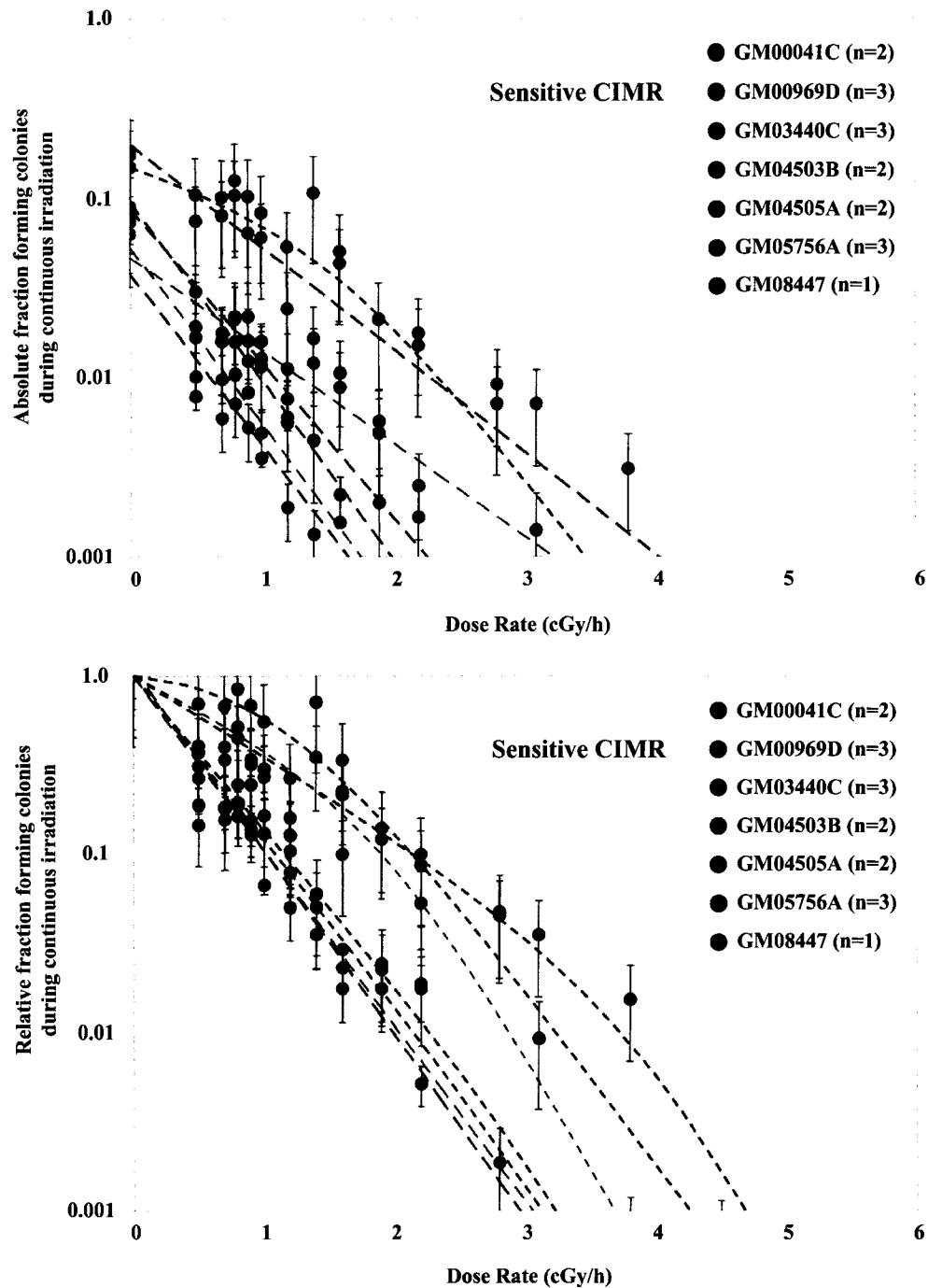


Figure 5.9. Results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from the moderately radiosensitive (GM03440C, GM05756A, and GM00969D) and hyper-radiosensitive (GM00041C, GM04503B, GM04505A, and GM08447) apparently normal CIMR controls (see Table 5.1). Individual strain error bars represent standard errors of the mean, n = number of experiments.

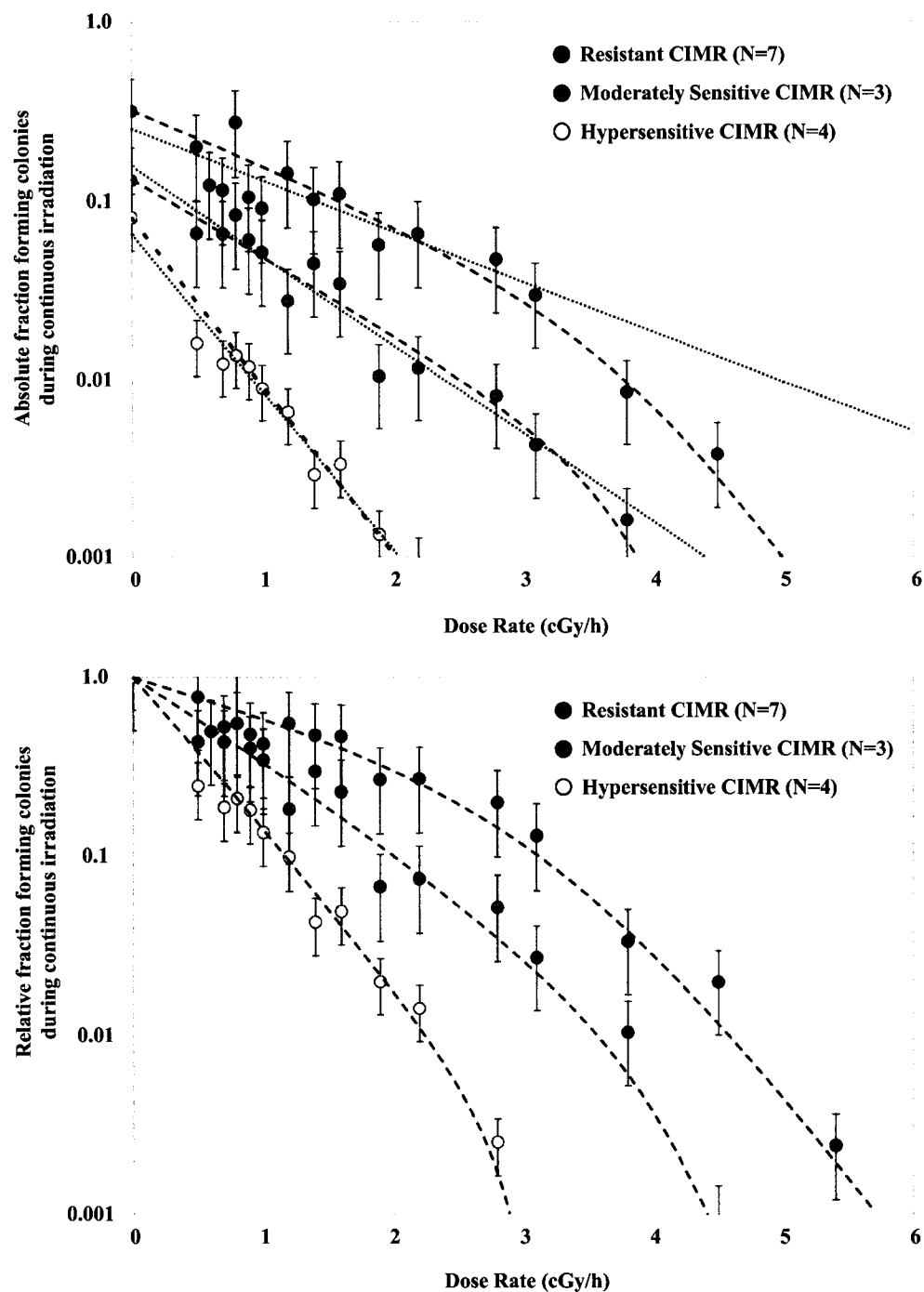


Figure 5.10. Group results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from the radioresistant (black-filled circles), moderately radiosensitive (grey-filled circles), and hyper-radiosensitive (open circles) CIMR controls (see Tables 5.1 and 5.3 and Figures 5.8 and 5.9). Group error bars represent standard deviations, N = number of fibroblast strains.

Figure 5.8 is a plot of the absolute and relative colony forming ability $\pm$ SEM for the seven radioresistant CIMR control fibroblast strains (strains requiring ≥ 4 cGy/h to reduce relative survival to 1%): AG01521, AG01522, GM02673, GM05757, GM06419, GM08333, GM08429. Figure 5.9 is a plot of the absolute and relative colony forming ability $\pm$ SEM for the three moderately radiosensitive CIMR control fibroblast strains (strains requiring ≥ 2.5 cGy/h but < 4 cGy/h to reduce relative survival to 1%) GM00969D, GM03440C, and GM05756A and the four hyper-radiosensitive CIMR control fibroblast strains (strains requiring < 2.5 cGy/h to reduce relative survival to 1%) GM00041C, GM04503B, GM04505A, and GM08447 (these latter two strains were also shown to have high G2 chromosomal radiosensitivity in Chapter 4). The average dose rates required to reduce relative survival to 10% and 1% were ~ 2.6 (range 2.2–3.2) and ~ 4.6 (range 4.2–5.2) cGy/h, respectively, for the 7 radioresistant CIMR control fibroblast strains; ~ 2.1 (range 1.9–2.1) and ~ 3.3 (range 2.9–3.7) cGy/h, respectively, for the 3 moderately radiosensitive CIMR control fibroblast strains; and, ~ 1.1 (range 1.0–1.2) and ~ 2.1 (range 2.0–2.3) cGy/h, respectively for the 4 hyper-radiosensitive CIMR control fibroblast strains.

Figure 5.10 is a plot of the average absolute and relative colony forming ability $\pm$ SD for the three groups of radioresistant, moderately radiosensitive, and hyper-radiosensitive CIMR control fibroblast strains. Both the overall best-fit line (heavier dashed lines) and the least-squares fit linear-exponential regression curve for the ≤ 3 cGy/h survival data (lighter dashed lines) are shown in the upper panel. The relative colony formation data in the lower panel has been normalized to the y -intercept (c) of the three linear-exponential regression lines in the upper panel since they were $> 5\%$ different

than the average measured zero-dose plating efficiencies for the groups (0.251 versus 0.311 for the radioresistant strains, 0.155 versus 0.131 for the moderately radiosensitive strains, and 0.066 versus 0.082 for the hyper-radiosensitive strains, respectively). This figure also demonstrates that the asymptotic decrease in cell survival as the dose rate approaches the proliferation-limiting LDR often only becomes evident in the relative survival plots (the detection limit for absolute survival in this assay is approximately 1 in 1000 or 0.001). Colonies were never recovered for any cell strain at dose rates higher than the asymptotic portions of the LDR survival curves. The dose rate at this portion of the survival curve was considered proliferation-limiting low dose-rate for that strain.

The effect of continuous LDR irradiation on the retention of proliferative capacity for the 14 apparently normal and *Rb1*^{+/-} Rb family member fibroblast strains identified in Fitzek, *et al.* (2002) and Table 5.2 are shown in Figures 5.11–5.15, separated by family. The average radioresistant CIMR control fibroblast strain response from Figure 5.10 is also plotted on Figures 5.11–5.15 to serve as a reference line for the typical “normal” response in this assay. The legend symbols for these figures are identically color-coded: fathers are blue circles, mothers are green circles, and the affected proband children (either sex) are red circles. The Rb family member fibroblast strain responses are summarized in Table 5.2, and the strains again ranked from least (1) to most (14) radiosensitive in this assay. The average dose rates required to reduce relative survival to 10% and 1% were ~1.6 (range 1.3–1.9) and ~3.0 (range 2.5–3.2) cGy/h, respectively, for the 7 moderately radiosensitive Rb family member fibroblast strains; and, ~1.1 (range 0.8–1.4) and ~2.0 (range 1.6–2.4) cGy/h, respectively for the 7 hyper-radiosensitive Rb family member fibroblast strains in all five Rb families.

Table 5.2. Results of the continuous LDR (0.5–8.4 cGy/h) irradiation colony formation assays for the 14 Rb family member fibroblast strains. Strains are ranked from least (1) to most (14) radiosensitive.

Table 5.2.

Fibroblast Strain	Sex	<i>Rb1</i> Genotype	No. Expts.	PE $\pm$ SEM _{PE} (Range)	Dose rate (cGy/h) for relative survival level of:		Rb Family Continuous LDR Rank	Continuous LDR Classification
					0.10	0.01		
MF-2M Rb Family I	F	+/+	5	0.110 $\pm$ 0.016 (0.013–0.198)	1.30 cGy/h	2.52 cGy/h	7	Moderately Radiosensitive
MF-3R Rb Family I	F	+/-	4	0.101 $\pm$ 0.019 (0.026–0.195)	1.32 cGy/h	2.58 cGy/h	6	Moderately Radiosensitive
MF-4F Rb Family I	M	+/+	3	0.448 $\pm$ 0.061 (0.267–0.632)	1.93 cGy/h	3.10 cGy/h	1	Moderately Radiosensitive
MF-6F Rb Family II	M	+/+	5	0.127 $\pm$ 0.024 (0.021–0.302)	0.80 cGy/h	1.60 cGy/h	14	Hyper-Radiosensitive
MF-7R Rb Family II	M	+/-	4	0.097 $\pm$ 0.022 (0.031–0.225)	1.50 cGy/h	2.80 cGy/h	5	Moderately Radiosensitive
MF-10R Rb Family III	M	+/-	4	0.278 $\pm$ 0.032 (0.112–0.422)	1.50 cGy/h	2.82 cGy/h	4	Moderately Radiosensitive
MF-11F Rb Family III	M	+/-	6	0.089 $\pm$ 0.015 (0.009–0.228)	1.70 cGy/h	3.20 cGy/h	3	Moderately Radiosensitive
MF-12M Rb Family III	F	+/+	4	0.037 $\pm$ 0.013 (0.005–0.113)	1.18 cGy/h	2.18 cGy/h	10	Hyper-Radiosensitive

Table 5.2. Continued.

Fibroblast Strain	Sex	<i>Rb1</i> Genotype	No. Expts.	PE $\pm$ SEM _{PE} (Range)	Dose rate (cGy/h) for relative survival level of:		Rb Family Continuous LDR Rank	Continuous LDR Classification
					0.10	0.01		
MF-13M Rb Family IV	F	+/+	5	0.130 $\pm$ 0.029 (0.027–0.372)	1.04 cGy/h	2.04 cGy/h	11	Hyper-Radiosensitive
MF-14R Rb Family IV	F	+/-	4	0.217 $\pm$ 0.034 (0.045–0.377)	1.78 cGy/h	3.22 cGy/h	2	Moderately Radiosensitive
MF-15F Rb Family IV	M	+/+	5	0.160 $\pm$ 0.015 (0.100–0.262)	1.24 cGy/h	2.42 cGy/h	8	Hyper-Radiosensitive
MF-16M Rb Family V	F	+/+	3	0.146 $\pm$ 0.064 (0.034–0.366)	1.44 cGy/h	2.18 cGy/h	9	Hyper- Radiosensitive
MF-17R Rb Family V	F	+/-	2	0.024 $\pm$ 0.005 (0.017–0.031)	1.08 cGy/h	1.88 cGy/h	12	Hyper-Radiosensitive
MF-18F Rb Family V	M	+/+	5	0.188 $\pm$ 0.061 (0.007–0.730)	1.00 cGy/h	1.70 cGy/h	13	Hyper-Radiosensitive

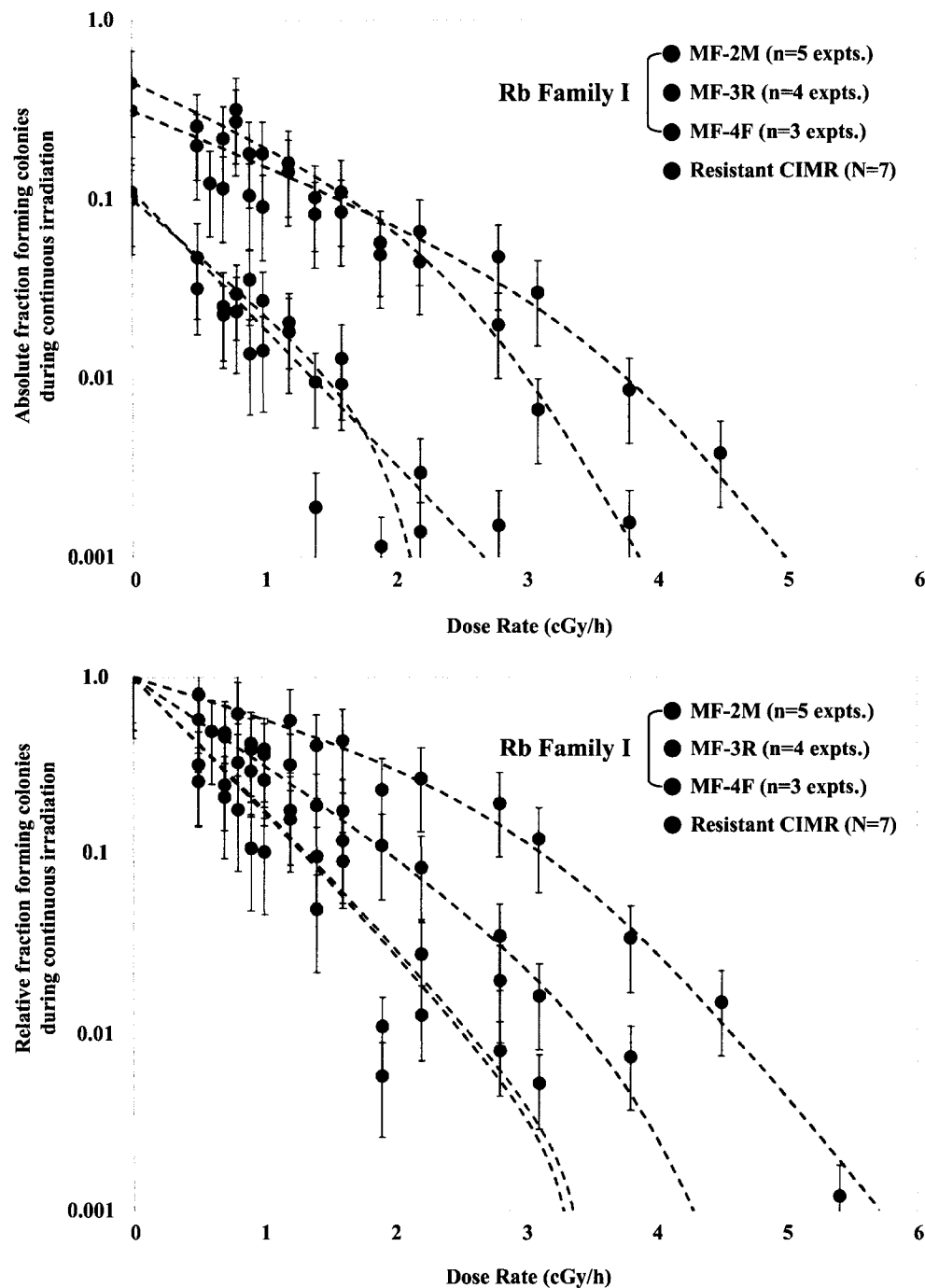


Figure 5.11. Results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from members of Rb Family I: MF-2M (green circles), MF-3R (red circles), and MF-4F (blue circles), compared to the average response of the 7 radioresistant CIMR control fibroblast strains (black circles; see Tables 5.2 and 5.3 and Figure 5.10). Individual strain error bars represent standard errors of the mean, n = number of experiments.

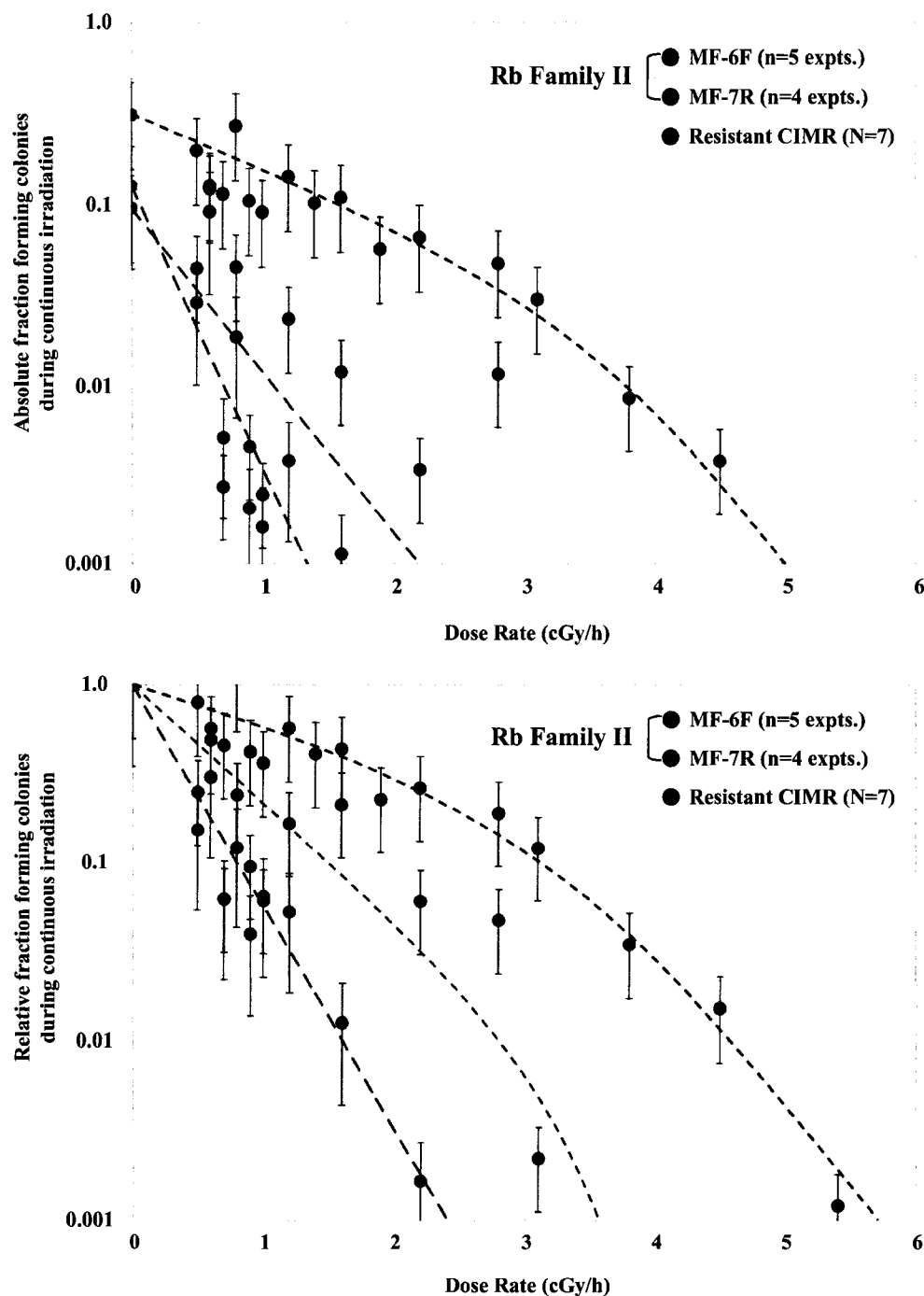


Figure 5.12. Results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from members of Rb Family II: MF-6F (blue circles) and MF-7R (red circles), compared to the average response of the 7 radioresistant CIMR control fibroblast strains (black circles; see Tables 5.2 and 5.3 and Figure 5.10). Individual strain error bars represent standard errors of the mean, n = number of experiments.

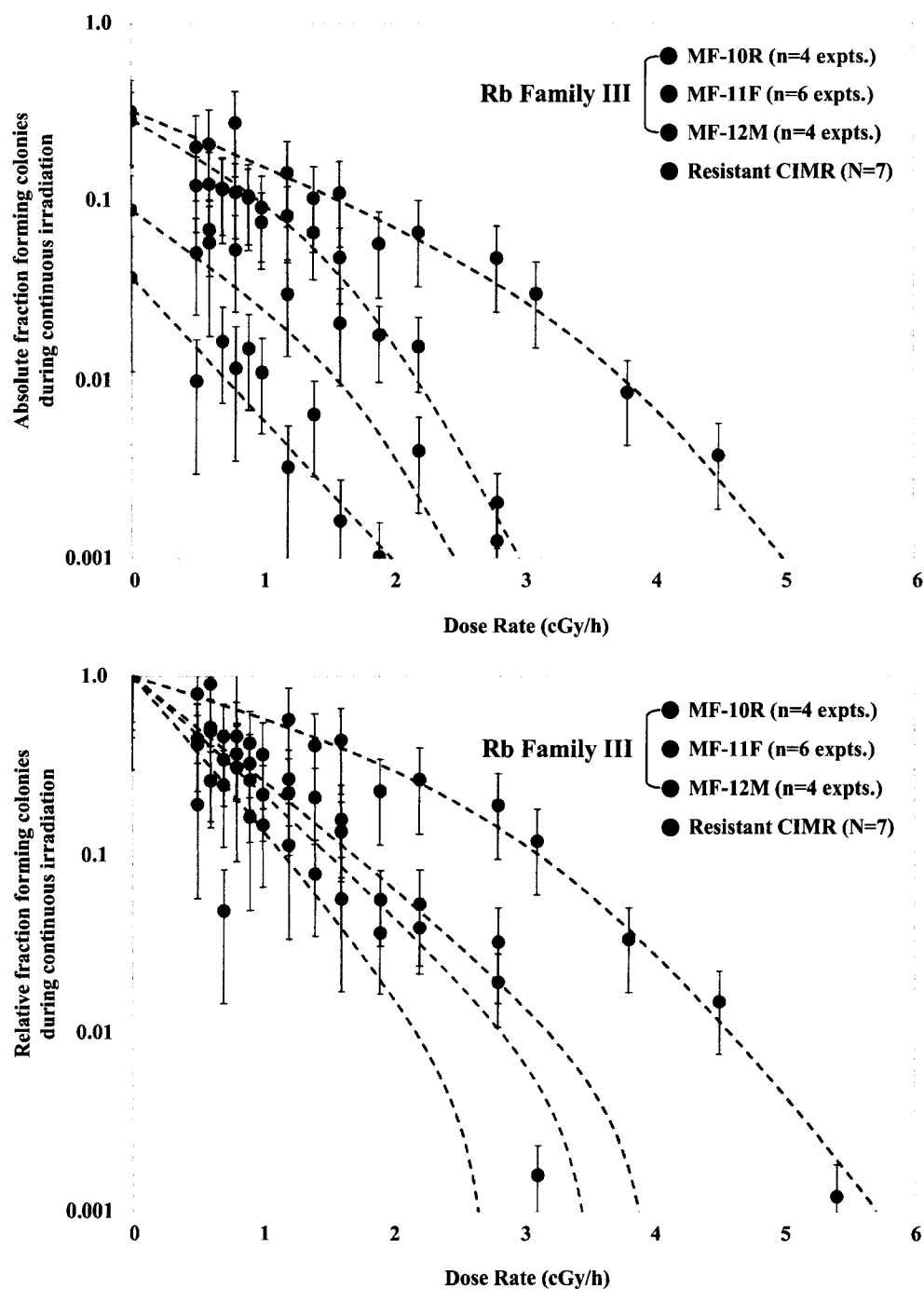


Figure 5.13. Results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from members of Rb Family III: MF-10R (red circles), MF-11F (blue circles), and MF-12M (green circles), compared to the average response of the 7 radioresistant CIMR control fibroblast strains (black circles; see Tables 5.2 and 5.3 and Figure 5.10). Individual strain error bars represent standard errors of the mean, n = number of experiments.

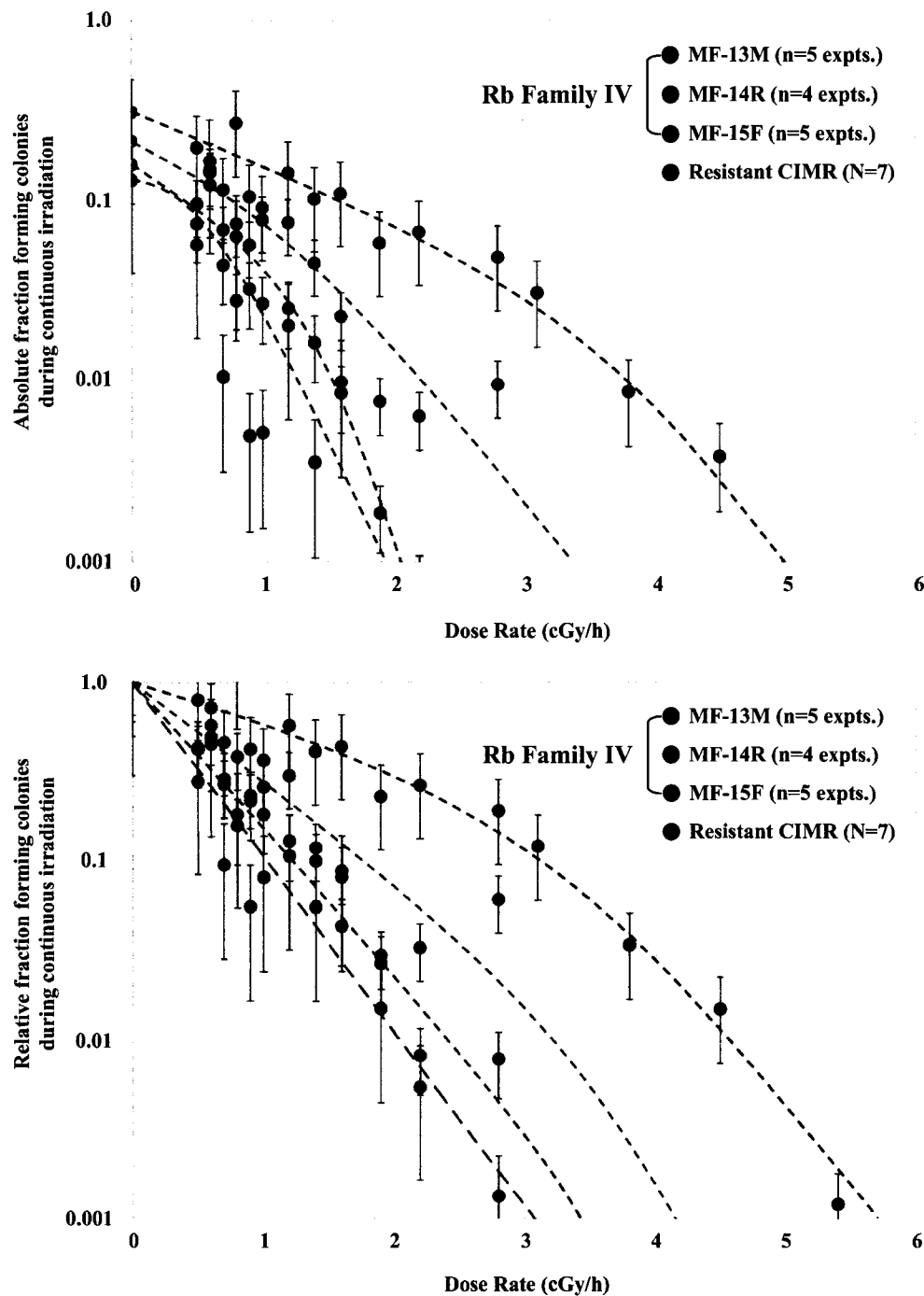


Figure 5.14. Results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from members of Rb Family IV: MF-13M (green circles), MF-14R (red circles), and MF-15F (blue circles), compared to the average response of the 7 radioresistant CIMR control fibroblast strains (black circles; see Tables 5.2 and 5.3 and Figure 5.10). Individual strain error bars represent standard errors of the mean, n = number of experiments.

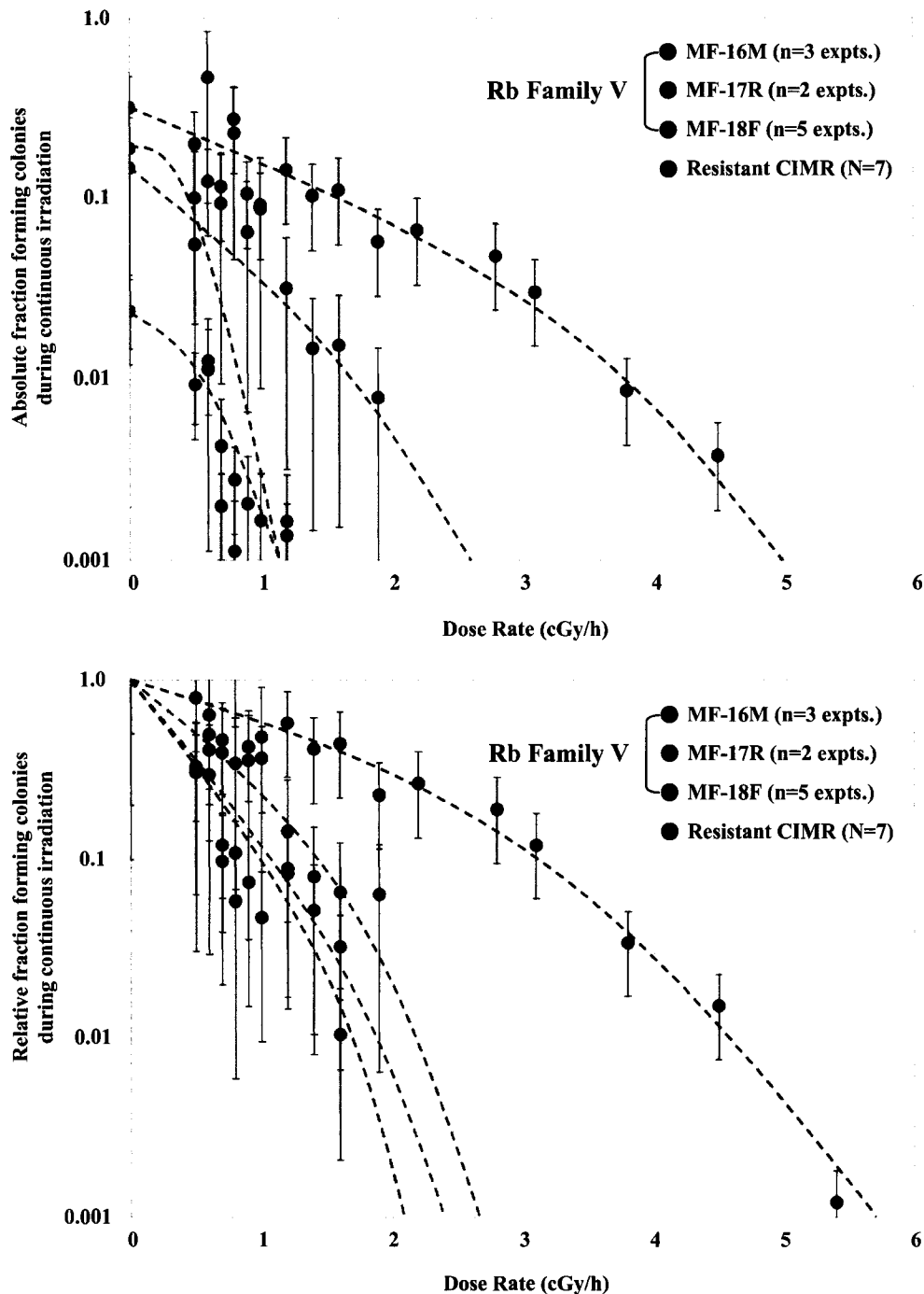


Figure 5.15. Results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from members of Rb Family V: MF-16M (green circles), MF-17R (red circles), and MF-18F (blue circles), compared to the average response of the 7 radioresistant CIMR control fibroblast strains (black circles; see Tables 5.2 and 5.3 and Figure 5.10). Individual strain error bars represent standard errors of the mean, n = number of experiments.

To examine the relationship between continuous LDR irradiation survival and acute (high dose-rate) irradiation survival, the 14 Rb family member fibroblast strains were grouped according to their acute irradiation survival D_0 values, as reported in Fitzek *et al.* (2002) and Table 1.1, in Figure 5.16. These groups include strains with D_0 values of 90–110 cGy (MF-7R, MF-10R, and MF-14R; all are affected probands), strains with D_0 values of 70–90 cGy (MF-2M, MF-3R, MF-11F, and MF-12M), and strains with D_0 values of 50–70 cGy (MF-4F, MF-6F, MF-13M, MF-15F, MF-16M, MF-17R, and MF-18F). No significant differences between these three groups based on acute irradiation survival response were observed (see also Table 5.4 and Figure 5.22).

The variation in plating efficiencies recorded in Tables 5.1 and 5.2 is to be expected during the execution of such a large number of experiments over two years, even despite the use of a single serum lot. Multiple plating efficiency experiments with flasks arranged throughout the LDR experimental setups (Figures 5.1 and 5.6) without irradiation did not yield any significant differences for the cell strains tested (both apparently normal and retinoblastoma family member fibroblast strains, data not shown).

The effects of continuous LDR irradiation on the retention of proliferative capacity for all 28 fibroblast strains identified in Tables 5.1 and 5.2 is summarized in Table 5.3. The CIMR and Rb family member strains are separately grouped by the three radiosensitivity categories (radioresistant, moderately radiosensitive, or hyper-radiosensitive), and also grouped together by the same categories (no Rb family member fibroblast strain was considered radioresistant). The Rb family member fibroblast strains are also grouped according to their acute (HDR) D_0 values as reported in Fitzek *et al.* (2002) and Table 1.1, as in Figure 5.16. The number of fibroblast strains, the range of

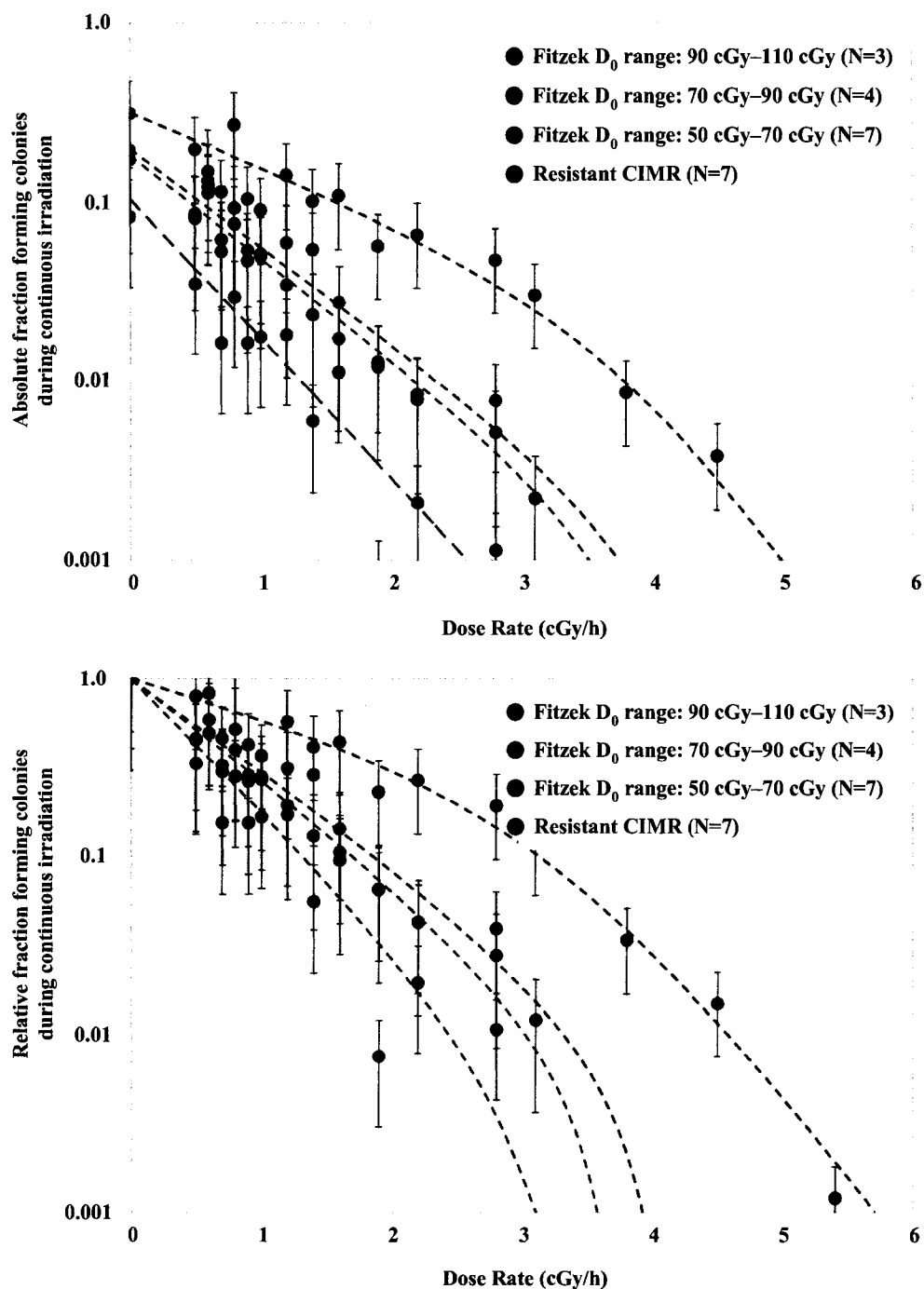


Figure 5.16. Group results of the continuous LDR irradiation colony formation assay for the 14 Rb family member fibroblast strains grouped according to their acute dose survival D_0 value as reported by Fitzek, *et al.* (2002) and Table 1.1, compared to the average response of the radioresistant CIMR control fibroblast strains (see Table 5.3 and Figures 5.10–5.15). Group error bars represent standard deviations, N = number of fibroblast strains.

Table 5.3. Summary of the continuous LDR (0.5–8.4 cGy/h) irradiation colony formation assay results for the 14 Rb family member and 14 CIMR control fibroblast strains.

Table 5.3.

Group	No. fibroblast strains	PE Range	Dose rate $\pm$ SD _{DR} (cGy/h) for relative survival level of:		Significant difference (<i>P</i> < 0.05) from reference normals
			0.10	0.01	
<i>Apparently Normal and Rb1^{+/-} CIMR Controls</i>					
Normal radiosensitivity (Radioresistant)	7	0.160–0.447	2.55 $\pm$ 0.36 cGy/h	4.56 $\pm$ 0.49 cGy/h	Referent
Moderately Radiosensitive	3	0.064–0.178	2.06 $\pm$ 0.14 cGy/h	3.28 $\pm$ 0.42 cGy/h	Yes
Hyper-Radiosensitive	4	0.075–0.090	1.07 $\pm$ 0.07 cGy/h	2.11 $\pm$ 0.13 cGy/h	Yes
<i>Retinoblastoma Family Members</i>					
Moderately Radiosensitive	7	0.089–0.448	1.57 $\pm$ 0.23 cGy/h	2.95 $\pm$ 0.36 cGy/h	Yes
Hyper-Radiosensitive	7	0.024–0.188	1.11 $\pm$ 0.20 cGy/h	2.00 $\pm$ 0.29 cGy/h	Yes
<i>Combined Radiosensitive Apparently Normal CIMR Controls and Retinoblastoma Family Members</i>					
Moderately Radiosensitive	10	0.064–0.448	1.68 $\pm$ 0.31 cGy/h	3.05 $\pm$ 0.39 cGy/h	Yes
Hyper-Radiosensitive	11	0.024–0.188	1.10 $\pm$ 0.16 cGy/h	2.04 $\pm$ 0.24 cGy/h	Yes

Table 5.3. Continued.

Group (Fibroblast Strains)	No. fibroblast strains	PE Range	Dose rate $\pm$ SD _{DR} (cGy/h) for relative survival level of:		Significant difference (<i>P</i> < 0.05) from reference normals
			0.10	0.01	
<i>Fitzek, et al. (2002) Acute Dose Irradiation Survival D₀ Ranges</i>					
90 cGy–110 cGy (MF-7R, MF-10R, MF-14R)	3	0.097–0.278	1.59 $\pm$ 0.16 cGy/h	2.95 $\pm$ 0.24 cGy/h	Yes
70 cGy–90 cGy (MF-2M, MF-3R, MF-11F, MF-12M)	4	0.037–0.110	1.37 $\pm$ 0.22 cGy/h	2.62 $\pm$ 0.42 cGy/h	Yes
50 cGy–70 cGy (MF-4F, MF-6F, MF-13M, MF-15F, MF-16M, MF-17R, MF-18F)	7	0.024–0.448	1.22 $\pm$ 0.37 cGy/h	3.06 $\pm$ 0.35 cGy/h	Yes

plating efficiencies (PE), and the average dose rate $\pm$ SD required to reduce relative survival to 10% and 1% are listed. The moderately radiosensitive, hyper-radiosensitive, and D_0 groups are all significantly ($P < 0.05$) more radiosensitive than the average response of the radioresistant CIMR control fibroblast strains. All *individual* moderately radiosensitive and hyper-radiosensitive fibroblast strains (the Rb family members and the seven sensitive CIMR controls) are also significantly ($P < 0.05$) more radiosensitive than the average response of the radioresistant CIMR control fibroblast strains.

Representative examples of crystal violet-stained T-75 flasks seeded with 500 cells from the radioresistant apparently normal CIMR control strain AG01521, the hyper-radiosensitive Rb family member strain MF-6F (the father in Rb family II), and the moderately radiosensitive Rb family member strain MF-7R (the affected proband child in Rb family II) are shown in Figure 5.17, panel A. Note the loss of colony formation ability as the dose-rate increases and the poorer overall plating efficiency of the retinoblastoma family members. Clonal size heterogeneity is also evident in the unirradiated flasks and increasingly so in the irradiated flasks as the dose-rate increases.

In Figure 5.17 panel B, the results of the continuous LDR irradiation colony formation assay summarized in Table 5.3 are plotted. The three radiosensitivity groups and the numbers of Rb family member and CIMR control fibroblast strains per group are indicated by the grey-shaded areas. The upper and lower limits of response for the CIMR and Rb family member groups are indicated by the solid and dashed lines, respectively. The upper end of the Rb family member response is almost identical to the average response of the moderately radiosensitive CIMR control strains plotted in Figure 5.10. The average dose rates required to reduce relative survival to 10% and 1% were ~ 2.6 and

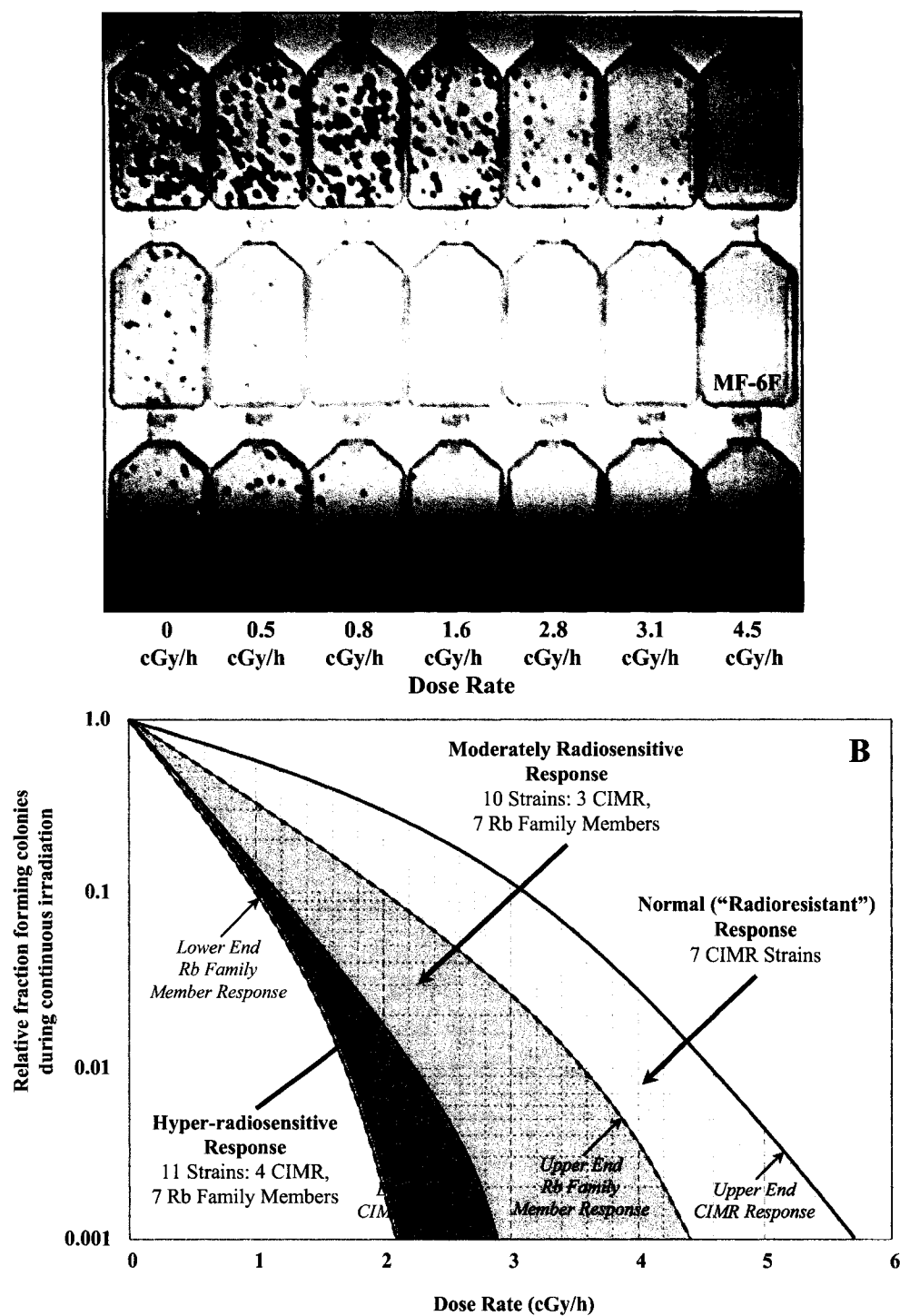


Figure 5.17. A. Representative stained T-75 flasks (500 cells seeded) for strains AG01521 (radioresistant), MF-6F (hyper-radiosensitive), and MF-7R (moderately radiosensitive) after two weeks of growth in the continuous LDR irradiation colony formation assay. B. Summary of the results of the continuous LDR irradiation colony formation assay for fibroblast strains derived from the Rb family members and apparently normal CIMR controls.

~4.6 cGy/h, respectively, for the 7 radioresistant CIMR fibroblast strains; ~1.7 and ~3.1 cGy/h, respectively, for the 10 moderately radiosensitive Rb family member and CIMR strains; and, ~1.1 and ~2.0 cGy/h, respectively for the 11 hyper-radiosensitive Rb family member and CIMR strains.

Interestingly, Figures 5.8–5.17 demonstrate that the proliferative capacity of all three radiosensitivity groups drop to ~30–80% of unirradiated control levels by 0.5 cGy/h, the lowest dose-rate examined for the MRB012 series of LDR experiments. This was somewhat unexpected since the survival of rodent cells at this dose rate is identical to unirradiated levels (Priestly, *et al.*, 1998; Chapter 6). Seven experiments were performed at even lower dose rates (0.06–0.6 cGy/h), supplied by the lead-shielded MRB008 source depicted in Figure 5.6, using two radioresistant CIMR control strains AG01521 and GM02149), a moderately radiosensitive CIMR control strains GM03440C, and three hyper-radiosensitive CIMR control strains GM00041C, GM04503B, and GM04505A (which display the most dramatic decrease in relative survival by 0.5 cGy/h in the MRB012 LDR irradiation facility) to better characterize the dose rate response in this region.

The results of these very low dose-rate (VLDR) experiments are shown in Figure 5.18. The radiosensitive strains repeatedly (3–4 experiments per strain) maintained a flat hypersensitive dose-rate response, indicating even continuous exposure of these sensitive strains to 0.06 cGy/h is sufficient to limit proliferation by ~50%. However, the dose rates employed in these experiments were apparently still too high to characterize a dose rate response of the radiosensitive CIMR strains for relative survival levels of >70%. The two radioresistant CIMR strains demonstrated a smooth linear decrease in this relative

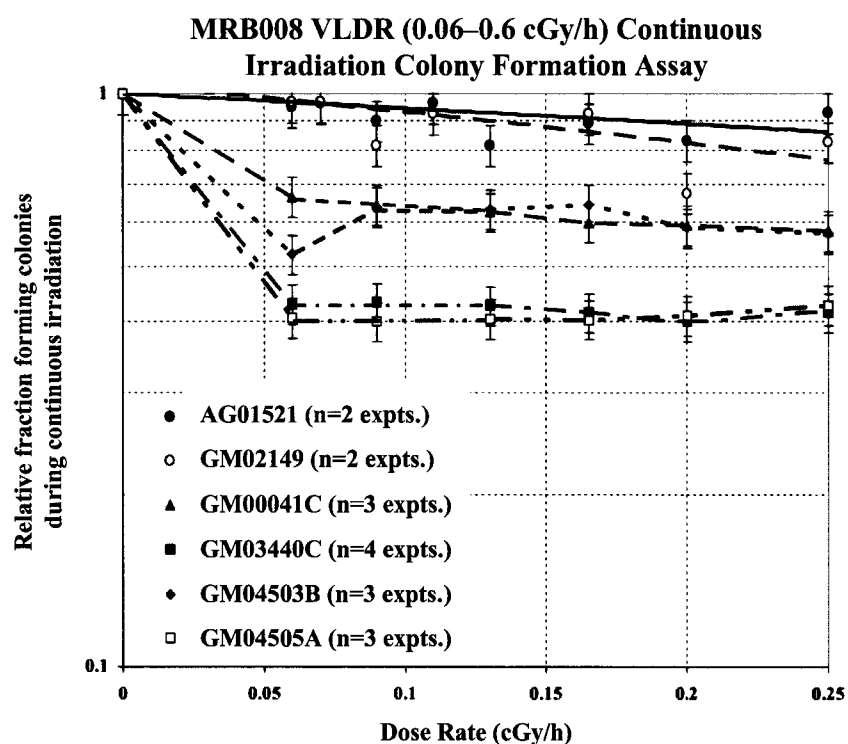


Figure 5.18. Results of the very low dose-rate (0.06–0.6 cGy/h) continuous irradiation colony formation assays conducted with two radioresistant apparently normal CIMR strains (AG01521 and GM02149), a moderately radiosensitive apparently normal CIMR strain (GM03440C), and three hyper-radiosensitive apparently normal CIMR strains (GM00041C, GM04503B, and GM04505A) using the lead-shielded MRB008 setup depicted in Figure 5.6.

survival range at these very low dose-rates, which coincided exactly with their MRB012 LDR responses beginning at 0.5 cGy/h shown in Figure 5.8.

Histograms of the continuous irradiation dose rates required to reduce relative survival to 10% and 1% for the 14 Rb family member and 14 CIMR control fibroblast strains are shown in Figures 5.19 and 5.20, respectively (the “MF,” “AG,” and “GM” prefixes have been omitted). Both distributions appear bimodal, with a noticeable separation between the radioresistant CIMR control strains and the moderately and hyper-radiosensitive Rb family member and remaining CIMR control strains. Figure 5.20 also shows the individual fibroblast strains in each of the three radiosensitivity categories: hyper-radiosensitive strains (dose rates <2.5 cGy/h, 11 total) in the first two “bins,” moderately radiosensitive strains (dose rates ≥ 2.5 cGy/h but <4 cGy/h, 10 total) in the following three bins, and the radioresistant strains (dose rates ≥ 4 cGy/h, 7 CIMR) in the final three bins. Interestingly, there seems to be a preponderance of apparently normal female fibroblast strains (both Rb family mothers and female CIMR controls) in the more radiosensitive groups.

In Figure 5.21, the dose rates required to reduce relative survival to 10% and 1% are compared with plating efficiency (PE) values for the 28 human fibroblast strains examined in the continuous LDR irradiation colony formation assay (see Tables 5.1 and 5.2). The continuous irradiation dose rates required for both the 10% and 1% relative survival levels are both significantly correlated ($P = 0.00011$ and 1.95×10^{-5} , respectively) to PE. The ability of cells to reattach to the cell culture flask is not only tested during the initial plating of these cells, but at each cell division as the daughter cells reattach to the culture dish upon the completion of mitosis and cytokinesis.

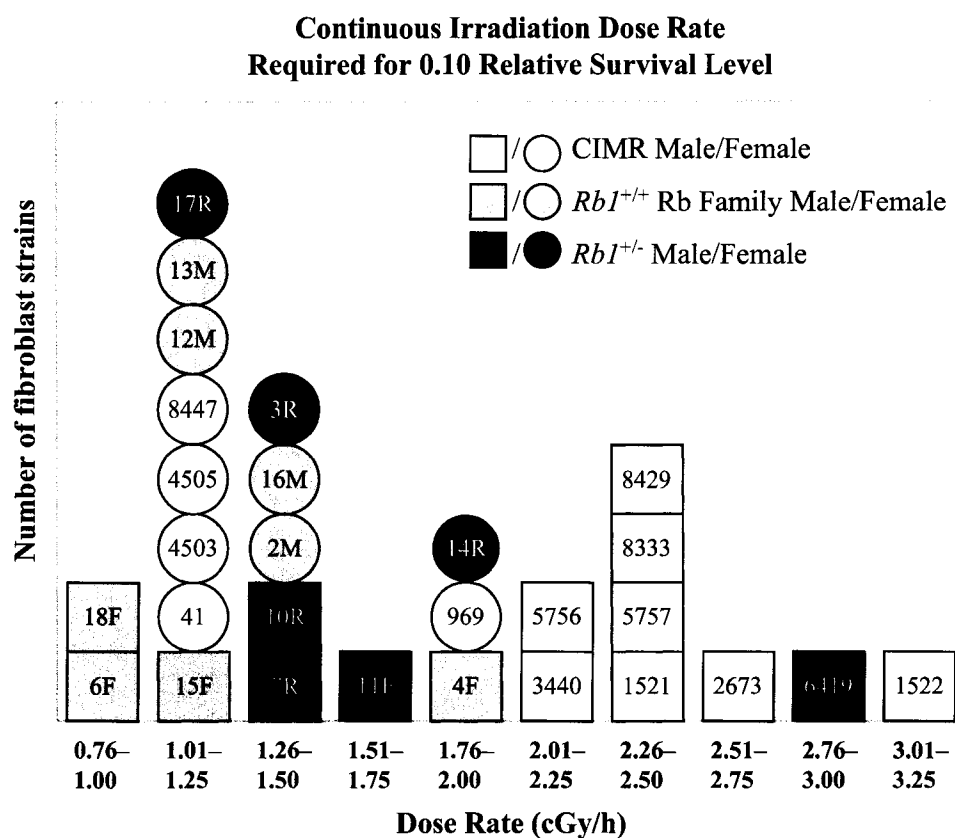


Figure 5.19. Distribution of the dose-rates (in cGy/h) required to reduce relative survival to 10% in the continuous LDR irradiation colony formation assay for the 14 Rb family member and 14 CIMR control fibroblast strains (see Tables 5.1 and 5.2).

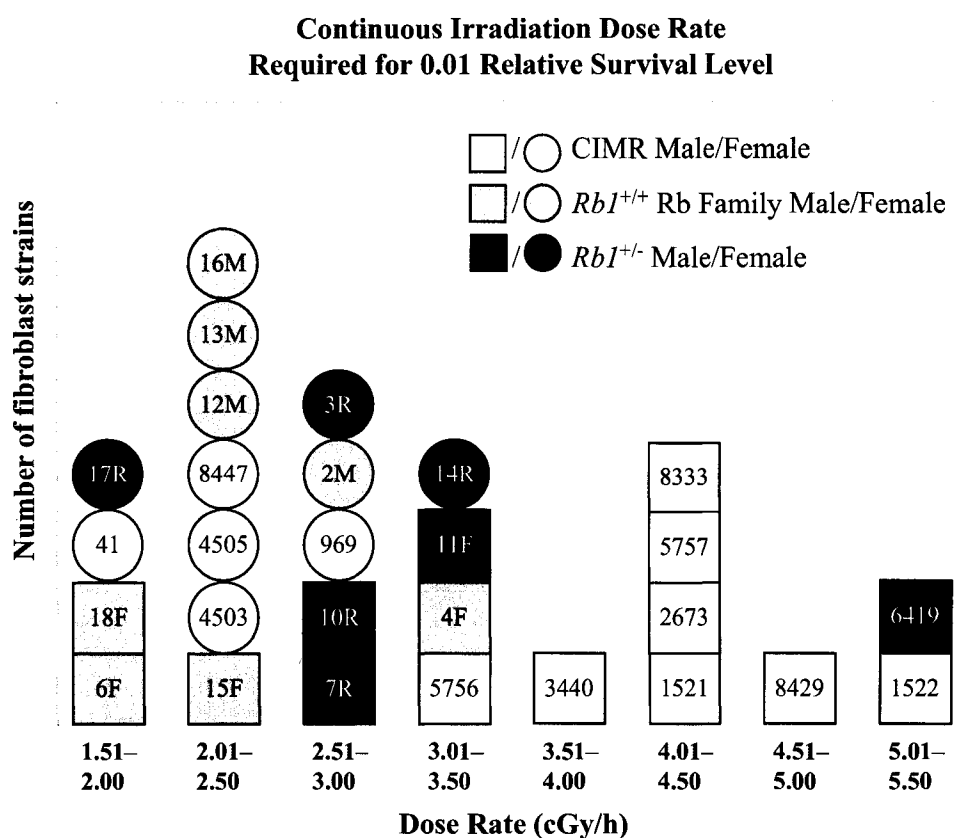


Figure 5.20. Distribution of the dose-rates (in cGy/h) required to reduce relative survival to 1% in the continuous LDR irradiation colony formation assay for the 14 Rb family member and 14 CIMR control fibroblast strains (see Tables 5.1 and 5.2).

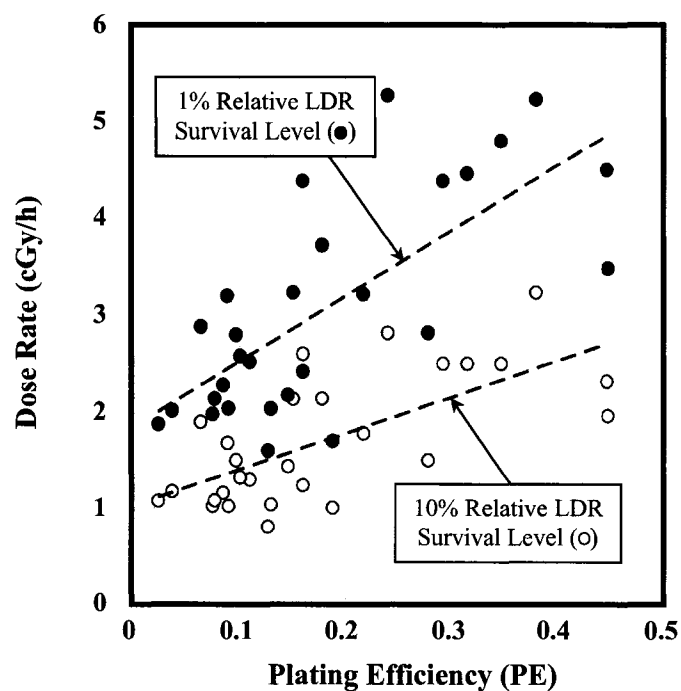


Figure 5.21. Plot of dose rates (in cGy/h) required to reduce relative survival to 10% (open circles) and 1% (filled circles) compared to plating efficiency (PE) values for the 28 human fibroblast strains examined in the continuous LDR irradiation colony formation assay (see Tables 5.1 and 5.2). The continuous irradiation dose rates required to reduce relative survival to 10% and 1% and the PE values are significantly correlated ($P = 0.00011$ and 1.95×10^{-5} , respectively).

In Figure 5.22, the dose rates required to reduce relative survival to 10% and 1% (Table 5.2) are compared with the two acute irradiation survival parameters, D_0 and ${}_1D_0$, reported by Fitzek, *et al.* (2002; see Table 1.1) and Chapter 3 (Table 3.1), respectively, for the 14 Rb family member fibroblast strains. Only the continuous irradiation dose rates required to reduce relative survival to 1% and the Fitzek D_0 values are significantly correlated ($P = 0.022$). This lack of correlation between the two assays is reflective of the significant differences between them. In the acute irradiation survival assays, single cells are irradiated in a single, short high dose-rate exposure (completed usually in minutes) and then plated for colony formation without further radiation exposure to the growing colony. In the continuous LDR irradiation survival assay, single cells are irradiated continuously at low dose-rate as they attempt to divide and form colonies, with each successive round of daughter cells exposed to the same low dose-rate irradiation stress as their parental cells.

In Figure 5.23 panel A, the dose rates required to reduce relative survival to 10% and 1% (Tables 5.1 and 5.2) are compared to the frequencies of radiation-induced chromatid breaks and combined gaps and breaks recorded in the G2 chromosomal radiosensitivity assay in Chapter 4 (Table 4.4) for the 14 Rb family member, three radioresistant CIMR control (AG01521, GM02149, and GM06419), and two hyper-radiosensitive CIMR control (GM04505A and GM08447) fibroblast strains. The continuous irradiation dose rates and the frequencies of both groups of radiation-induced chromatid aberrations are all significantly correlated with one another ($P = 0.00014$ – 0.00052). In panel B, the pattern of correlation for the 19 individual fibroblast strains is identified, and this pattern is identical for the four data set comparisons in panel A. This

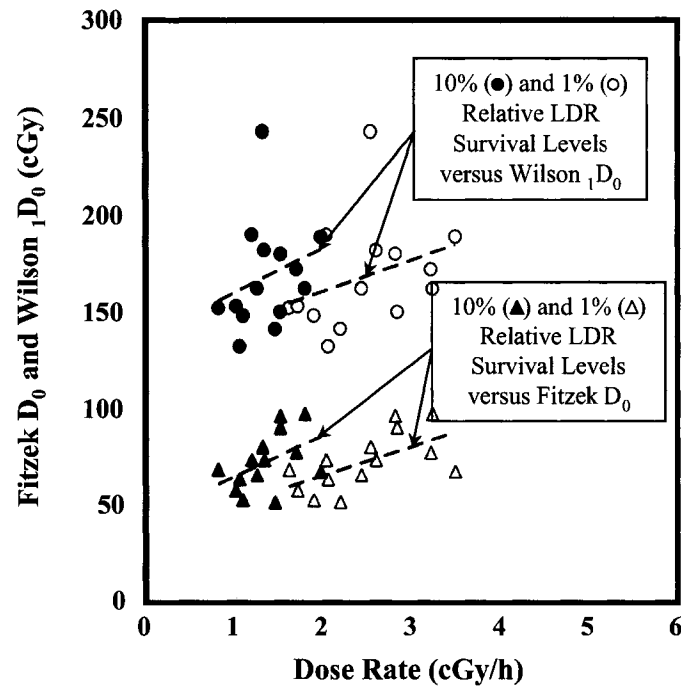


Figure 5.22. Plot of dose rates (in cGy/h) required to reduce relative survival to 10% (filled symbols) and 1% (open symbols) in the continuous LDR irradiation colony formation assay (see Tables 5.1 and 5.2) compared to acute irradiation survival D_0 values reported by Fitzek, *et al.*, 2002 (triangles, see Table 1.1) and ${}_1D_0$ values reported in Table 3.1 (circles) for the 14 Rb family member fibroblast strains. Only the continuous irradiation dose rates required to reduce relative survival to 1% and the Fitzek D_0 values (open triangles) are significantly correlated ($P = 0.022$).

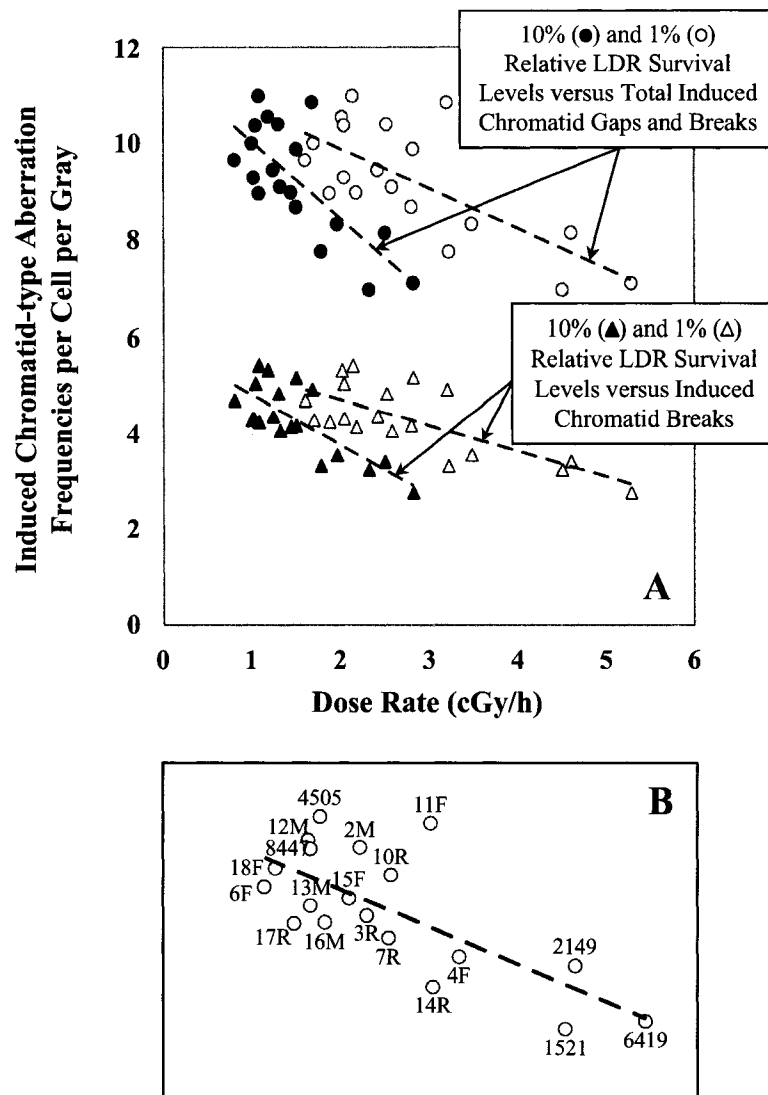


Figure 5.23. **A.** Plot of dose rates (in cGy/h) required to reduce relative survival to 10% (filled symbols) and 1% (open symbols) in the continuous LDR irradiation colony formation assay (see Tables 5.1 and 5.2) compared to the frequencies of radiation-induced chromatid breaks (triangles) and combined chromatid gaps and breaks (circles) in the G2 chromosomal radiosensitivity assay (see Table 4.4) for the 14 Rb family member fibroblast strains. The continuous irradiation dose rates required to reduce relative survival to 10% and 1% and the frequencies of both groups of radiation-induced aberrations are significantly correlated ($P = 0.00014$ – 0.00052). **B.** Pattern of response of the particular fibroblast strains (with the “AG,” “GM,” and “MF-” prefixes omitted) tested in both the G2 chromosomal radiosensitivity and continuous LDR colony formation assays. This pattern is identical for each of the four data set comparisons presented in panel A.

correlation was not unexpected as previous reports from this laboratory of the continuous LDR proliferative capability of various cell types indicated the failure of cells to proliferate as the dose-rate approached the proliferation-limiting LDR was due to cell cycle redistribution into the radiosensitive G2-phase of the cell cycle (Bedford and Mitchell, 1973, 1979; Mitchell, *et al.*, 1979a, 1979b; Stackhouse and Bedford, 1993, 1994).

5.5. Discussion

The benefit of the MRB008 and MRB012 irradiation facilities used in these experiments is the ability to tailor a range of low to intermediate dose-rates (<1 cGy/h to ~50 cGy/h) by using different numbers and combinations of sources, varying the distance to the source, and varying the rates of attenuation with liquid-filled flasks or lead shielding (see Figures 5.1, 5.6, 6.2 and 6.4). This allows the measurement of a variety of cellular endpoints in the dose-rate region where DNA damage signaling and repair capacities vary the most (Amdur and Bedford, 1990, Vilenchik and Knudson, 2000).

Although the shape of the continuous LDR colony formation assay dose-rate response appears similar to an acute irradiation colony formation assay dose response, a number of important differences can be appreciated. As cells attempt to replicate under low dose-rate radiation exposure, overall population doubling times can range from 24 hours to >200 hours (see Figure 5.7). This makes the calculation of the overall dose received by the original cell generating the colony quite difficult (Bedford, *et al.*, 1980); the total dose received to the *flask* could be easily calculated, but not to the *cells* in the flask due to the dose-rate-specific and cell strain-specific growth characteristics. As

such, survival data is plotted in the ordinate in these series of experiments against dose-rate, rather than dose, on the abscissa.

Another important difference is the shape of the dose response curve at higher dose rates. A traditional acute irradiation survival curve is assumed to exponentially decrease as a function of dose, implying that with enough cells plated (*i.e.*, billions) one could hope to recover a surviving colony at higher doses simply based on Poisson probabilities. The continuous LDR irradiation survival curve however, rapidly becomes asymptotic as the dose-rate approaches the proliferation-limiting LDR. This implies that even with such large numbers of cells plated, one would never expect to recover a surviving colony. Poisson probabilities still dictate the survival response of the irradiated cells (admittedly for smaller doses per unit time), but these probability functions apply to each successive pair of daughter cells *in every cell division* as they attempt to form a colony. The continuous LDR exposure survival curves are more similar in form to survival responses observed for cells treated with various chemical agents, for example, Fanconi anemia cells treated with the cross-linking agent MMC (*e.g.*, Tebbs, *et al.*, 2005), implying the possibility of a threshold for these types of chronic, LDR exposures.

With population doubling times approaching 100 hours or more at the higher dose rates, a cell may accrue large numbers of DSBs before attempting to undergo DNA replication or mitosis if the *rate* of DSB formation per hour is sufficiently low to fail to engage appropriate DSB response/repair and cell cycle control mechanisms (Vilenchik and Knudson, 2001, 2003; Collis, *et al.*, 2004, Ishizaki, *et al.*, 2004; also discussed in Chapter 2). As such, this quantity of DSBs may only signal for cell cycle arrest when registered at the G1/S- and G2/M-transition checkpoints as cells prepare for DNA

replication and mitosis. These unrepaired DSBs could prove to be lethal (akin to the concept of low dose hyper-radiosensitivity) or result in higher mutation rates (Bedford and Mitchell, 1977; Vilenchik and Knudson, 2001, 2003). It would be interesting to measure γ -H2AX foci, a sensitive measure of cellular DSBs in chromatin (Rothkamm and Löbrich, 2003), in irradiated cultures of the hyper-radiosensitive Rb family member and CIMR control fibroblast strains at these low dose-rates to test this theory, although such foci were shown to be significantly attenuated in their formation at 1.8 cGy/h in other normal, hTERT-immortalized, and malignant human cells (Collis, *et al.*, 2004; Ishizaki, *et al.*, 2004).

GM06419, a *Rb1* heterozygote strain obtained from the CIMR cell bank with a reported acute D_0 value of 85 cGy (Li, *et al.*, 1995) but very low G2 assay scores (Sanford, *et al.*, 1989, 1996; Chapter 4), was included in the group of resistant normal controls as its response in this assay was equivalent (if not better) than the other resistant normal controls. This apparent contradiction among GM06419's radiation responses in these different assays reinforces the fact that overall radiosensitivity is not related to *Rb1* genotype status and other genetic factors must be involved in the hyper-sensitive response of this cell line to single doses of acute irradiation. Likewise the similar degree of responses in the Rb families, some affected and some unaffected by retinoblastoma, casts doubt on a *direct* role of *Rb1* haploinsufficiency on individual radiation sensitivity.

The dose-rate region of ~0.1–10 cGy/h encompasses the very range of dose-rates where evidence for DNA repair signaling thresholds have been reported, cells begin to demonstrate adaptive responses to later challenge doses, and the inverse dose-rate effect for mutagenesis of dividing somatic and germ-line cells is most dramatic (see Chapter 2

for a brief review and references to these topics). The dramatic changes in survival and proliferative capacity at dose rates of 0.1 cGy/h to 10 cGy/h seen in the current experiments detailed herein provide additional support for a *critical* dose rate range for cellular response in the mGy/h and cGy/h region.

The shape of the dose response for the radiosensitive CIMR strains in the mGy/h region (Figure 5.18) could be further characterized by increasing the lead shielding thickness for the MRB008 cesium source and by using culture medium-based tritiated water β -particle exposures. The potential impact of such findings could influence various regulatory safety issues pertaining to very low dose-rate exposures of cycling cell populations for the general population. Likewise, such a study could be extremely informative for identifying other critical dose-rate DNA DSB signaling thresholds required to activate various cellular response mechanisms (Rothkamm and Löbrich, 2003; Collis, *et al.*, 2004; Ishizaki, *et al.*, 2004).

The potential for continuous low dose-rate irradiation as a complete cell-cycle genetic screen for radiosensitivity mutants has been confirmed with the Stackhouse and Bedford *irs-20* series, and much hope is placed on finding similarly useful results with the completion of the viral cDNA library screen of the Rb family member fibroblasts in the near future (see Chapter 7 for further discussion of this and other topics related to these experiments).

CHAPTER 6

Effects of Continuous Low Dose-Rate Irradiation on *atm* and *tp53*-Mutant MEF Cells and Cytogenetic and Immunocytochemical Analyses of *tp53*-Mutant MEF Cells

6.1. Abstract

Mouse embryo fibroblast (MEF) strains deficient for the tumor suppressor genes *tp53* (double-knockout homozygotes), conferring the most radioresistant phenotype yet observed in mammalian cells, or *atm* (double-knockout homozygotes and single-knockout heterozygotes), conferring (in the homozygote case) one of the most radiosensitive phenotypes yet observed, were used to establish the upper and lower limits of rodent cell radiation response in the continuous LDR irradiation colony formation assay (Stackhouse and Bedford, 1993; Priestly, *et al.*, 1998). Response of the *atm*-heterozygote MEF strains (10% relative survival at 3.5–5 cGy/h) was intermediate to that of the hyper-radiosensitive knockout strains (10% relative survival at 2–3 cGy/h) and the wild-type strains (10% relative survival at 13–14 cGy/h), indicating such a LDR experimental protocol may be useful in positively identifying human A-T heterozygote individuals. The frequency of surviving colonies composed of $\geq 50\%$ (often approaching 100%) giant, aneuploid cells in the radioresistant *tp53*^{-/-} cell population (10% relative survival at 15.2 cGy/h) was increased by continuous LDR exposures in a dose-rate-dependent manner, and not by initial acute doses up to 10 Gy.

A series of cytogenetic and immunocytochemical investigations were conducted with the *tp53*^{-/-} MEF cell line to further examine the phenotype of spontaneous and radiation-induced massive chromosomal aberrations in the giant, aneuploid cells observed in these cultures. Severely aneuploid karyotypes with extensive chromosomal rearrangements were observed with multiplex fluorescence *in situ* hybridization (mFISH) analysis, and a novel telomere dysfunction phenotype termed “telomere fission” involving large numbers of extra-chromosomal telomeric repeats (ECTRs) and telomeric double-minute chromosomes (TDMs) was observed with telomere FISH analysis. Immunocytochemical analyses of these giant cells demonstrated acentric and centric interphase micronuclei and multi-lobed nuclear bodies, large numbers of γ -H2AX foci (a marker for DNA DSBs), and aberrant centrosomes (number and morphology). The phenotypes of the unstable *tp53*^{-/-} cells represent a new class of telomere dysfunction and spontaneous genomic instability, driven by excessive telomere *fission*-driven (rather than telomere *fusion*-driven) recombinational chromosome bridge-fusion-breakage (BFB) cycles suggesting the activity of hyper-recombinational (possibly ALT-associated) DNA repair and deregulated cell cycle control mechanisms, permissive only in the *tp53*^{-/-} genetic background. Genetic analysis of this mutant cell line may further increase our understanding of the direct and indirect roles the tp53 protein (or the associated role of an as-yet unidentified protein partner) plays in DNA repair and the overall maintenance of genomic stability.

6.2. Introduction

This chapter reports a series of experimental measurements of continuous low dose-rate (LDR) irradiation colony formation ability of *atm*^{-/-}, *atm*^{+/-}, and *atm*^{+/+} mouse embryo fibroblast (MEF) strains derived from 129S6/SvEvTac (hereafter referred to as 129S6) mice provided by the laboratory of Dr. Michael Weil at Colorado State University; and a sub-cloned line (Nagasawa clone 6) of an immortalized *tp53*^{-/-} MEF line derived from C57BL/6;129/Sv mice as first reported in Donehower, *et al.* (1992). All cell cultures reported in this series were first initiated by Dr. Hatsumi Nagasawa, either directly from the 129S6 *atm* group of mice, or subcultured from the original cell line in the case of the *tp53*^{-/-} clone 6. These MEF strains were chosen to demonstrate the limits of rodent somatic cell response in the continuous LDR irradiation colony formation assay first described by Stackhouse and Bedford (1993) and later expanded by Priestly, *et al.* (1998), with *atm*-knockout cells being the most radiosensitive and *tp53*-knockout cells being the most radioresistant.

The Priestly report examined the continuous LDR (≤ 15 cGy/h) colony formation ability of CHO 10B2 (wild-type) and *prkdc*^A *irs*-20 (DNA-PKcs-deficient) cells, C57BL/6 mouse fibroblasts, and a *scid prkdc*^{-/-}/*tp53*^{-/-} MEF cell line SGCR11. These data are included in Figure 6.8 and Table 6.2 for discussion purposes. The CB17 mouse *scid* cell line SGCR11 was considered only to be *prkdc*-deficient in the Priestly report. However, SGCR11 was later shown to carry a homozygous T-to-G transversion mutation in both *tp53* alleles at nucleotide 572 (Araki, *et al.*, 1999; Jimenez, *et al.*, 1999). This mutation results in a replacement of leucine residue with an arginine residue at codon 191, located in the critical *tp53* DNA binding domain. Interestingly, normal levels of

DNA-damage induced phosphorylation and some residual DNA binding activity has been reported for the expressed mutant *tp53* protein in this cell line (Woo, *et al.*, 1998; Burma, *et al.*, 1999). Jimenez, *et al.* (1999) observed the SGCR11 *tp53* mutation resulted in a small increase in acute post-irradiation survival of this cell line compared to other hyper-radiosensitive *prkdc*-knockout MEFs. It is likely that such a small shift toward radioresistance would be expected of SGCR11's continuous LDR survival response as well, as reported by Priestly, *et al.* (1998), compared to isogenic *prkdc*-knockout mouse cells.

The *tp53*^{-/-} cell line used in this work was originally generated by gene targeting efforts using a plasmid vector containing 3.7 kilobases (kb) of the genomic *tp53* gene, interrupted in exon 5 by a *PoIII-neo* expression cassette, in mouse embryonic stem (ES) cells (Donehower, *et al.*, 1992). The insertion of the *neo* cassette via a homologous recombination event was accompanied by a deletion of a 450-bp DNA fragment containing 106 nucleotides of exon 5 and ~350 nucleotides of intron 4 in the DNA binding domain region of the wild-type *tp53* protein. A successfully targeted G418-resistant ES clone was used to generate chimeric mice by ES cell microinjection into 3.5-day old C57BL/6 blastocysts and implantation into pseudopregnant C57BL/6 x CBA F1 hybrid females. Chimeric offspring (evident by ES cell-derived 129/Sv strain Agouti pigmentation) were intercrossed, and the expected ~25% of *tp53*^{-/-} offspring were successfully generated (surprisingly, since knockout of both alleles of the *tp53* gene was anticipated to be an embryonic lethal condition). MEFs derived from these animals (including the cell line used in this report) were negative for detectable *tp53* mRNA levels by Northern and RT-PCR analysis, and for detectable levels of expressed *tp53*

protein by Western immunoblotting and metabolic labeling analyses (also confirmed by Western immunoblotting analysis in this laboratory by Dr. Y. Peng and P. Wilson, data not shown).

Earlier passages of the *tp53*-knockout MEF cell line has previously been shown to be resistant to DNA damaging agents and to be genetically unstable, showing high degrees of aneuploidy, chromosome abnormalities, centrosome amplification, and giant cell formation, upon continued passaging in culture (Donehower, *et al.*, 1992; Harvey, *et al.*, 1993b; Sands, *et al.*, 1995; Fukasawa, *et al.*, 1996). Likewise, *in vivo* experiments have shown that spleen and bone marrow cells obtained from *tp53*-deficient mice have 20-fold higher spontaneous levels of chromosome aberrations and >10-fold higher levels of radiation-induced hyperploidy (induction of chromosomal aberrations by radiation was similar to wild-type controls; Bouffler, *et al.*, 1995). Loss-of-heterozygosity (LOH) events mediated by large-scale interstitial or whole chromosome deletions are the most common mutation of the wild-type allele of *tp53* in *tp53*^{+/-} heterozygote mouse and human tumor cells (Jacks, *et al.*, 1994; Shao, *et al.*, 2000).

Genomic instability, evidenced by the amplification of chromosomal-scale genomic sequences [often including the *c-myc*, dihydrofolate reductase (*DHFR*), and carbamoyl-phosphate synthetase-aspartate transcarbamoyl-dihydroorotase (*CAD*) genes], chromosomal rearrangements and structural aberrations, centrosome amplification, and aneuploidy, are also commonly seen in cells derived from *tp53*-deficient mice and human *tp53*-deficient Li-Fraumeni syndrome (LFS) patient and tumor cells (Livingstone, *et al.*, 1992; Yin, *et al.*, 1992; Bouffler, *et al.*, 1995; Fukasawa, *et al.*, 1997; Boyle, *et al.*, 1998, 2002; Zhu, *et al.*, 2002). Consistent with the essential role of the *tp53* protein in

preventing tumorigenesis, *tp53*^{-/-} mice develop normally but are susceptible to multiple types of spontaneous neoplasms, particularly lymphomas and sarcomas, by 6 months of age (Donehower, *et al.*, 1992; Harvey, *et al.*, 1993a; Jacks, *et al.*, 1994).

Several sub-clones of the *tp53*^{-/-} MEF cell line (passage >60–100) were isolated by Dr. Hatsumi Nagasawa for good growth characteristics and culture homogeneity, with minimal evidence of giant cells or aberrant mitotic figures. However, many of these sub-clones rapidly became heterogeneous upon continued culturing, and one sub-clone in particular (clone 6) was observed to again display a phenotype of aberrant mitoses, aneuploid giant cells and massive chromosome aberrations in approximately 15–20% of the cells examined (Nagasawa *et al.*, manuscript in preparation). The frequency of this unstable subpopulation was enhanced in a dose-dependent manner by low dose (≤ 4 Gy) radiation exposure, but reached a plateau of $\sim 35\%$ in cells collected in the first post-irradiation mitosis. The *tp53*^{-/-} MEF cell line has maintained its radioresistant phenotype at these higher culture passages, as measured by acute dose irradiation survival measurements of isoleucine-deprived G0/G1-phase cultures (Nagasawa *et al.*, manuscript in preparation, and this work).

The phenotype of giant cell formation in this cell line was further explored in the current series of studies by employing higher doses of acute (high dose-rate, up to 10 Gy) and continuous LDR irradiation (1–15 cGy/h) ¹³⁷Cs γ -irradiation. Acute γ -ray doses of up to 10 Gy were incapable of stimulating the giant cell phenotype in exposed cultures, but interestingly, exposure to continuous LDR irradiation (~ 10 –15 cGy/h) resulted in a significant increase in the numbers of giant cells in exposed cultures. Cytogenetic and immunocytochemical investigations, with and without irradiation, were undertaken to

characterize this clone and possibly identify patterns of chromosome dysfunction involved in the generation of the massive chromosome aberration and giant cell phenotypes in this cell line. It was hypothesized that telomeric end-to-end fusions or genomic amplification could be responsible for generating the long chromosomes observed in the metaphase preparations of the giant cells. This would be evident by the existence of interstitial telomere sequences at the point of fusion using telomere fluorescence *in situ* hybridization (FISH; Bailey, *et al.*, 1999), or long stretches of homogenously staining regions (HSRs) on the longer chromosomes “painted” a single color by 24-color multiplex FISH (mFISH) chromosome painting (Bartova, *et al.*, 2003).

Neither of these phenotypes were observed in the unstable *tp53*^{-/-} cells. Rather a new phenotype of “telomere fission,” excessive numbers of extra-chromosomal telomeric repeats (ECTRs) and telomeric double-minute chromosomes (TDMs) was observed using telomere FISH. This was coupled with extensive Robertsonian non-reciprocal translocations, complex rearrangements and aberrations, and chromosome fragmentation, as revealed by mFISH analysis. Immunocytochemical analyses demonstrated two classes of giant cells containing either acentric micronuclei or centric micronuclei and multi-lobed nuclei (linked by anaphase bridges). Both types of micronuclei contain large numbers of γ -H2AX foci (generally considered to be a marker for the presence of DNA double-strand breaks), and aberrant centrosome numbers and morphology (previously observed for these cells; Fukasawa, *et al.*, 1996). These phenotypes contribute to the excessive genomic instability that is evident in this unstable sub-cloned line of *tp53*-knockout MEF cells, the extensive degree of which, including the phenotypes of telomere

fission and massive chromosomal rearrangements, has not previously been reported for this well-described cell line in the literature.

6.3. Materials and Methods

6.3.1. Cell Culture and Irradiations

All MEF cell strains were grown in α MEM cell culture medium (GIBCO/Invitrogen) supplemented with 15% FBS (Hyclone), 100 μ g/ml streptomycin sulfate, 100 U/ml penicillin G (both Sigma), and 1X GlutaMAXTM-I (*l*-alanyl-*l*-glutamine, GIBCO/Invitrogen) in a 37°C incubator supplied with 95% air/5% CO₂ mixture. Cultures were routinely passaged at 1:4 dilutions prior to density-inhibited contact inhibition. Isoleucine-deficient MEM (GIBCO/Invitrogen formula 04-5033EL; see Nagasawa, *et al.*, 1989) was supplemented with 5% 3X dialyzed FBS (Hyclone), 100 μ g/ml penicillin G, 100 U/ml streptomycin sulfate (both Sigma), and 1X GlutaMAXTM-I (GIBCO/Invitrogen). A single lot of FBS was used in all the experiments to reduce experimental variability.

MEF cultures, initially obtained by Dr. Hatsumi Nagasawa, were initiated from liquid nitrogen-frozen stocks by rapid thawing of the frozen ampule in a 37°C water bath. One to two ml of the thawed MEF suspensions in 10% DMSO/90% cell culture cryomedium were supplemented with 5 ml of fresh cell culture medium and centrifuged at 1000 rpm for five minutes. The supernatant was then aspirated and the cell pellet broken up and resuspended in one ml of fresh cell culture medium. These suspensions were then aliquoted into T-25 tissue culture flasks (Greiner Bio-One) filled with 5 ml of pH-equilibrated cell culture medium (by placement of the medium-filled flask in the

37°C incubator for a minimum of one hour). The cell culture medium was replaced the next day with 5 ml of fresh cell culture medium. Cultures were passaged until sufficient cell numbers were available for experimental analysis.

For the acute dose irradiation survival experiments, cells were irradiated in the G0/G1-phase using isoleucine-deficient medium (Nagasawa, *et al.*, 1989) or in asynchronously cycling cultures plated ~16–18 hours prior to exposure. For the isoleucine-deprivation method, MEFs in T-25s were allowed to grow to near confluency, usually within two to three days following subculture. The cell culture medium was then replaced twice at three day intervals with 5 ml of isoleucine-deficient culture medium to achieve a >95% G0/G1 population (as monitored by long-term BUdR incorporation experiments). Alternatively, extra flasks of cells plated at low density for the low dose-rate studies (see below) were irradiated 16–18 hours after plating in complete cell culture medium (when cells are in asynchronous growth).

On the day of irradiation, the flasks were capped tightly and transported to the MRB470 irradiation facility. Cells were irradiated at room temperature with graded doses of 0 (sham) to 1000 cGy of ^{137}Cs γ -rays at a dose rate of 250 cGy/min, supplied by a J. L. Shepard and Associates Mark I-68A beam irradiator with an approximate activity of 3170 Ci (as of September 2004). The asynchronously growing cultures were then returned to the 37°C incubator, with caps loosened, for approximately 10 days of growth. After 10 days, the flasks were aspirated, rinsed with 0.9% sodium chloride and fixed with 100% ethanol. The flasks were then stained with a 0.1% crystal violet/methanol solution and colonies with >50 viable cells were counted as survivors.

Following irradiation of the isoleucine-deficient cultures, the medium was aspirated and the cells were immediately detached with 0.25% trypsin/EDTA (GIBCO/Invitrogen) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (Dulbecco's PBS-A formulation) for three minutes at 37°C. 500 μl of the well-mixed single cell suspension was diluted into 9.5 ml of PBS and counted with a Coulter Z-1 cell counter (Beckman-Coulter, Inc.). Cells were then plated at low density in T-25 flasks containing pH-equilibrated complete medium (placed for a minimum of 1 hour in the 37°C incubator) to achieve approximately 50–100 viable colonies per dose following 10 days of growth. After plating, the flasks were returned to the incubator and the medium was fully replaced in both sets of flasks after the first week of growth. After 10 days, the flasks were removed from the incubator, the medium aspirated, and the cells fixed and stained as described above.

Continuous low dose-rate (LDR) colony formation assays were performed similarly to those described in Chapter 5 for the human fibroblast studies, and by Priestly, *et al.* (1998). Briefly, 200–2000 cells were plated from asynchronous cultures of the *tp53*^{-/-} MEF clone 6 cell line and the 129S6 *atm* MEF groups, as above, into T-75 flasks filled with 25 ml of complete cell culture medium supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (GIBCO/Invitrogen) to ensure pH equilibration (~7.2–7.3) without supplied CO₂. The flasks were capped tightly and placed in the 37°C warm room overnight for plating. After 16–18 hours, the flasks were transferred to the MRB008 and MRB012 low dose-rate facilities and arranged according to the setups depicted in Figures 6.2 and 6.4. The flasks were irradiated for 5–7 days, after which the medium was exchanged and the flasks were returned for an additional 5–7 days of irradiation. At the end of the exposure period, the flasks were

removed from the irradiation facility, the medium aspirated, and the cells fixed and stained as described above.

Dosimetry of the MRB012 and MRB008 experimental setups depicted in Figures 6.2 and 6.4 was performed exactly as described in Chapter 5, and is reported in Figures 6.3 and 6.5, respectively. A higher initial dose rate of 15.3 cGy/h was achieved at the first flask position by exposing six even-numbered sources (numbered 2, 4, 6, 8, 10, and 12) rather than four as in Chapter 5, and lowering the shelf from 56 cm to 46 cm above the sources. Also, using flasks filled with 100 ml (rather than 25 ml) water or having them completely water-filled resulted in a more rapid rate of attenuation of the dose rate that could be achieved in only 7 T-75 flask heights (rather than 35).

6.3.2. Data and Statistical Analyses

Estimates for absolute survival in both the acute irradiation and continuous LDR irradiation experiments were calculated as the average number of colonies counted per number of cells plated per dose or dose rate. The logarithm of these survival estimates were averaged for 2–4 experiments conducted per MEF strain, and fit with a linear-quadratic ($y = \alpha x^2 + \beta x + c$) least-squares-weighted regression function, where y is the average absolute survival for a dose or dose rate x (SigmaPlot). The calculated intercept (c) was used as the zero-dose plating efficiency to calculate the relative surviving fraction (*i.e.*, the absolute surviving fractions corrected for the zero-dose plating efficiencies). Standard errors of the mean (SEM) were calculated by combining the standard deviations of the absolute surviving fractions by the square root of the sum-of-the-squares method (Bevington and Robinson, 1992) and dividing this value by the square root of the number

of experiments, and constitute the error bars for Figures 6.1, 6.6, and 6.7. SEM values for the PE, y -intercept c , SF_2 , α and β values reported in Table 6.1 were provided by the software regression analyses (SigmaPlot).

As no parameter-based radiobiological model exists for the continuous LDR exposures that accounts for variable cell growth and differential *dose rate-dependent* cell cycle-phase sensitivities (such studies have classically only used density-inhibited, G0/G1-phase cell populations), the best-fit lines for the data sets in Figures 6.6–6.8 have been drawn for illustrative purposes only. The lines were drawn using the least squares fit linear-quadratic regression curve (assuming an asymptotic decrease in survival as the dose rate approaches the proliferation-limiting LDR). This asymptotic decrease in survival is similar to the shape of dose response curves seen for many chemical agents and was modeled after the LDR rodent cell responses in the continuous LDR colony formation assay described by Priestly, *et al.* (1998). The only radiosensitivity parameter reported for the individual MEF strains and groups thereof in Table 6.2 is the continuous irradiation dose rate required to reduce relative survival to 10% and 1%. Statistical differences were tested by Student *t*-tests for two independent sample distributions (SigmaPlot; Tarone, *et al.*, 1983).

6.3.3. Cytogenetic and Immunocytochemical Experiments

The clonal line (Nagasawa clone 6) derived from the C57BL/6;129/Sv *tp53*^{-/-} MEF cell line originally described in Donehower, *et al.* (1992), Harvey, *et al.* (1993a, 1993b), Sands, *et al.* (1993), and Fukasawa, *et al.* (1996) was used for these series of experiments. Analyses of centric and acentric micronuclei and fragmented macronuclei,

telomere fluorescence *in situ* hybridization (FISH), and multiplex FISH (mFISH) were performed with cytogenetic preparations of isoleucine-deprived G0/G1 cultures, prepared as described above, released into the cell cycle by subculture into complete medium and collected by 0.1 $\mu\text{g/ml}$ colcemid (GIBCO/Invitrogen) treatment at the first mitosis (as verified by “harlequin”/FPG staining techniques; Perry and Wolff, 1974) in four hour intervals beginning 20 hours after subculture into complete medium. Immunocytochemical staining of centrosomes and γ -H2AX foci was conducted with cytogenetic preparations of asynchronous cultures grown in complete medium. All cytogenetic and immunocytochemical experiments were conducted with unirradiated cultures except the micronuclei dose response analyses.

Centrosome and γ -H2AX Immunocytochemistry

For measurements of centrosome status, the cell culture medium from asynchronously growing cultures was transferred to 15 ml conical tubes to collect any detached mitotic cells. The remaining cells were released from the culture dish with 0.25% trypsin/EDTA (GIBCO/Invitrogen) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (Dulbecco’s PBS-A formulation) for three minutes at 37°C. Colcemid treatment was not used. This cell suspension was likewise transferred into the same 15 ml conical tubes and centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated and the cell pellet was gently broken up and resuspended in 5 ml of PBS. The cell suspensions were again centrifuged, aspirated, and resuspended in 5 ml PBS twice more. Following the final centrifugation, the cell pellet was resuspended in a final volume of 2 ml PBS.

These cell suspensions were then loaded into a Shandon CytoSpin[®]-2 cyto-centrifuge and spun onto clean glass microscope slides at 2000 rpm. The slides were then immediately fixed in 4% paraformaldehyde and ice-cold 100% methanol for three minutes each. Following two rinses in PBS, the slides were then blocked for a half hour with FBS. The slides were rinsed quickly in PBS and 30 μ l of 1:50 mouse anti- β -tubulin (Sigma) and 30 μ l of 1:50 goat anti-pericentrin (Santa Cruz Biotechnology, Inc.) was applied to the slide, covered with a 24-mm x 60-mm glass coverslip (VWR), and incubated at 37°C for a half hour in a humid chamber. Following this incubation, the coverslip was removed and the slide rinsed quickly in PBS. 30 μ l of 1:50 Cy3-labeled anti-mouse IgG (Sigma) and 30 μ l of 1:50 Alexa-488-labeled anti-goat IgG (Molecular Probes/Invitrogen) was applied to the slide, overlaid with a new coverslip, and incubated at 37°C for a half hour in a humid chamber. Following this incubation, the coverslip was removed and the slide rinsed quickly in PBS. 50 μ l of SlowFade[™] Light (Molecular Probes/Invitrogen) with 0.5 μ g/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) was applied to the slide and covered with a 22-mm x 40-mm glass coverslip (VWR). The slides were then viewed under 345-nm, 488-nm, and 545-nm excitation wavelengths with an Olympus AX-70 microscope system and digital images captured by the MacProbe image analysis system (Applied Imaging Systems).

The immunocytochemistry protocol for γ -H2AX foci staining is essentially identical to that described above for the centrosome staining, with a few important differences. Cells were grown directly on one or two-well Lab-Tek[™] microscope chamber slides (Nalge Nunc International); and after achieving ~50% cell population density, the cell culture medium was aspirated and the chambers removed. After fixation

and blocking as above, 30 μ l of 1:50 mouse anti-phospho-histone H2A.X (phospho-Ser139; Upstate Cell Signaling Solutions) was applied to the slide as the primary antibody, and 30 μ l of 1:50 Alexa-488 anti-mouse IgG (Molecular Probes/Invitrogen) was applied to the slide as the secondary antibody. The slides were then processed and imaged as described above in the centrosome staining protocol.

Micronuclei, Telomere PNA-FISH, and mFISH Analyses

Micronuclei, telomere peptide nucleic acid (PNA) fluorescence *in situ* hybridization (FISH), and multiplex FISH (mFISH) analyses were all performed with the same series of cytogenetic preparations of isoleucine-deprived G0/G1 cultures of *tp53*^{-/-} MEF clone 6 cells released into the cell cycle by 1:4 subculture into complete medium. Mitotic cells were collected using 0.1 μ g/ml colcemid treatment every four hours beginning 20 hours following subculture, and were processed by standard cytogenetic fixation protocols (see below). An additional series of experiments were later performed at identical time points without colcemid treatment for the micronuclei analyses to ensure the measured frequencies were not affected by microtubule destabilization. Telomere PNA-FISH and mFISH procedures are described in the next paragraphs.

The presence of centromeric DNA was visualized by mounting slides with SlowFade[™] antifade solution (Molecular Probes/Invitrogen) containing 0.5 μ g/ml DAPI (Sigma), an AT-rich preferential DNA stain. Centromeres are composed of high copy numbers of AT-rich repetitive sequences, and appear as bright spots under fluorescent illumination (Haugland, 2005). Images were captured using an Olympus AX-70 microscope system under 345-nm illumination, equipped with a 63X and 100X objective,

and the MacProbe image analysis system (Applied Imaging, Inc.). The images were then color inverted, making the black background appear white and the brightly fluorescing centromeric regions appear as dark spots in the light grey nuclear bodies. The frequencies of acentric and centric micronuclei and fragmented, multi-lobed macronuclei compared to the normal morphology (circular or oval shaped) nuclei were recorded for each mitotic collection window, with and without colcemid treatments (see Figure 6.12).

The cytogenetic samples were processed using standard techniques. Briefly, the cell culture medium was removed into 15 ml conical tubes (Greiner Bio-One) to ensure collection of any detached and rounded mitotic cells and the cells were detached with 0.25% trypsin/EDTA (GIBCO/Invitrogen) in Ca^{2+} / Mg^{2+} -free PBS (Dulbecco's PBS-A formulation) for three minutes at 37°C. The detached single cell suspensions were then transferred into the 15 ml conical tubes with the cell culture medium and centrifuged for 4 minutes at 1000 rpm. After pouring off the supernatant, the cell pellet was gently broken up and resuspended in 10 ml of 37°C 75mM KCl hypotonic buffer. The tubes were then incubated for 7.5 minutes in a 37°C water bath, after which 2 ml of freshly prepared 3:1 methanol:acetic acid (Carnoy's fixative) was added and the tubes gently inverted to mix the solutions. The tubes were incubated for 2.5 minutes at room temperature and then centrifuged for four minutes at 1000 rpm. The supernatant was poured off into a hazardous waste container and the cell pellet was very gently broken. The cell suspensions were then fixed by the dropwise addition of fresh fixative to a volume of 3–4 ml and mixed by air-bubbling the suspensions with the pipette. The cell suspensions were then placed into the refrigerator or cold room.

Days or weeks later, the cell suspensions in fixative (now methyl acetate, more technically a preservative) were allowed to return to room temperature prior to being centrifuged at 1000 rpm for five minutes. The supernatant was poured off into a hazardous waste container, and the cell pellet was gently broken up, fixed by the dropwise addition of freshly prepared Carnoy's fixative to a volume of 3–4 ml and mixed by gently air-bubbling the suspensions with the pipette. After five minutes, the suspension was again centrifuged and the fixation process repeated. Following the final centrifugation, the suspensions were resuspended in 0.5–1.5 ml of fresh fixative depending upon the volume of the cell pellet and 60 μ l of cell suspension was dropped onto clean, cold and wet microscope slides (VWR). After air-drying, the slides were placed in a desiccation chamber in the 37°C warm room overnight.

Only freshly prepared slides (less than two days old) were used for the telomere PNA-FISH and mFISH procedures. Both protocols were adapted slightly from the manufacturer's recommendation and were quite similar, so they will be described together. For the telomere PNA-FISH protocol, a master hybridization mix composed of 15 μ l formamide, 9.5 μ l sterile dH₂O, 3 μ l 20X SSC pH 6.3, and 2.5 μ l G-rich (TTAGGG₃) Cy3-conjugated telomeric PNA probe (Applied Biosystems) per slide was prepared in a 200 μ l PCR tube. For the mFISH protocol, 8–10 μ l per slide of 21X mouse mFISH hybridization mix (MetaSystems GmbH) was aliquoted into a 200 μ l PCR tube. These hybridization mixes were heated for 10 minutes at 76°C and held 30–60 minutes at 37°C using a Hybaid PCR thermocycler during the following slide preparation steps.

Slides were rinsed in RT PBS for 2 minutes and RT 2X SSC for 2 minutes. The slides were then incubated in 100 μ g/ml RNase (Sigma, boiled for 10 minutes at 95°C to

remove any residual DNase activity) in 2X SSC for 10 minutes at 37°C. The slides were then rinsed twice in RT 2X SSC for 2 minutes each, and then incubated for 5 minutes in 0.1 μ g/ml proteinase K (Sigma) suspended in 20 mM Tris-HCl/2 mM CaCl₂ (pH 7.4) at 37°C. The slides were rinsed twice for 2 minutes each in RT PBS, fixed with 3.7% formaldehyde in PBS (must be prepared fresh before each use) for 2 minutes at RT, and rinsed twice for two minutes each in RT PBS. The slides were then dehydrated in a series of 70%, 85%, and 100% ethanol rinses and denatured for two minutes in 70% formamide/2X SSC at 72°C. After denaturation, the slide was then immediately placed in ice-cold 70% ethanol for two minutes, followed by two additional two minutes rinses in 85% and 100% ethanol. The slide was dried rapidly with an air jet and placed at 37°C for ~15 minutes. Ten to thirty μ l of pre-warmed 37°C hybridization mix (see above) was added to the slide, which was covered with a coverslip and sealed with rubber cement. The slide was then hybridized at 37°C in the dark for 16–48 hours.

Following the hybridization period, the rubber cement and coverslip was gently removed and the slides rinsed for 10 minutes in 0.4X SSC/0.3% Tween-20 (Sigma) at 45°C. The slides were then washed twice in 2X SSC/0.1% Tween-20 for two minutes each at room temperature. The telomere PNA slides were rinsed in 1X PN buffer and PBS for two minutes each, mounted with 30 μ l of SlowFade™ antifade solution (Molecular Probes/Invitrogen) containing 0.5 μ g/ml DAPI (Sigma) and a coverslip. The mFISH slides were further processed for secondary antibody detection and signal amplification using the MetaSystems GmbH B-tect™ detection kit as described in the manufacturers protocol and mounted as above. Both the telomere PNA-FISH and mFISH slides were imaged with the Olympus AX-70 microscope system equipped with

the appropriate filter sets and the 100X objective; telomere PNA-FISH images were captured with the MacProbe™ program (Applied Imaging, Inc.) and the mFISH images were captured with the PowerGene™ mFISH program (Applied Imaging, Inc.).

6.4. Results

6.4.1. Results of the Survival Experiments

The results of the acute dose irradiation survival measurements made with the C57BL/6;129/Sv *tp53*^{-/-} MEF clone 6 cell line are reported in Table 6.1 and plotted in Figure 6.1. The measured and calculated (by linear-quadratic regression curve-fitting) plating efficiency values (PE and the *y*-intercept *c*, respectively) and survival parameters [relative surviving fraction at 2 Gy (SF₂), α , β] for both the isoleucine-deprived G0/G1-phase cultures and asynchronous cultures are shown in Table 6.1. These data confirmed previous survival measurements of this cell line made by Dr. Hatsumi Nagasawa (this isoleucine-deprived *tp53*^{-/-} MEF clone 6 and wild-type MEF A20 clone data were included in Table 6.1 and Figure 6.1 for comparison purposes) and a recent report by Alsbeih, *et al.* (2004) using isogenic, non-transformed strain C57BL/6;129Sv *tp53*^{-/-}, *tp53*^{+/-}, and wild-type MEF cell lines (the same genetic background as the Nagasawa clone 6 cells).

All three data sets (Wilson, Nagasawa, Alsbeih) clearly identify C57BL/6;129/Sv *tp53*-knockout MEF cells as radioresistant to acute dose irradiation compared to wild-type controls, a phenotype generally associated with *tp53*-deficiency in many other normal, transformed, and tumor cells (see Discussion). Similar survival responses were obtained by immediate plating of irradiated isoleucine-deprived G0/G1-phase cultures

Table 6.1. Plating efficiencies, measured survival parameters, and linear-quadratic ($S = e^{-\alpha D - \beta D^2}$) fit-calculated survival parameters ($\pm$ SEM) for the series of acute dose survival experiments completed with C57BL/6;129/Sv *tp53*^{-/-} MEF clone 6 cells. Cells were irradiated in G0/G1-phase cultures established by isoleucine deprivation or as asynchronously growing single cells plated at low density ~16–18 hours prior to irradiation (in conjunction with the continuous LDR irradiation colony formation experiments presented in Figure 6.6 and Table 6.2).

Table 6.1.

Cell Type/ Culture Type	No. Expts.	Average PE ± SEM _{PE} (Range)	Average Relative SF2 ± SEM _{SF2} ^a (Log-Average Relative SF2 ± SEM _{SF2})	Linear-Quadratic Fit: $S = e^{-\alpha D - \beta D^2}$			
				PE _{LQ} ± SEM _{PE LQ}	SF _{2 LQ} ± SEM _{SF2 LQ}	$\alpha \pm \text{SEM}_\alpha$ (cGy ⁻¹)	$\beta \pm \text{SEM}_\beta$ (cGy ⁻²)
<i>tp53</i> ^{-/-} MEF clone 6/ Isoleucine-deficient medium (G0/G1)	3	0.281 ± 0.004 (0.267–0.296)	0.861 ± 0.008 (0.857 ± 0.004)	0.300 ± 0.035	0.700 ± 0.089	0.0014 ± 0.0005 cGy ⁻¹	1.9 x 10 ⁻⁶ ± 5.0 x 10 ⁻⁷ cGy ⁻²
<i>tp53</i> ^{-/-} MEF clone 6/ Asynchronous, complete medium	2	0.366 ± 0.001 (0.365–0.367)	0.722 ± 0.014 (0.721 ± 0.017)	0.369 ± 0.036	0.741 ± 0.075	0.0009 ± 0.0004 cGy ⁻¹	3.0 x 10 ⁻⁶ ± 4.2 x 10 ⁻⁷ cGy ⁻²
WT MEF A20 clones/ Isoleucine-deficient medium (G0/G1) ^b	2	Not reported	0.522 ± 0.087	ND ^c	0.430 ± 0.066	0.0035 ± 0.0006 cGy ⁻¹	3.6 x 10 ⁻⁶ ± 5.6 x 10 ⁻⁷ cGy ⁻²

^a SF₂ = relative surviving fraction at 2 Gy.

^b Survival data for isoleucine-deprived C57BL/6;129/Sv wild-type (WT) MEF A20 clones data graciously supplied by Dr. H. Nagasawa for comparison purposes.

^c ND = not determined.

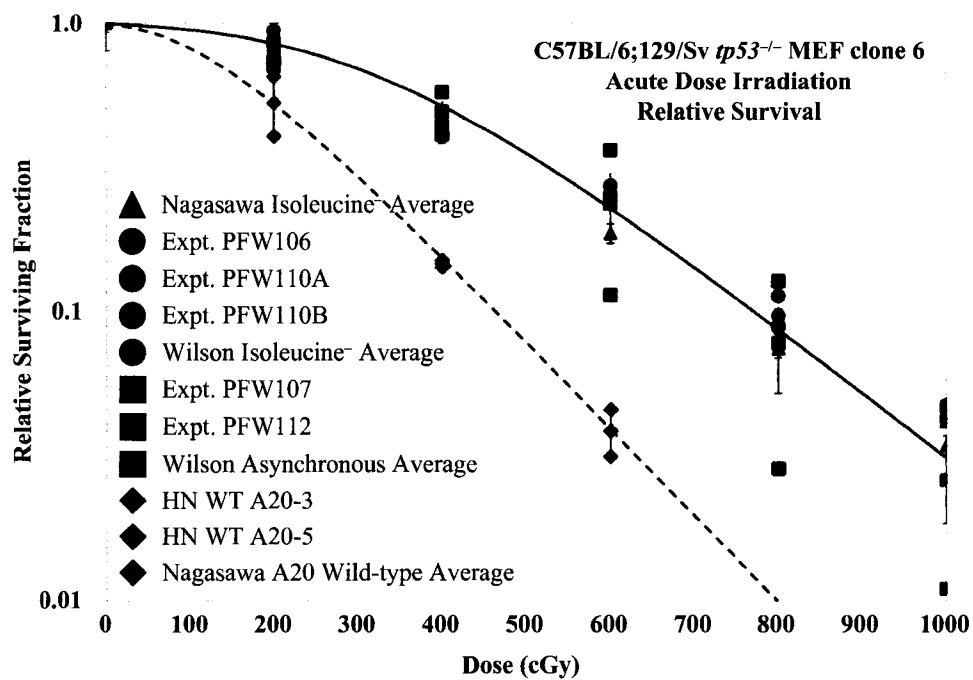


Figure 6.1. Acute dose irradiation survival assay results for C57BL/6;129/Sv *tp53*^{-/-} MEF clone 6 cells (see Table 6.1). Cultures were irradiated in G0/G1 with the use of isoleucine-deficient medium (circles) or irradiated asynchronously 16–18 hours after plating at low density in complete medium (squares). Survival data for this cell line (triangle) and the wild-type control C57BL/6;129/Sv MEF strain A20 (diamonds) irradiated in G0/G1 with the use of isoleucine-deficient medium were kindly provided by Dr. H. Nagasawa, and are included for comparison purposes. Error bars represent standard errors of the mean.

and by irradiating asynchronous cultures approximately 16–18 after plating at low density (in conjunction with the low dose-rate survival experiments discussed next). The average plating efficiency for the isoleucine-deprived MEM cultures (0.28) is ~25% lower than that for asynchronous cultures supplied with complete α MEM medium (0.37).

The MRB012 and MRB008 low dose-rate facility experimental setups used in the MEF continuous LDR irradiation colony formation assays are shown in Figures 6.2 and 6.4, respectively. Dosimetry of these experimental setups (ion chamber exposure rate measurements and dose-rate calculations) was completed exactly as described in Chapter 5 for the human fibroblast LDR experiments (the modified T-150-encased ion chamber pancake probe used for the exposure rate measurements is shown in the upper right panel of Figure 6.2). Even with two additional sources exposed (the six even-numbered sources versus sources 1, 4, 7, and 10 in Chapter 5), a more rapid attenuation of the dose-rate in MRB012 was achieved with T-75 flasks filled with 100 ml of water or having them completely filled with water, rather than using only 25 ml of water. A higher initial dose rate of 15.3 ± 0.6 cGy was achieved on the first shelf/flask row (lowered to 46 cm), and the setup was constructed to reduce the dose rate by ~3 cGy/h in the subsequent cell flask positions. Dose rates <3 cGy/h were achieved using the unshielded MRB008 source shown in Figure 6.4. This setup was constructed on shelves 2–4 of the MRB008 37°C incubator (shelf 1 was removed) using T-75 flasks filled with 25 ml of water to reduce the dose rate by ~0.5 cGy/h in the subsequent cell flask positions.

Isodose-rate contour plots for the MRB012 and MRB008 MEF LDR experimental setups are shown in Figures 6.3 and 6.5, respectively. Exposure measurements taken across the plane of the experimental area in each facility demonstrate no more than $\pm 6\%$

MEF/Human Fibroblast LDR Irradiation Setup: MRB012

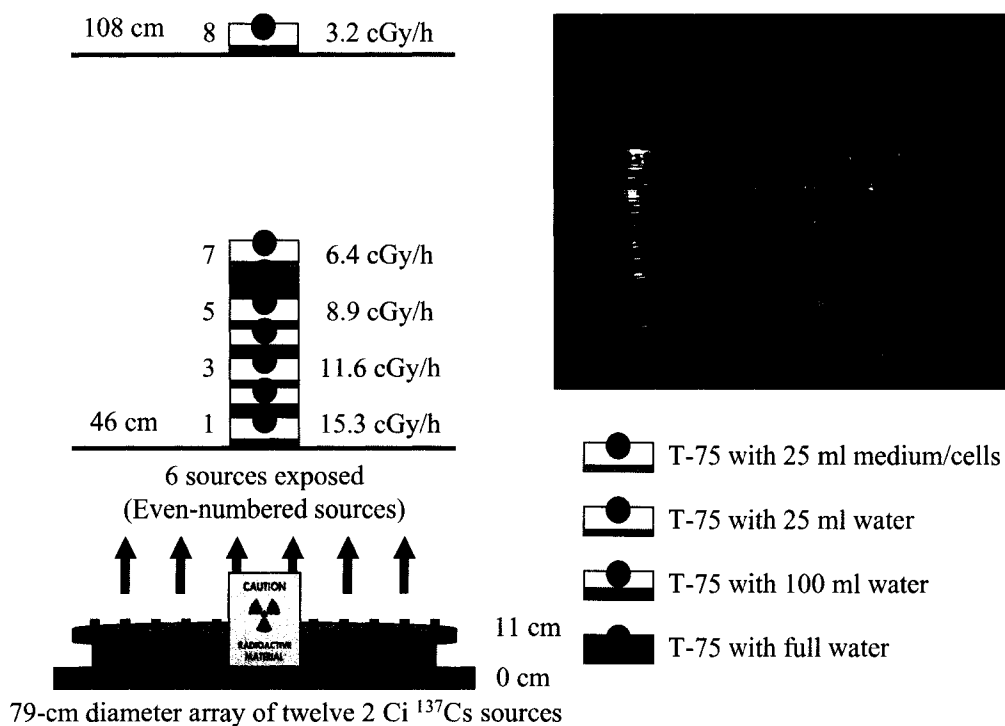


Figure 6.2. Experimental setup for the MRB012 low dose-rate irradiation facility discussed in this chapter.

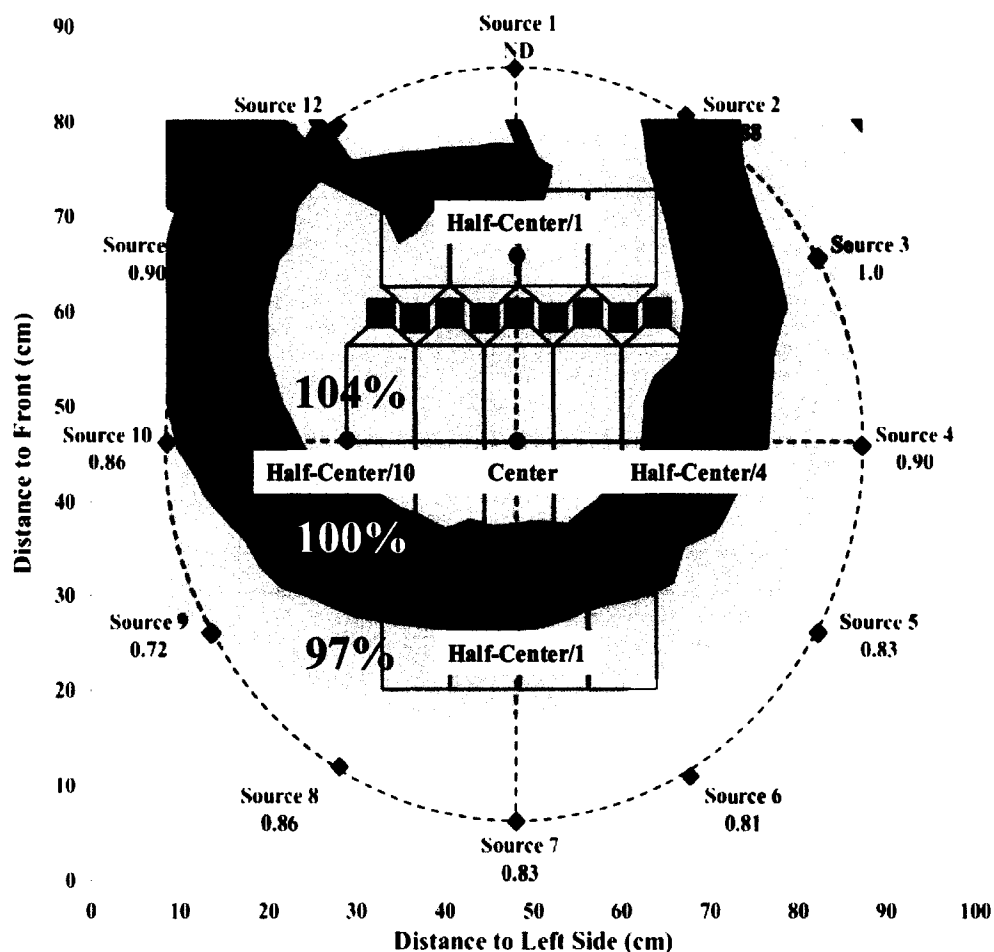


Figure 6.3. Isodose-rate contour plot of the flask position 1 (15.3 ± 0.6 cGy/h) field plane with six MRB012 sources (2, 4, 6, 8, 10 and 12) exposed in the experimental setup detailed in Figure 6.2. Individual source strengths (relative to the strongest source, source 3) are denoted below each source label. ND = not determined.

MEF/Human Fibroblast LDR Irradiation Setup: MRB008

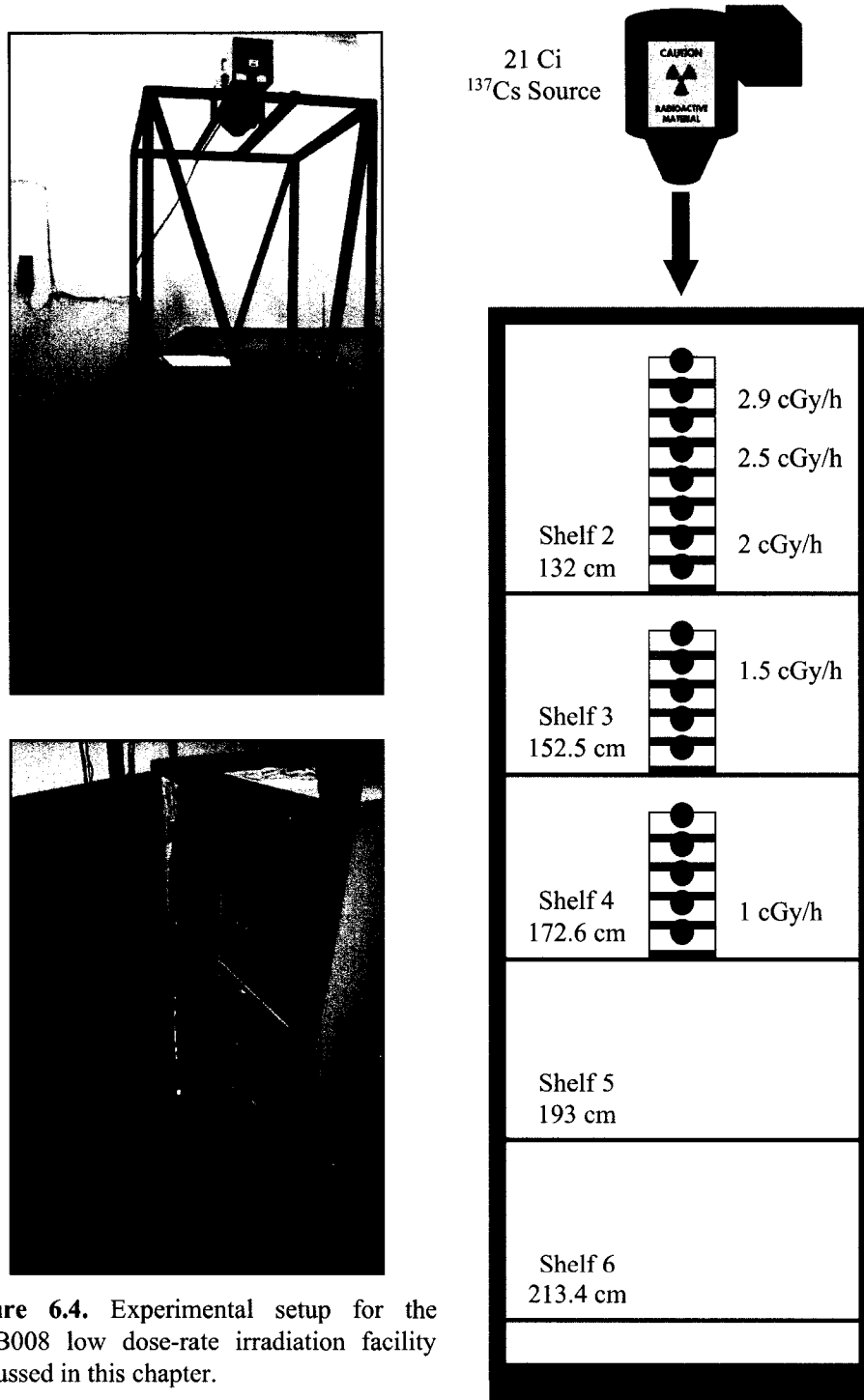


Figure 6.4. Experimental setup for the MRB008 low dose-rate irradiation facility discussed in this chapter.

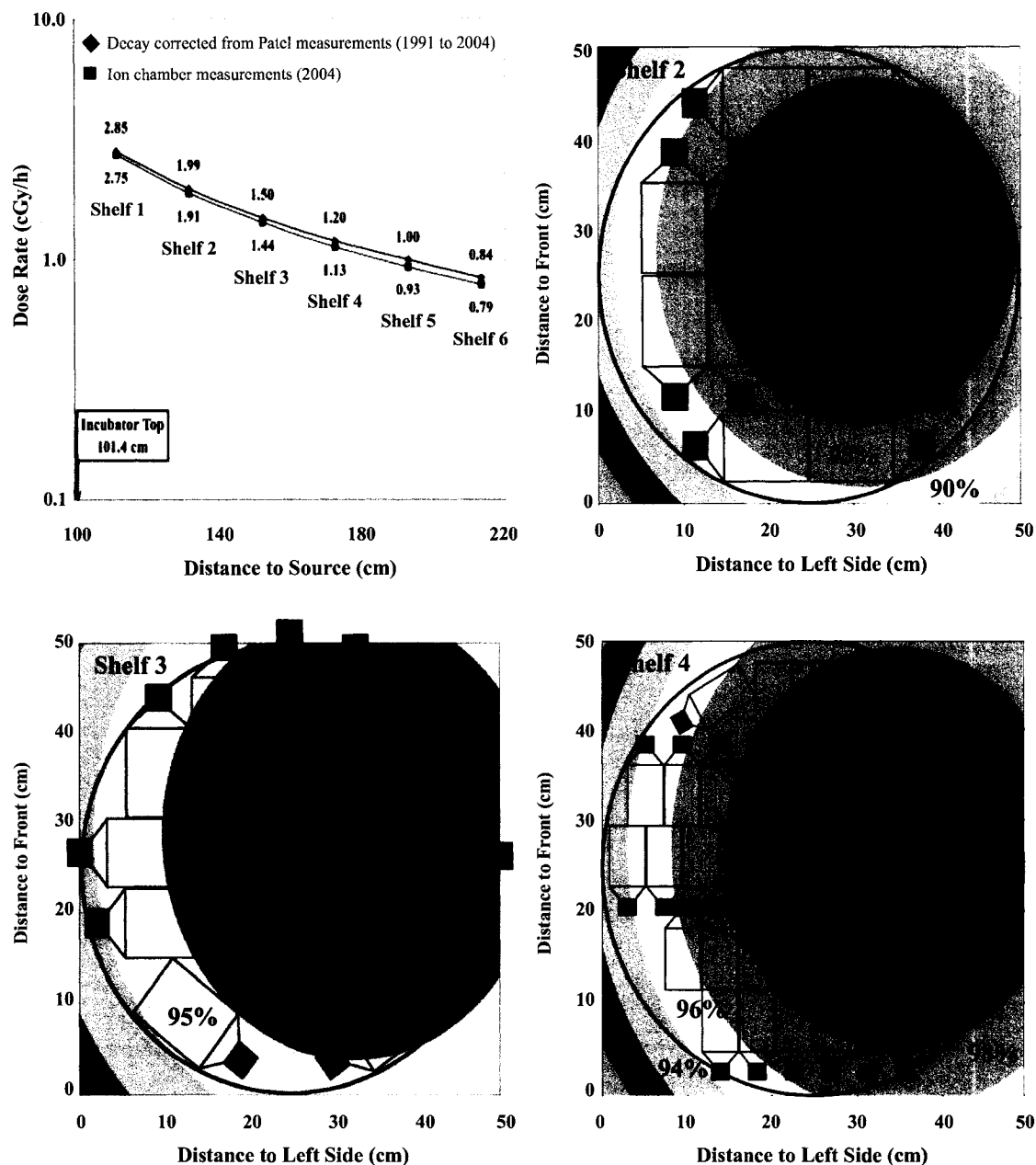


Figure 6.5. Semi-logarithmic plot of MRB008 dose rates (in cGy/h) with the source exposed versus shelf position in the incubator. Isodose-rate contour plots of shelves 2–4 of the MRB008 incubator, seen in the experimental setup detailed in Figure 6.4, are shown with the three different T-75 and T-25 flask layouts used in these continuous LDR irradiation experiments.

relative variation in dose-rate for any given flask level. Figure 6.3 is an isodose-rate contour plot of the MRB012 experimental plane for flask row 1 (15.3 ± 0.6 cGy/h) with the six ~ 2.2 mCi sources exposed, with each gray level representing a 3–4% relative change in dose-rate. The relative contribution from each of the twelve MRB012 sources exposed independently are reported as a ratio relative to the strongest source (source 3) below the source labels in Figure 6.3 (see Chapter 5). This variation in the source strengths accounts for the slight skewing of the dose-rate field toward sources 2 and 12 when the six sources are exposed.

The first panel of Figure 6.5 is a semi-logarithmic plot of dose rates (in cGy/h) versus MRB008 incubator shelf position, as seen in Figure 6.4. The remaining panels of Figure 6.5 are isodose-rate contour plots for shelf 2 (1.9 ± 0.1 cGy/h), shelf 3 (1.4 ± 0.1 cGy/h), and shelf 4 (1.1 ± 0.1 cGy/h) of the MRB008 incubator, with each gray level representing a 2–5% relative change in dose-rate. These panels also show the three T-25/T-75 flask orientations used in the experiments discussed in Chapters 5 and 6. The patterns of the dose-rate fields in both LDR facilities are closely maintained at every flask level within the experimental setups.

The effect of continuous LDR irradiation on the retention of proliferative capacity for the C57BL/6;129/Sv *tp53*^{-/-} MEF clone 6 cell line and the 129S6 wild-type, heterozygote, and homozygote *atm*-knockout MEF strains are plotted in Figures 6.6 and 6.7, respectively, and reported in Table 6.2. The experimental, average and log-transformed average survival data for 2–4 independent experiments per MEF strain are plotted in Figures 6.6 and 6.7. As in Chapter 5, the only radiosensitivity parameters reported in Table 6.2 are the dose rates required to reduce relative survival to 10% and

Table 6.2. Summary of rodent cell continuous LDR irradiation colony formation data reported in this chapter and Priestly, *et al.*, 1998.

Cell Line or Strain	Rodent Strain	Genotype of Interest	No. Expts.	PE $\pm$ SEM _{PE} (Range)	Dose rate (cGy/h) for relative survival level of:	
					0.10	0.01
<i>Wilson Measured Parameters</i>						
Weil Strain 347	129S6 mouse	<i>atm</i> ^{-/-}	2	0.055 $\pm$ 0.002 (0.052–0.058)	3 cGy/h	3.5 cGy/h
Weil Strain 417	129S6 mouse	<i>atm</i> ^{-/-}	3	0.014 $\pm$ 0.002 (0.011–0.019)	2 cGy/h	2.6 cGy/h
Weil Strain 344	129S6 mouse	<i>atm</i> ^{+/-}	2	0.038 $\pm$ 0.012 (0.021–0.055)	5 cGy/h	8 cGy/h
Weil Strain 345	129S6 mouse	<i>atm</i> ^{+/-}	3	0.030 $\pm$ 0.003 (0.022–0.036)	3.5 cGy/h	5.6 cGy/h
Weil Strain 390	129S6 mouse	WT	3	0.041 $\pm$ 0.002 (0.036–0.049)	13.2 cGy/h	16 cGy/h (estimated)
<i>tp53</i> ^{-/-} MEF clone 6/ Subclone 6W1A	C57BL/6;129/Sv mouse	<i>tp53</i> ^{-/-}	4	0.372 $\pm$ 0.021 (0.296–0.489)	15.2 cGy/h	17.5–18 cGy/h (estimated)
<i>Priestly, et al. (1998) Reported Parameters</i>						
SCGR11 <i>scid</i> fibroblasts	CB17 mouse	<i>prkdc</i> ^{-/-} , <i>tp53</i> ^{-/-}	Not reported		2.6 cGy/h	3.2 cGy/h
C57BL/6 fibroblasts	C57BL/6 mouse	WT	Not reported		13.3 cGy/h	14.7 cGy/h
CHO <i>irs</i> -20 (10B2-derived)	Chinese hamster	<i>prkdc</i> ^A	Not reported		4.6 cGy/h	6 cGy/h
CHO 10B2	Chinese hamster	WT	Not reported		14 cGy/h	15.5 cGy/h

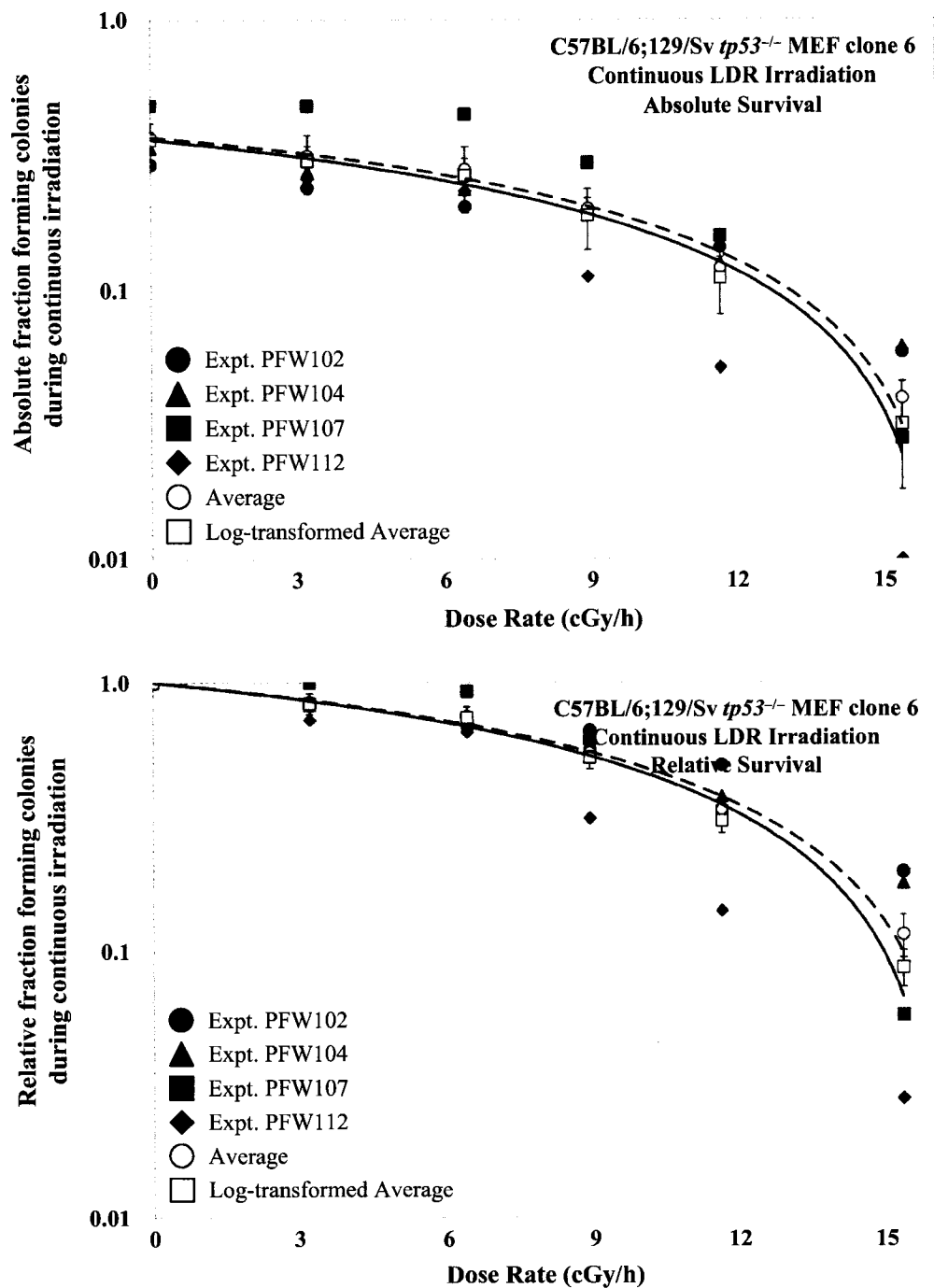


Figure 6.6. Results of the continuous low dose-rate colony formation assay performed with the C57BL/6;129/Sv *tp53*^{-/-} MEF clone 6 cell line (see Table 6.2). Error bars represent standard errors of the mean.

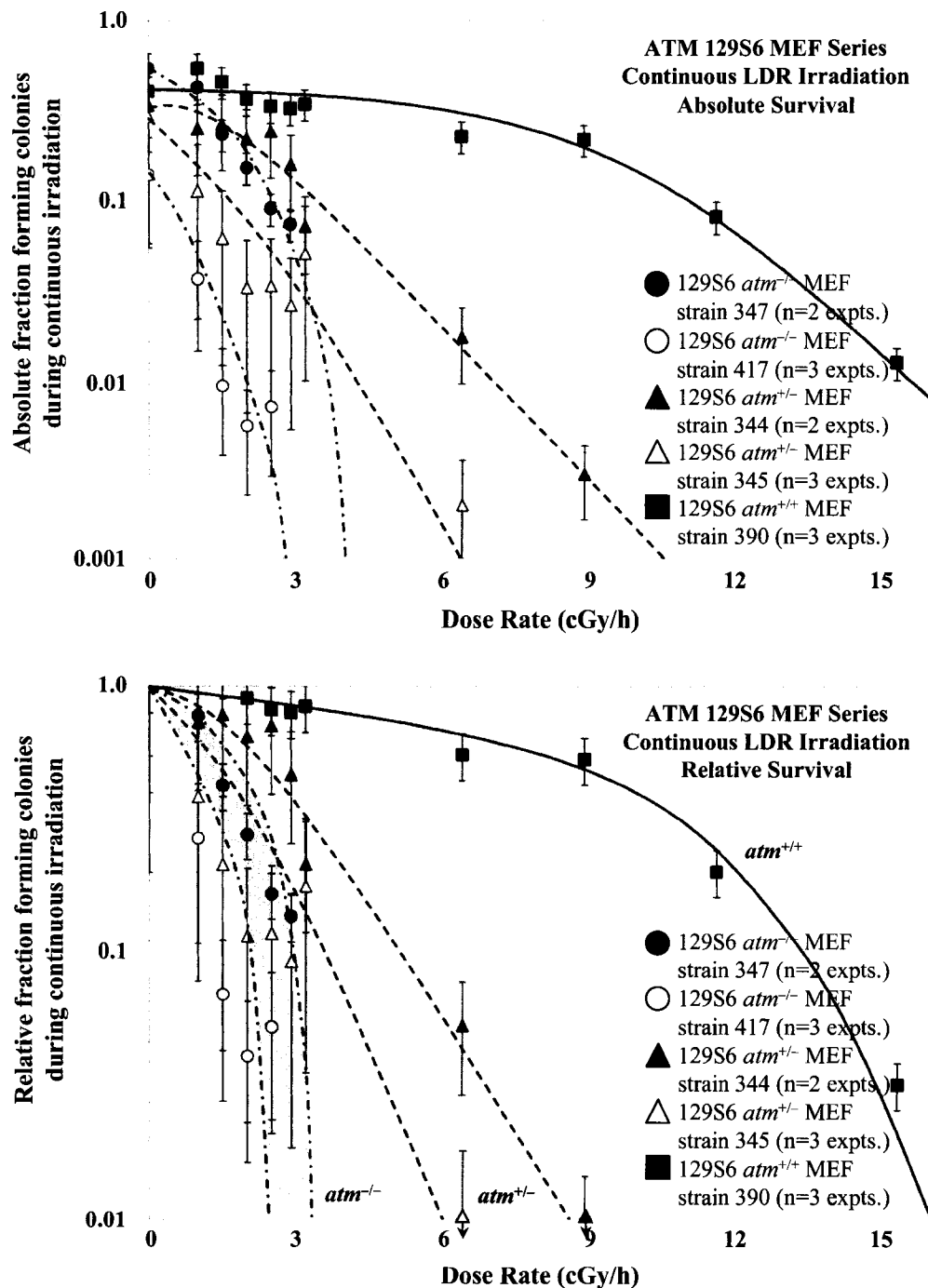


Figure 6.7. Results of the continuous low dose-rate colony formation assay performed with the 129S6 *atm*^{-/-} (circles), *atm*^{+/-} (triangles), and wild-type (square) MEF cell strains (see Table 6.2). Error bars represent standard errors of the mean.

1% (obtained by linear-quadratic curve-fitting of the survival data). In Figure 6.8, these survival data (black lines) are compared to the published results of Priestly, *et al.* (1998), using wild-type CHO 10B2 and *prkdc*-deficient *irs*-20 cells, and wild-type C57BL/6 mouse fibroblasts and a *scid prkdc^{-/-}/tp53^{-/-}* MEF cell line, SGCR11 (colored lines).

As shown in Figures 6.6 and 6.8 and Table 6.2, the C57BL/6;129/Sv *tp53^{-/-}* MEF clone 6 cells (10% relative survival at 15.2 cGy/h and 1% relative survival at ~17.5–18 cGy/h) are more radioresistant than wild-type C57BL/6 mouse fibroblasts (10% relative survival at 13.3 cGy/h and 1% relative survival at 14.7 cGy/h) and CHO 10B2 cells (10% relative survival at 14 cGy/h and 1% relative survival at 15.5 cGy/h). This radioresistant LDR response was expected considering the radioresistant HDR irradiation response shown in Figure 6.1 and Table 6.1. Likewise, the response of the wild-type 129S6 MEF strain 390 cells (10% survival at 13.2 cGy/h and 1% survival at 16 cGy/h) seen in Figure 6.7 is very consistent with the wild-type CHO 10B2 and C57BL/6 mouse fibroblast responses reported by Priestly, *et al.* (1998).

As shown in Figure 6.7, the continuous LDR irradiation colony formation assay was capable of clearly identifying the highly radiosensitive *atm*-knockout homozygote MEF strains, but more importantly, a significant and large difference between the *atm*-heterozygote MEF strains and the wild-type control cells. Significant differences between the post-irradiation survival of these two latter groups were previously unattainable with LDR exposures of *confluent* cultures of human A-T heterozygote and apparently normal fibroblasts, as discussed in Weeks, *et al.* (1991), Nagasawa, *et al.* (1992), and Chapter 2. A dose-rate of 2–3 cGy/h will reduce relative survival to 10% survival and 2.6–3.5 cGy/h is sufficient to reduce relative survival to 1% in the *atm^{-/-}*

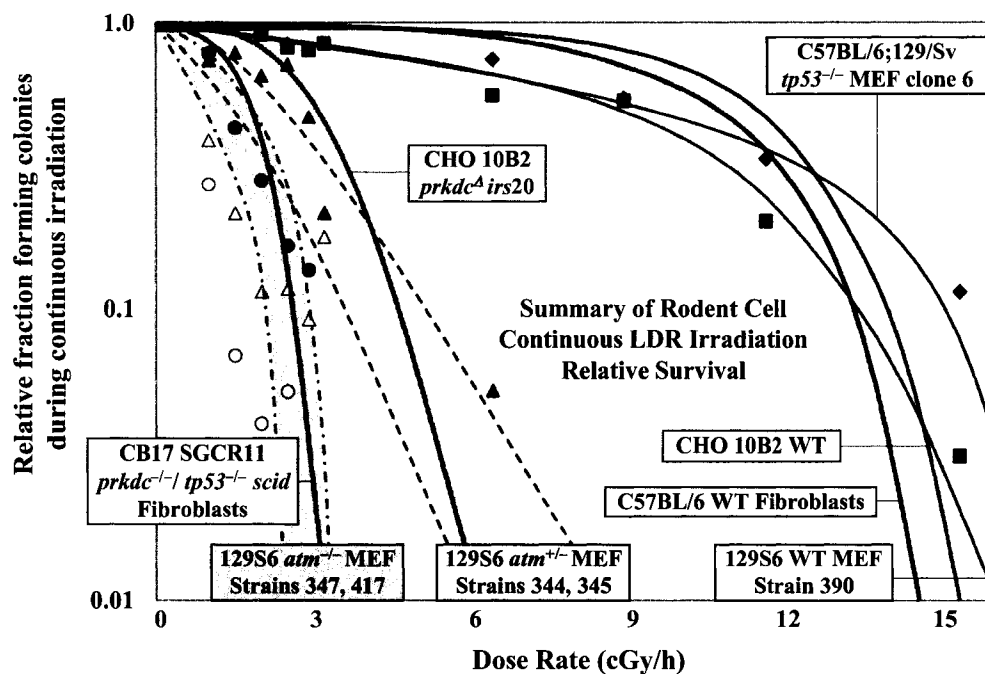


Figure 6.8. Summary of the continuous LDR irradiation colony formation assay results for various rodent (mouse and Chinese hamster) cell strains measured in this chapter (symbols and solid/dashed black lines, see Table 6.2 and Figures 6.6 and 6.7) and reported by Priestly, *et al.*, 1998 (colored lines; see Table 6.2).

MEF strains (347 and 417). The dose-rates required to reduce relative survival to 10% and 1% for the *atm*^{+/-} MEF strains (344 and 345) are 3.5–5 cGy/h and ~6–8 cGy/h, respectively. MEF strains of both *atm* haplotypes are significantly ($P < 0.0001$) more radiosensitive than the wild-type control MEF strain (390), and from each other. Much hope is placed on the ability of the continuous LDR irradiation colony formation assay to significantly differentiate the LDR survival of asynchronous cultures of *human* A-T heterozygote fibroblast strains.

The giant cell phenotype observed in the unstable *tp53*^{-/-} MEF clone 6 cultures afforded a readily observable indicator to record effects that radiation exposure might have on the incidence of this phenotype (*i.e.*, whether radiation exposure is capable of increasing the baseline frequency of 10–15% giant cells in this cell line). The acute irradiation (0–10 Gy) and low dose-rate irradiation (0–15 cGy/h) survival experiments described above provided a large number of surviving colonies to examine for this purpose. In Figure 6.9, examples of three types of colonies observed after 12 days of continuous LDR irradiation are shown: colonies with $\leq 5\%$ giant cells, colonies with $> 5\%$ but $\leq 50\%$ giant cells, and colonies with $\geq 50\%$ giant cells. A decrease in overall proliferative capacity (the number of cells per colony) is evident as the dose-rate is increased from 0–15 cGy/h in the left-hand to the right-hand panels.

Approximately 10% of unirradiated colonies from both experimental series contained $\geq 50\%$ giant cells, as expected from the previous studies with this cell line. As shown in the right-hand plot of Figure 6.9, this frequency of colonies with $\geq 50\%$ giant cells (black bars) was not significantly increased by acute radiation exposures with single doses up to 10 Gy. However, colonies recovered from the continuous LDR radiation

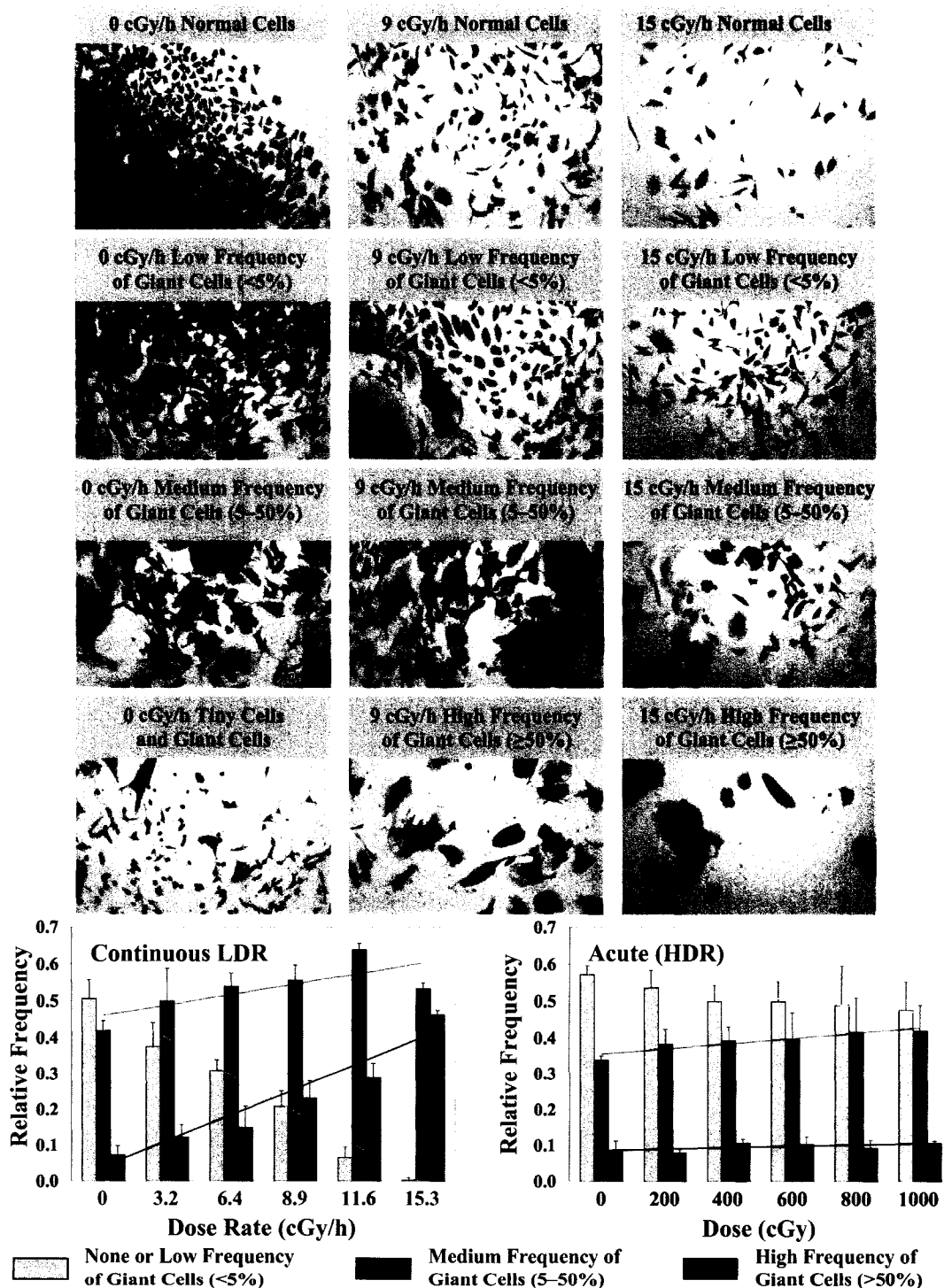


Figure 6.9. Cell size phenotypes observed in *tp53*^{-/-} clone 6 colonies following two weeks of growth under LDR irradiation at dose rates of 0–15 cGy/h or after an initial acute dose of 0–1000 cGy. The frequency of colonies with low (<5%), medium (5–50%), or high (>50%) percentages of giant cells are plotted for both radiation delivery schemes; only continuous LDR irradiation induced the giant cell phenotype, in a dose-rate responsive manner. Colonies of >50 giant cells were *consistently* observed, *i.e.*, such cells *are* capable of continued growth.

exposures demonstrated a *significant* increase in the proportion of colonies with $\geq 50\%$ giant cells in a linear, dose-rate-responsive manner ($\sim 2\%$ increase in this proportion per cGy/h), as shown in the right-hand plot of Figure 6.9. Approximately half of the colonies recovered after growth at 15 cGy/h had $\geq 50\%$ giant cells. Strangely enough, the colonies recovered at these higher dose rates seemed to consistently contain *almost* 100% giant cells, at the nearly complete expense of colonies with minimal ($< 5\%$) giant cells. Current paradigms of the transmissibility of lethal chromosome aberrations and their effect on cell survival seem to be at odds with the apparent proliferative capacity of these giant cells under continuous LDR irradiation (see Discussion below).

To further explore this unexpected finding, colonies of *tp53*^{-/-} clone 6 cells were grown from single cells for one week at 15 cGy/h in MRB012 and, following a medium change, an additional week without irradiation. Thirty-five randomly chosen colonies (from Figure 6.9, approximately 50% of colonies have 5–50% giant cells and the other 50% have $\geq 50\%$ giant cells) were cloned into 6-well plates with complete medium using sterile 0.25% trypsin-soaked 1-cm Whatman filter paper discs (as recommended by Dr. Nagasawa). The growth of these sub-clones was monitored for the following week prior to analysis.

The results of this *tp53*^{-/-} MEF clone 6 LDR giant cell cloning experiment are shown in Figure 6.10. Fifteen of the 35 sub-clones (43%) became confluent in the well (marked by purple circles numbered 3, 4, or 5) within one week and were subsequently subcultured into additional T-25 flasks and frozen in liquid nitrogen. The well labeled “5” was confluent within two days of growth after cloning, wells labeled “4” became confluent in 3–4 days, and wells labeled “3” became confluent in 5–6 days. After one

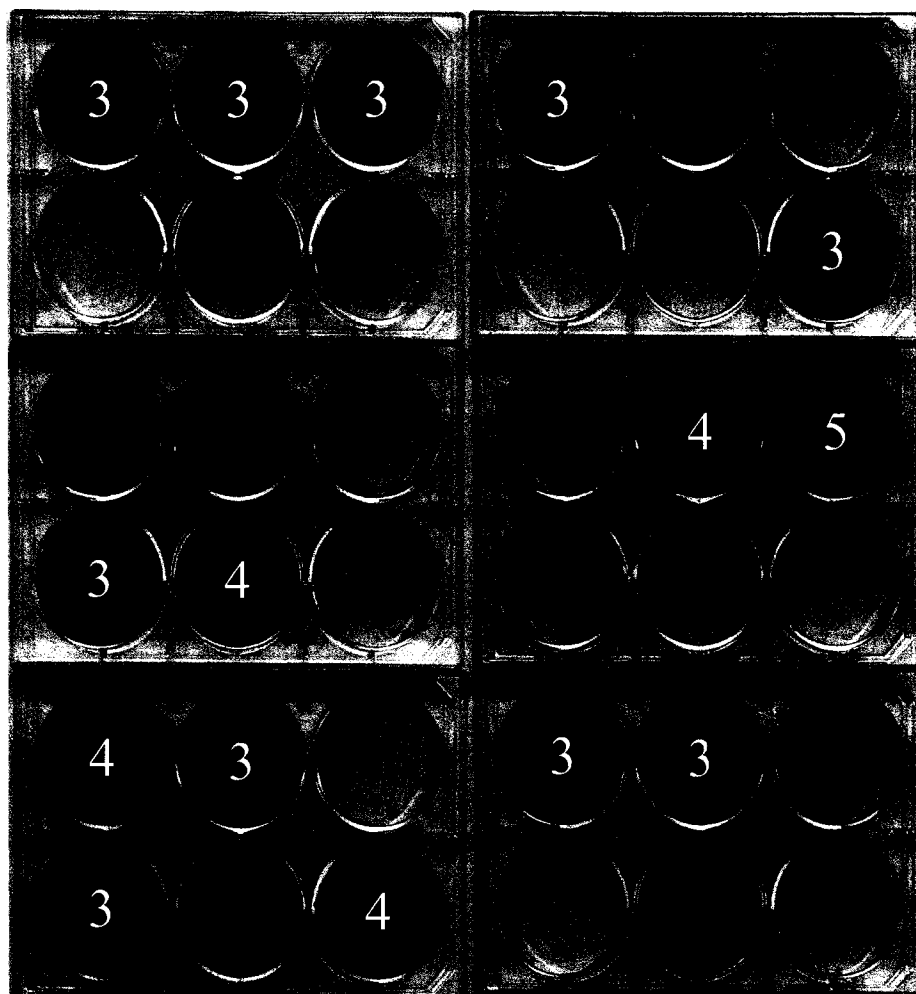


Figure 6.10. Clones of *tp53*^{-/-} MEF clone 6 survivors following growth at 15 cGy/h. Colonies were initially plated as single cells and grown under 15 cGy/h continuous irradiation for one week, the medium replaced and grown for an additional week without irradiation, and 35 colonies were randomly chosen and cloned into the six-well plates photographed above. Following one week growth, colonies in wells labeled as **3** (confluent in 5–6 days), **4** (confluent in 3–4 days), or **5** (confluent in 2 days) became confluent and were subcultured into T-75 flasks. The growth of colonies in wells labeled as **1** or **2** was noticeably poorer than the more aggressive **3**, **4**, and **5** wells. Approximately 43% of the colonies recovered following initial growth under 15 cGy/h were capable of continued proliferation similar to unirradiated control cultures.

week, the remaining colonies in the 6-well plates were fixed with ethanol and stained with crystal violet. The wells labeled “2” or “1” contain poor-growing colonies that were not considered survivors. Large numbers of giant cells in the poor growing “1”-labeled wells and some of the “2”-labeled wells were noted. Presumably, the giant cells were incapable of proliferating without the continuous LDR radiation exposure needed to initiate additional giant cells from the diploid cell population or, alternatively, to provide a selective growth condition advantageous to the existing giant cells (by supplying enough radiation-induced DSBs and genomic “plasticity” to provide genetic variants capable of continued proliferation). The colonies observed in the remainder of the “2”-labeled wells contained nearly 85–90% diploid cells (and only 10–15% giant cells), as in the unirradiated parental cultures. These cells presumably failed to proliferate from the delayed effects of the continuous LDR radiation exposure.

Unirradiated colonies of C57BL/6;129/Sv *tp53*^{-/-} MEF clone 6 cells after 1 week of growth in 0.5% soft agar are shown in the first two panels (A, B) of Figure 6.11. This is a classic feature of the tumorigenic phenotype (Hall, 2000; Hanahan and Weinberg, 2000). Within 36 hours, very rapid outgrowth of larger colonies picked from the soft agar cultures and plated (without disaggregation) into 6-well plates with complete medium was observed (Figure 6.11, panel C). As with the parental cultures, *all* the sub-cloned cell cultures contained both giant, multi-nucleated attached (interphase) cells and large, multipolar mitotic cells at a frequency of ~10–20% (Figure 6.11, panel D). These giant cells were presumed to contain the highly aneuploid chromosome complements observed by the Donehower and Fukasawa groups and Dr. Nagasawa.

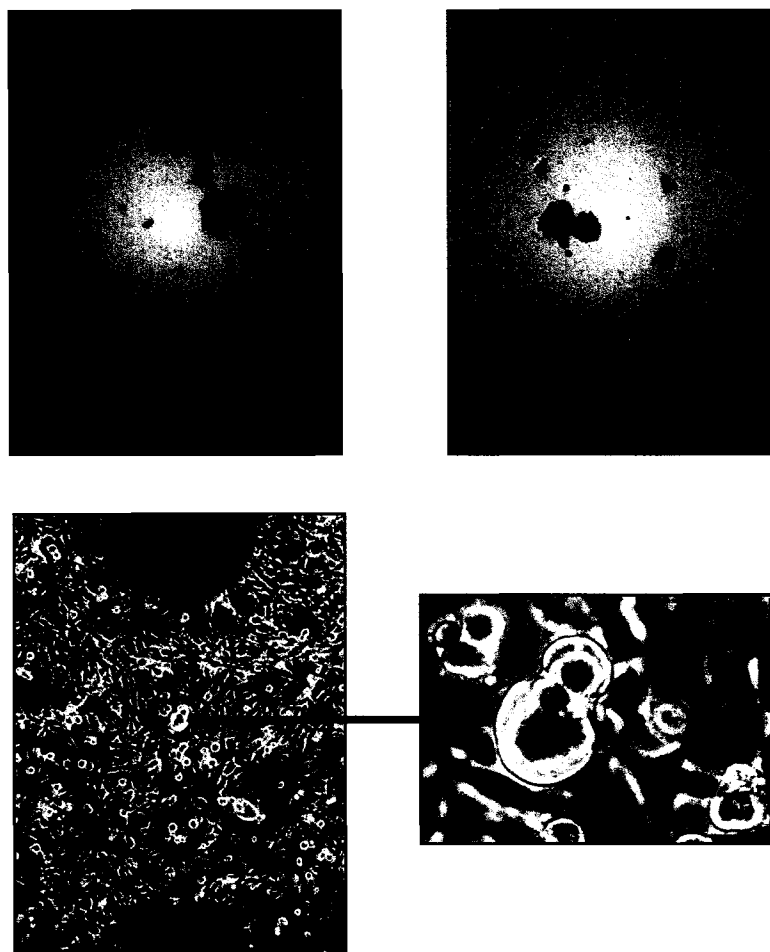


Figure 6.11. Colonies of *tp53*^{-/-} MEF clone 6 cells growing in soft agar, a classic feature of the tumorigenic phenotype. Single cells were plated into 0.5% soft agar and MEM/10% FBS medium by Dr. H. Nagasawa and colonies were photographed with a Nikon Eclipse TS100 microscope equipped with a 10X objective after one week of growth (panels **A** and **B**). Numerous subclones were then picked and placed directly (without disaggregation) into 6-well plates supplied with α MEM/15% FBS medium, all of which grew aggressively and were successfully passaged further into T-25 flasks. The rapid outgrowth from the soft agar-cloned colony seen in panel **C** was noted *after only 36 hours* of culture and was replete with aberrant (multipolar, multinucleated) mitotic bodies, as seen in panel **D** and as first noted in the parental cultures.

These cloning experiments suggest the instability phenotype could not be “cloned out” of the ~85–90% diploid cells in the parental cultures of the *tp53*^{-/-} MEF cells, *i.e.*, sub-clones *without* the giant cell phenotype present in ~10–15% of the cell population could not be isolated. This suggests it is a defect present in the entire population (in the “normal” diploid cells) affecting DNA repair, mitotic fidelity, or cell cycle control, and is probably recessive in nature, becoming unmasked in a spontaneous or radiation-induced LOH event typical of *tp53*-deficient mutant cells. The phenotype is stimulated by continuous LDR radiation exposures, but not by acute radiation exposures, indicating DNA damage incurred in each cell division at these low dose-rates (on the order of a few DSBs/hour) may be sufficient to generate additional giant cells at a higher frequency.

6.4.2. Results of the Cytogenetic Experiments

The following series of cytogenetic observations were conducted after the initial observation of massive chromosomal aberrations in the *tp53*^{-/-} MEF clone 6 giant cells by Dr. Hatsumi Nagasawa. Unirradiated metaphase spreads displayed very long, apparently fused (“noodle”) chromosomes often lacking an obvious primary constriction (centromere), large numbers of chromosome and chromatid-type aberrations, and severe chromosomal fragmentation with excessive double minute chromosomes (see Figure 6.12, panels E–H). The frequency of this phenotype in unirradiated cultures was ~15%, increasing to ~30% after 2 Gy and ~35% after 2 Gy, indicating a steady-state plateau had been reached (Figure 6.12, lower panel plot). Total chromosome aberrations in the ~85% of the diploid cells in these cultures obeyed an expected linear or curvilinear radiation dose response.

Figure 6.12. Frequencies per cell of micronuclei and chromosomal aberrations in *tp53*^{-/-} MEF clone 6 cells following acute irradiation with doses of 0, 2, and 4 Gy. Micronuclei observed in cytogenetic preparations included both acentric and centric forms (centromeres stain brightly with DAPI and, once inverted, appear as dark black spots in interphase nuclei). Acentric micronuclei (**A, B**) result from chromosome breaks releasing acentric fragments from the chromosome, and are induced both spontaneously and by radiation. Micronuclei containing centric fragments and nuclear bodies connected by thin chromatin bridges (**C, D**) result from failure of the mitotic apparatus due to the presence of multipolar spindles. Giemsa-stained metaphase spreads with massive chromosomal aberrations (**E–H**) in the same cytogenetic preparations were also frequently observed, the frequencies ($\pm$ SEM) of both total (chromosome and chromatid-type in near-diploid normal cells) and massive chromosomal aberrations (frequency of total metaphases) per cell were kindly provided by Dr. H. Nagasawa. While the induction of both total interphase and metaphase aberrations obey an obvious radiation dose response, the frequencies of centric micronuclei and massive chromosomal aberrations appear to plateau, indicating other factors besides direct radiation-induced chromosome breakage may influence the generation of these phenotypes.

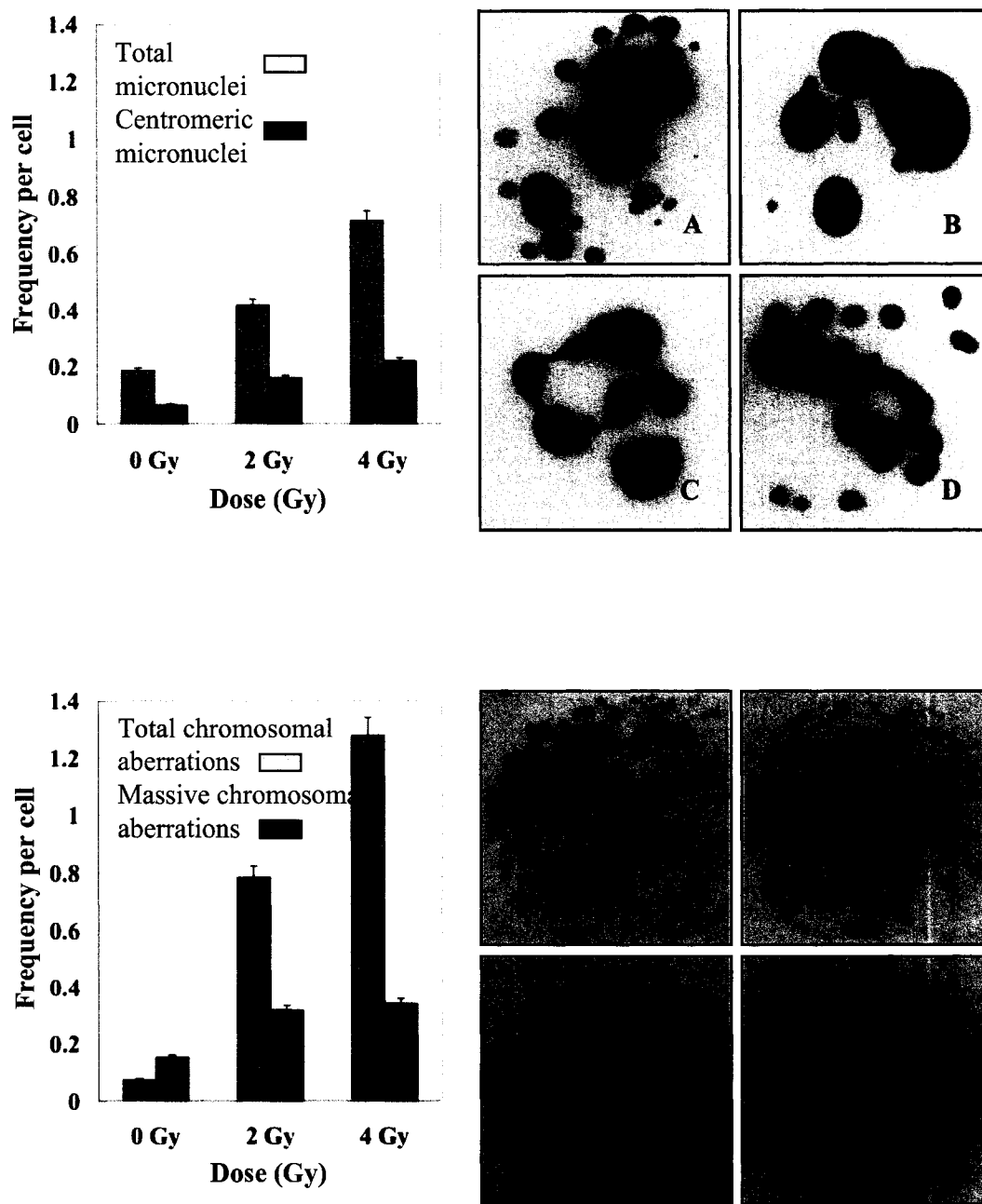


Figure 6.12.

Using the same slides from these original analyses (colcemid-based metaphase collections) and freshly-prepared slides (without colcemid), the frequencies of aberrant interphase nuclei were examined in the *tp53*^{-/-} MEF clone 6 cell line using reverse DAPI staining, as shown in Figure 6.12, panels A–D and upper panel plot. DAPI is a DNA stain that preferentially binds A/T-rich regions of DNA, such as those found at the centromeres of chromosomes; these centromeres fluoresce very brightly under 345-nm illumination (Haugland, 2005). By inverting (“reversing”) the DAPI image with the image analysis software, the bright centromere spots become dark, as shown in Figure 6.12, panels A–D.

Two types of aberrant cell nuclei in Figure 6.12 were immediately apparent: large, multi-lobed nuclei surrounded by many detached micronuclei without centromere signals (*acentric* micronuclei, panels A and B) or with centromere signals (*centric* micronuclei, panels C and D). A large percentage of the acentric micronuclei, typical of those seen after radiation exposure that result from acentric fragment losses, would probably be lost in following mitosis. The loss of critical genes on these fragments is probably lethal to these future daughter cells. The centromeric kinetochore regions of the centric micronuclei, however, could properly bind spindle fibers and be pulled randomly to either pole (or *multiple* poles, see below) during anaphase. Since many of these centric micronuclei are still attached by thin chromatin bridges (see Figure 6.12, panels C and D), these dicentric (or polycentric) chromosomes will be pulled in both directions at once (or more than two directions for cells with ≥ 3 centrosomes) by the spindle apparatus in mitosis, breaking the bridged chromosomes. These unrepaired chromosome ends in the daughter cells are “fusogenic” and chromosomal recombinational repair mechanisms

(primarily NHEJ) could drive further breakage-fusion-bridge (BFB) cycles in later generations (Maser and DePinho, 2002; Pihan and Doxsey, 2003).

The larger size of these aberrant nuclei (and micronuclei) and the degree of rearrangements seen in the interphase nuclear bodies in Figure 6.12, panels C and D suggest they are indeed the same cells that contain the massive chromosome aberrations observed in the metaphase preparations in panels E–H. Frequencies of centric micronuclei obeyed a similar pattern to their metaphase counterparts (metaphase spreads with massive chromosome aberrations), approximately doubling in frequency from ~10% in unirradiated cultures to ~20% after doses of 2 Gy and 4 Gy. The frequencies of acentric micronuclei increased with a linear dose response (~0.15/Gy), similar to the frequencies of total (typical) radiation-induced chromosome and chromatid-type aberrations (~0.3/Gy) in the metaphase preparations of the diploid cells in the population. Colcemid treatment did not affect these interphase results.

Consistent with the large degree of spontaneous chromosome rearrangement observed during the cytogenetic analyses, immunocytochemical analyses of these unstable *tp53*^{-/-} MEF clone 6 cells using anti-phospho-Ser139 histone H2AX antibodies revealed large numbers of γ -H2AX foci in the cells, as shown in Figure 6.13. Histone H2AX is a variant of histone H2A (accounting for ~10–15% of total histone H2A content in the cell nucleus) with a unique C-terminal extension containing a conserved serine phosphorylation residue at position 139 (Rogakou, *et al.*, 1998). This serine residue is phosphorylated after certain classes of DNA damage by ATM, ATR, and DNA-PK (Rogakou, *et al.*, 1998, Burma, *et al.*, 2001; Ward and Chen, 2001; Stiff, *et al.*, 2004; Wang, *et al.*, 2005) in megabase domains surrounding a DNA DSB, forming a γ -H2AX

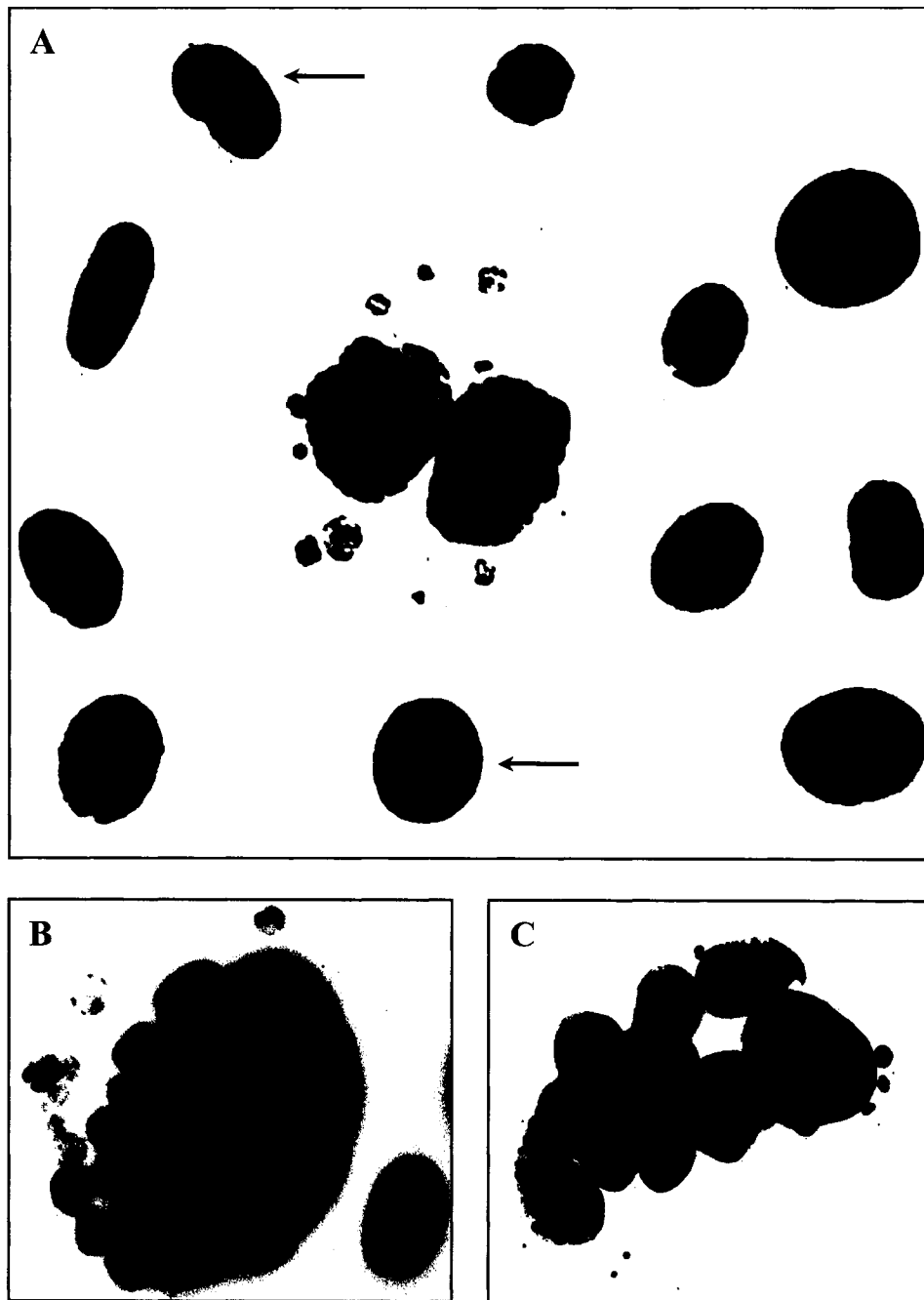


Figure 6.13. Spontaneous γ -H2AX foci observed in unstable *tp53*^{-/-} MEF clone 6 cells. Panel A demonstrates the low number of γ -H2AX foci in the smaller diploid cells (arrows denote probable S-phase cells with ≤ 5 foci) and a much larger bridged nucleus with micronuclei and large numbers of γ -H2AX foci (center). Panel B shows a large aberrant nucleus with acentric micronuclei (see Figure 6.12, panels A and B) and panel C shows a large multipolar nucleus (see Figure 6.12, panels C and D). Both nuclei in panels B and C have hundreds of γ -H2AX foci, a testament to the high frequency of spontaneous chromosome breakage in these unstable cells.

focus whose function is to recruit an ever-growing list of additional DNA repair and processing proteins to the site of damage (Rogakou, *et al.*, 1999; Paull, *et al.*, 2000; Thompson and Limoli, 2003; Bradshaw, *et al.*, 2005). Experiments employing both low LET (γ rays) and high LET radiation sources [HCPs, ^{125}I -labeled 5-iododeoxyuridine ($^{125}\text{IUdR}$), and ^{125}I -triplex forming oligonucleotides] have demonstrated a 1:1 correlation between γ -H2AX foci and DNA DSB induction at low doses, and data for pulsed field-gel electrophoresis (PFGE) experiments at higher doses (Rothkamm and Löbrich, 2003; Karlsoon and Stenerlow, 2003; Sedelnikova, *et al.*, 2002, 2004). The tens to hundreds of γ -H2AX foci observed in *all* the $tp53^{-/-}$ MEF clone 6 giant cells examined are a testament to the large number of *spontaneous* DNA DSBs occurring in their genomes.

As shown in Figure 6.14, the giant cells observed in asynchronous cultures of the $tp53^{-/-}$ MEF clone 6 cell line display a variety of centrosome disruption phenotypes (previously reported by Fukasawa, *et al.*, 1996 for earlier passages of these cells). The size of the cell nucleus was correlated with the number of centrosome signals observed (*i.e.*, larger cells had higher numbers of centrosomes). Also, smaller aberrant cells with 3–5 centrosome signals seem to have larger centrosome bodies than the larger cells with >10 signals, so it is possible that the centrosomes have become “fragmented” in the larger, more aneuploid giant cells (Nigg, 2002; Hut, *et al.*, 2003).

Fluorescence *in situ* hybridization (FISH) analysis with a telomere G-rich peptide nucleic acid (PNA) probe (Applied Biosystems) was employed to ascertain whether the long fusion chromosomes resulted from telomere-to-telomere end fusion events, which would be evident by the existence of interstitial telomeric sequences (Bailey, *et al.*, 1999, 2004). Instead of telomere fusion events being observed, telomere “fission” events, large

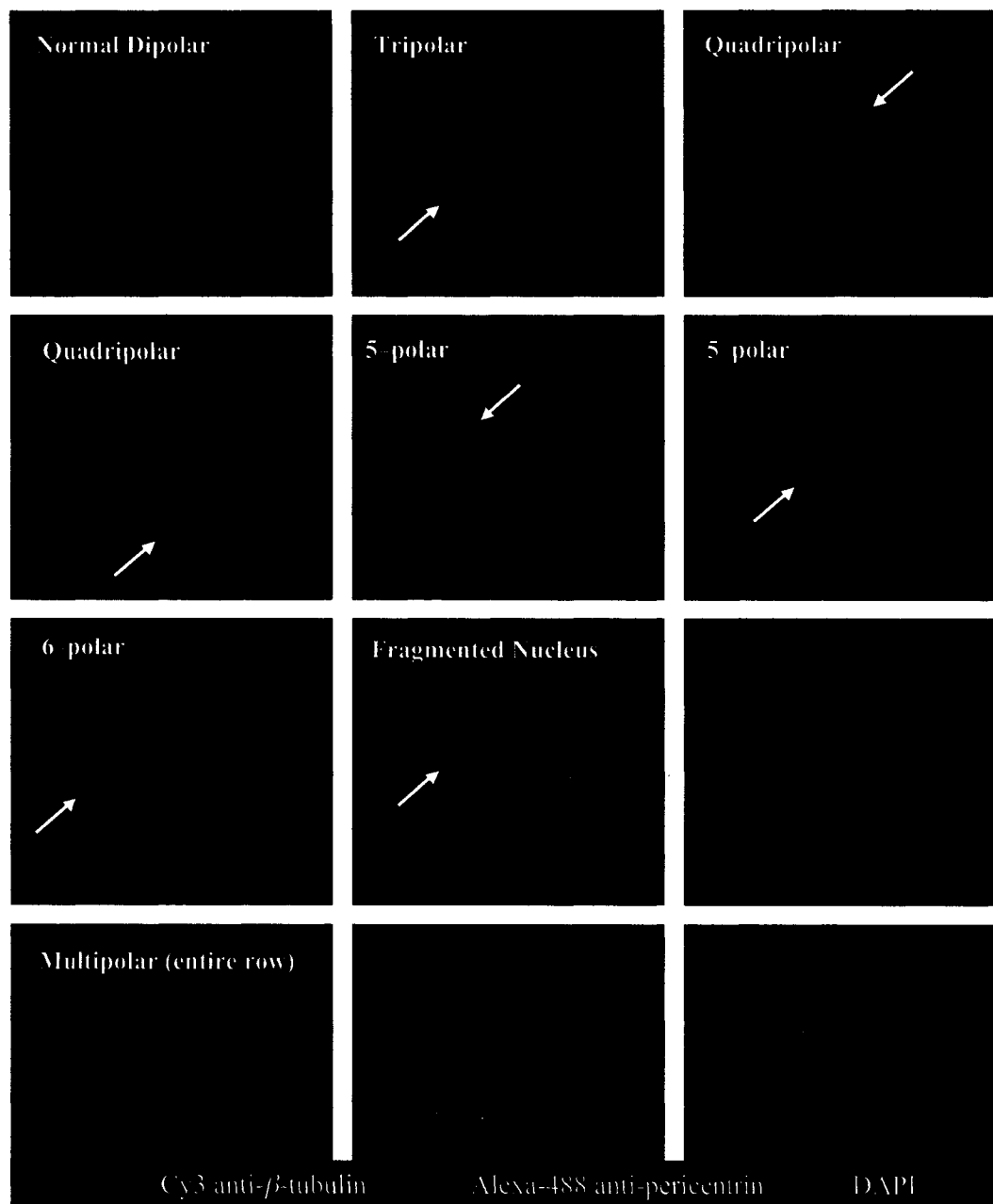


Figure 6.14. Centrosome phenotypes observed in unstable *tp53*^{-/-} MEF Clone 6 cells. From top to bottom, the panels demonstrate increasing numbers of centrosome signals in the larger unstable nuclei of the giant cells. Colocalization of the red and green signals merges into a yellow color. Aberrant centrosome number and morphology was observed in all giant cells examined in this study.

numbers of extra-chromosomal telomeric repeats (ECTRs), telomeric double minutes (TDMs) and larger, acentric telomere-capped fragments (on both ends), were observed. Two representative examples of this phenotype are shown in Figure 6.15. Many of the long chromosomes seen in the Giemsa-stained massive chromosome aberration metaphase spreads (Figure 6.12, panels E–H) lacked both telomeric and centromeric signals or were multicentric (visualized with reverse DAPI staining as above). Quantitative analyses of the degree of telomere fission in these cytogenetic preparations were not attempted; however, *all* metaphase spreads with massive chromosome aberrations displayed this telomere fission phenotype (although some to a more dramatic extent than others). Only very limited occurrences of interstitial telomeric FISH signals, indicating the site of a telomere end-to-end-fusion or telomeric sequence “capture” event, were observed.

In the final cytogenetic analysis performed, slides prepared for the telomere FISH studies were hybridized with the 21X mouse mFISH kit (MetaSystems GmbH) as described above. Representative metaphase spreads are shown in Figure 6.16. Unfortunately, the FITC channel did not register (due to an unrecognized software error) and fluorescent signals for 9 different chromosomes (1 painted solely with FITC, 8 others using FITC in combination with the 4 other fluorochromes) were unavailable (I do not believe this detracts from the results). Metaphase spreads of unirradiated “normal” diploid and tetraploid cells (as in Figure 6.16, panel D; mouse cells in culture frequently become tetraploid) demonstrated no evidence of chromosome rearrangements and the individual chromosomes were pseudo-colored by the software program as single colors only. In striking contrast, metaphase spreads with massive chromosomal aberrations

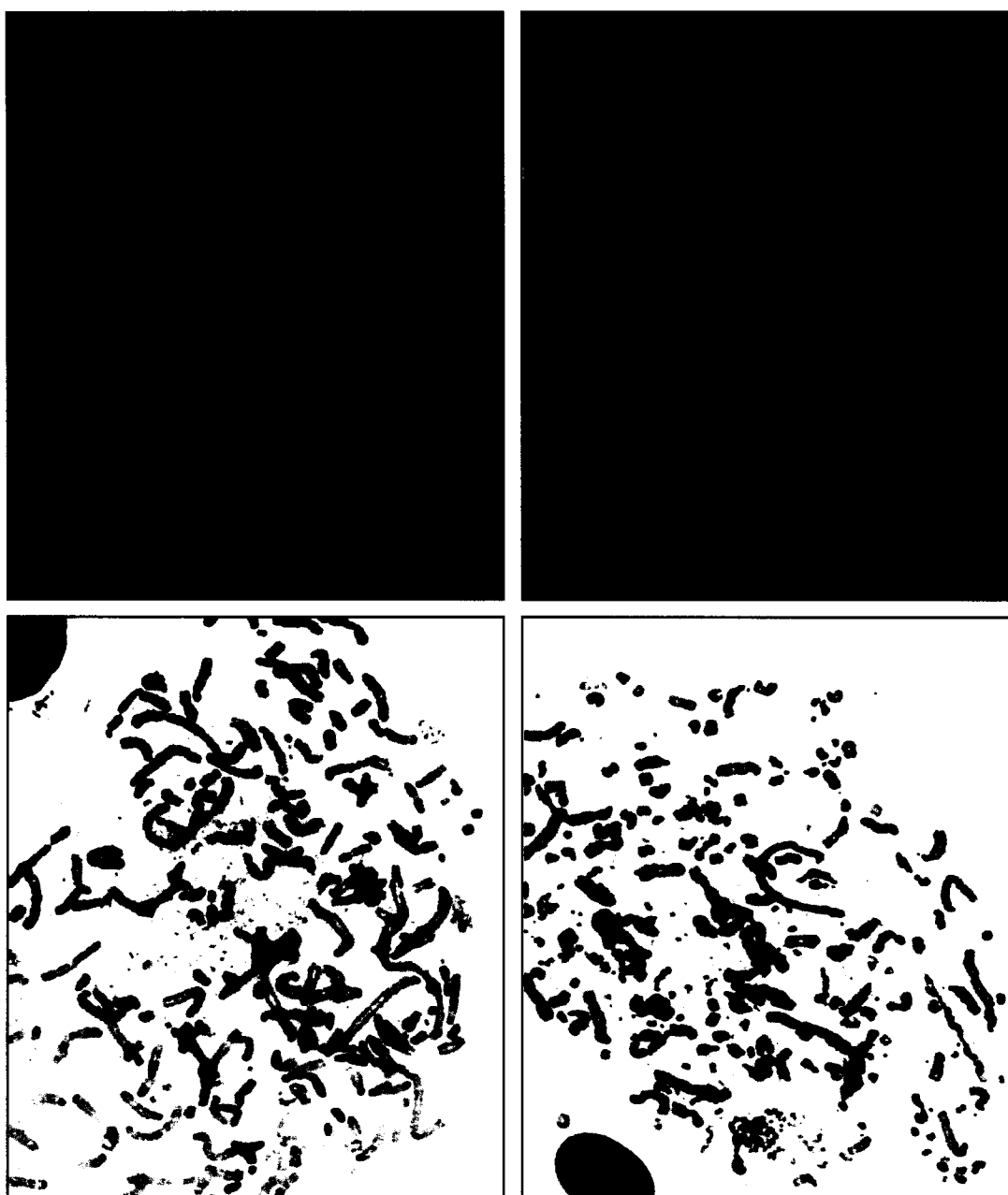


Figure 6.15. Spontaneous telomere fission in unstable *tp53*^{-/-} MEF clone 6 cells. Large numbers of extra-chromosomal telomeric repeats (ECTRs) and telomeric double minutes (TDMs) are evident in the two top panels, hybridized with G-rich telomere PNA probe (Applied Biosystems) and counterstained with DAPI. The two bottom panels have been inverted to identify the chromosomes by reverse-DAPI staining (see Figure 6.12). Centromeres are the darker black spots on (some of) the chromosomes. Many acentric and polycentric chromosomes, chromosome aberrations, and fragmented chromosome material are evident in the bottom panels.

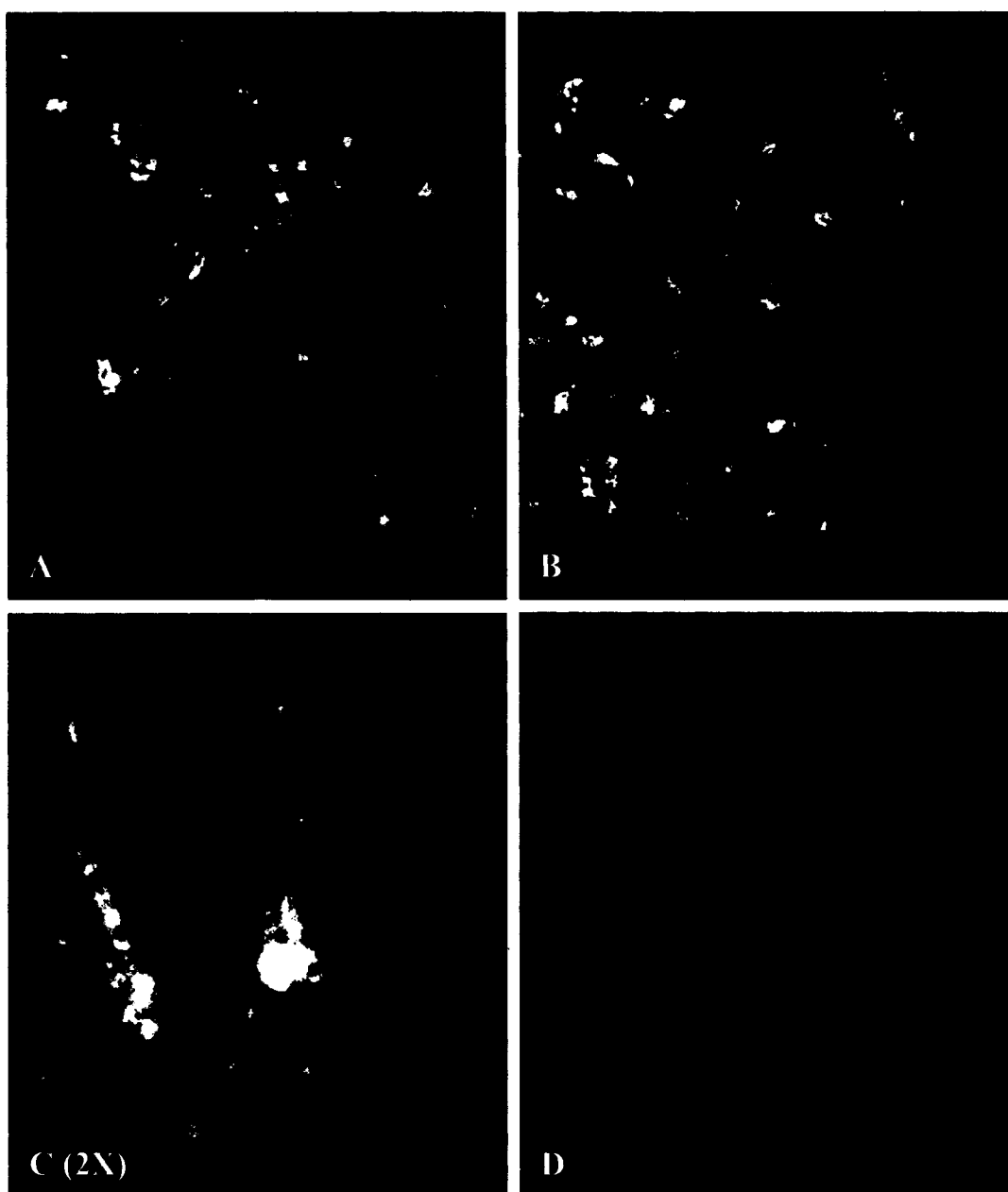


Figure 6.16. Massive chromosomal rearrangements in unstable *tp53*^{-/-} MEF clone 6 cells. Panels A–C are representative images of metaphase spreads from unstable cells hybridized with the 21X mouse mFISH kit (MetaSystems GmbH; panel C is enlarged 2X). Panel D is a representative image of a normal MEF metaphase spread (although near-tetraploid in chromosome complement) from the same slide, false-colored by the Applied Imaging mFISH software. Only 4 of the 5 combinatorial fluorescent planes were captured and processed due to technical difficulties, but this does not detract from the large numbers of chromosome translocations and high degree of spontaneous chromosomal instability obvious in panels A–C.

revealed extensive chromosome rearrangements with chromosome exchanges, fusions, and complex non-reciprocal translocations [looking almost rainbow-colored, as seen in mBAND analyses (these *intrachromosomal*, band-level multiplex FISH probes kits are also available through MetaSystems GmbH)], as shown in panels A–C of Figure 6.15. *Pairs* of homologous chromosomes (*e.g.*, the two largest chromosomes in panel B, painted aqua-yellow-green-blue-red-orange from top to bottom) were frequently observed in these mFISH experiments, definitive evidence that these giant, unstable *tp53*^{-/-} MEF cells are capable of multiple cell divisions in culture (in accord with the results of the survival experiments discussed above).

6.5. Discussion

6.5.1. Discussion of the Survival Experiments

The functionality of wild-type *tp53* protein is a requirement for proper genomic maintenance, mitotic fidelity, and the engagement of crucial DNA repair and cell cycle control mechanisms following DNA damage (where *tp53* plays many direct and indirect roles, see Chapter 2). This functionality may be affected by either loss of both wild-type alleles (*e.g.*, LOH events in LFS patient tumor cells) or the mutation of the wild-type allele. Mutant *tp53* alleles can sometimes result in the expression of mutant *tp53* protein with potentially deregulated activity; loss-of-function mutations in certain domains (DNA binding, transactivation, tetramerization) resulting in a dominant-negative effect (as in the *scid* cell line, SGCR11, discussed above) or gain-of-function mutations endowing novel protein function or altered protein-protein interactions and subcellular localization.

The results of the acute irradiation and continuous LDR irradiation survival experiments with the *tp53*^{-/-} MEF cell line presented in this chapter support the increasing body of evidence that *tp53* mutation results in increased radioresistance (extensively reviewed in Bristow, *et al.*, 1996; Cuddihy and Bristow, 2003). The radioresistant phenotype was first observed in *tp53*-mutant fibroblasts derived from patients with Li-Fraumeni syndrome, a rare autosomal dominant syndrome caused by *tp53* heterozygosity and characterized by soft tissue sarcomas appearing in infancy or childhood and a high incidence of familial clustering of early-onset (<45 years) sarcomas, breast cancers, brain tumors, and adrenocortical carcinomas (Li and Fraumeni, 1969; Blattner, *et al.*, 1979; Malkin, *et al.*, 1990). The list of radioresistant *tp53*-mutant or deficient cells has grown to include a large number of human and rodent normal cell types infected with viruses that inactivate Tp53 function (human papilloma virus E6, polyoma virus SV40 large T antigen, adenovirus E1A), transformed cells, and tumor cells (Bristow, *et al.*, 1996; Cuddihy and Bristow, 2003; Alsbeih, *et al.*, 2004; Böhnke, *et al.*, 2004). Studies examining the correlation of *tp53* mutation in human tumors and the efficacy of radiotherapy have reported decreased local control of tumors harboring mutations in *tp53* (also reviewed in Bristow, *et al.*, 1996; Cuddihy and Bristow, 2003).

Radioresistance during LDR (66 cGy/h) exposures was observed in nearly confluent cultures of fibroblasts derived from Li-Fraumeni affected individuals, as compared to the LDR survival of fibroblasts derived from apparently normal controls (Sproston, *et al.*, 1996). In Bristow, *et al.* (1998), mutant *tp53*-expressing rat embryo fibroblasts (REFs) were shown to be radioresistant following LDR irradiation and, interestingly, these cells were more proficient at repairing DNA double-strand breaks

induced during continuous LDR irradiation, as measured the single-cell gel electrophoresis Comet assay.

The continuous LDR irradiation colony formation assay was successful in identifying a significant difference between the response of the *atm*^{+/-} heterozygote and wild-type control MEF strains in these experiments. The high degree of radiosensitivity of the *atm*^{-/-} MEF strains in this assay was expected from the responses of the *prkdc*-deficient rodent cells reported in Priestly, *et al.* (1998). It remains to be seen if this type of asynchronous low dose-rate irradiation assay could positively identify *human* A-T heterozygote fibroblasts as well. Earlier studies reported by Weeks, *et al.*, (1991) and Nagasawa, *et al.* (1992) were unable to positively identify A-T heterozygotes in *confluent* cultures of human fibroblasts exposed at low dose-rates. Perhaps the current “whole cell cycle” asynchronous culture approach could be more effective. Unfortunately, translation of this assay into the clinic (*e.g.*, for identifying radiosensitive breast cancer cases or screening for A-T heterozygote individuals in the general population, as discussed in Chapter 2) would be difficult for general-use purposes.

6.5.2. Discussion of the Cytogenetic and Immunocytochemical Experiments

Centrosomes function as the primary microtubule-organizing center (MTOC) of the cell, providing the anchor points (poles) of the mitotic spindle apparatus (reviewed in Nigg, 2002; Badano, *et al.*, 2005). A single G1-phase centrosome duplicates in the cytoplasm in a coordinated fashion with DNA replication in the nucleus in S-phase, after which one daughter centrosome migrates to the opposite pole of the nucleus to orient the bipolar spindle for mitosis. The tp53 protein has been shown to directly interact at the

centrosome (Tarapore, *et al.*, 2001; Tritarelli, *et al.*, 2004). Centrosome hyper-amplification has been observed for *tp53*-, CDKN1A- (p21^{Waf1/Cip1}), and cyclin E- (the cyclin partner for CDK2, complexes of which drive S-phase progression) deficient normal and tumor cells, and in tumor cells over-expressing Mdm2, *tp53*'s negative regulator (Fukasawa, *et al.*, 1996, 1997; Pihan, *et al.*, 1998; Carroll, *et al.*, 1999; Mussman, *et al.*, 2000; Tarapore, *et al.*, 2001; Borel, *et al.*, 2002; D'Assoro, *et al.*, 2002, 2004; Tarapore and Fukasawa, 2002).

Multi-polarity of the centrosome-mediated spindle apparatus has been shown to result in chromosome fragmentation and the induction of G1-phase arrest in wild-type *tp53* cells carrying damaged chromosomes (Mussman, *et al.*, 2000; Tarapore, *et al.*, 2001; Borel, *et al.*, 2002; Tarapore and Fukasawa, 2002; Nigg, 2002; Badano, *et al.*, 2005). These fractured chromosomes are recombinogenic and “fusogenic,” becoming dicentric chromosomes following DSB repair that are likely to become an anaphase bridge at the following mitosis. These dicentric chromosomes break again at this mitosis, and these “breakage-fusion-bridge” (BFB) cycles can continue for many cell generations in certain genetic backgrounds (*tp53*-deficient, tumor-derived cells; Maser and DePinho, 2002; Pihan and Doxsey, 2003). This BFB concept traces its roots directly to the pioneering work of Dr. Hermann Muller and his work on X-ray-induced mutations in *Drosophila* (1927) and Dr. Barbara McClintock and her work in *Zea mays* (maize; 1939, 1941).

To explain the newly described “telomere fission” phenotype associated with these unstable *tp53*-knockout MEF cells, it is hypothesized this phenomenon of the generation of extra-chromosomal telomeric repeats (ECTRs) and telomeric double-minutes (TDMs) may be associated with a recombinational alternative lengthening of

telomeres (ALT)-type mechanism or other hyper-recombinational DNA repair/processing defect (reviewed in Henson, *et al.*, 2002). The phenotype of ECTRs/TDMs has been observed in:

- Telomerase-negative immortalized ALT fibroblast lines KMST-6, WI38 strain VA13, Saos-2, SUSM-1, U2 OS and MeT-4A (Tokutake, *et al.*, 1998; Wang, *et al.*, 2004)
- CHO cells, and primary and SV40-transformed Chinese hamster embryo cells (Regev, *et al.*, 1998)
- A-T patient fibroblasts (AG03058, AG04405, GM02052, GM05823; Hande, *et al.*, 2001; Tchirkov and Lansdorp, 2003)
- *atm*^{-/-} 129/SvEv mouse fibroblasts (Hande, *et al.*, 2001) and C3H/He mouse embryo fibroblasts (Undarmaa, *et al.*, 2004)
- Primary and SV40-transformed telomerase-negative human fibroblasts (Stavropoulos, *et al.*, 2002).
- Fanconi anemia (complementation group A) patient lymphocytes (Callén, *et al.*, 2002)
- *ercc1/xpf*^{-/-} mouse embryo fibroblasts (Zhu, *et al.*, 2003)
- Likely candidate: Immortal, telomerase-negative sub-clones of Li-Fraumeni syndrome patient fibroblasts (MDAH 087 LCS-AF series; Tsutsui, *et al.*, 2003) – 14% of metaphase spreads have double-minute chromosomes but telomere FISH painting was not employed.

ECTRs/TDMs were not observed in *telomerase-positive* human fibroblasts (WI38, GM00639, AG10076; Stavropoulos, *et al.*, 2002) or tumor cells examined (HeLa cervical carcinoma, HT1080 fibrosarcoma, and BT2E hepatocarcinoma cells; Tokutake, *et al.*, 1998; Regev, *et al.*, 1998; Tsutsui, *et al.*, 2003), implying a correlation of ECTRs/TDMs to ALT-based recombinational mechanisms. ECTRs ranging in size from 0.7 to 56.8 kb were observed in electron microscopy preparations of telomere-enriched nuclear fractions of GM00847 (normal and *hTert*-immortalized strains) and WI38 strain VA13 ALT cells,

which corresponded with the telomere restriction fragment (TRF) lengths determined by two-dimensional pulsed-field gel electrophoresis (PFGE) measurements (Cesare and Griffith, 2004).

However, the extensive degree of massive chromosomal rearrangement in the unstable $tp53^{-/-}$ MEF clone 6 cells evidenced by Figure 6.16 has not been observed in relation to the phenotype of ECTRs/TDMs in telomerase-negative ALT cell lines or in other $tp53$ -knockout cell lines. Similarly, such *large* numbers of ECTRs/TDMs in these cells has not been reported (the cell types listed above have been observed with a single or a few ECTRs/TDMs per cell), indicating the phenotype of telomere fission is a new telomere dysfunction phenotype, probably permissible only in a $tp53^{-/-}$ genetic background. A similar example of a reverse DAPI-stained, severely aneuploid metaphase spread of a spleen cell (“frequently” observed according to the author) obtained *in vivo* from $tp53^{-/-}$ C57BL/6 mice, with similar appearance to the Giemsa and reverse DAPI-stained metaphase preparations seen in Figures 6.12 and 6.15, was presented in the final figure of Fukasawa, *et al.* (1997). This indicates these instability phenotypes may not be limited to $tp53^{-/-}$ cells grown *in vitro*.

From the telomere FISH and mFISH analyses, the apparent breakpoints of these chromosomal rearrangements appear to be sub-telomeric, since very few cases of interstitial telomere FISH signals in the long, fused chromosomes were observed. Sub-telomeric FISH analysis (see Henegariu, *et al.*, 2001) could be potentially informative for further analysis of the chromosomal break-point regions in these chromosomes. Cytogenetic analyses of human normal and tumor cells, with and without irradiation, have shown sub-telomeric chromosomal regions may account for a significant percentage

of chromosome rearrangements that show telomere FISH signals at the breakpoint. Such interstitial FISH signals may actually result from small, “cryptic” translocations (*i.e.*, smaller than the Mb-scale resolution of FISH analysis) of sub-telomeric sequences (Slijepcevic, *et al.*, 1998). The high frequency of intrachromosomal duplication and rearrangement and interchromosomal recombination of subtelomeric regions may have also provided significant genetic diversity during primate evolution (Yunis, *et al.*, 1980; Olson and Varki, 2003; Linardopoulou, *et al.*, 2005).

Invoking a hyper-recombinational ALT mechanism as the basis of the large number of chromosomal rearrangements in the unstable $tp53^{-/-}$ MEFs is problematic as well. While a hyper-recombinational phenotype has been observed for ALT cells (Henson, *et al.*, 2002), it is shown to be restricted to telomeric sequences at the ends of mammalian chromosomes (Londoño-Vallejo, *et al.*, 2004) and interstitial telomeric sequences occurring naturally in, or engineered into, Chinese hamster chromosomes (Meyne, *et al.*, 1990; Kilburn, *et al.*, 2001). Interstitial telomeric sequences in CHO cells are “hotspots” of chromosomal fragility, breakage, and rearrangement, occurring at a 4–8-fold higher frequency spontaneously, directly after ionizing radiation exposure and treatment with restriction endonucleases, and even delayed many generations in the descendants of the cells originally exposed to radiation (a phenotype of radiation-induced genomic instability, RIGI; Alvarez, *et al.*, 1993; Slijepcevic, *et al.*, 1996, 1998; Day, *et al.*, 1998; Kilburn, *et al.*, 2001; Bouffler, *et al.*, 2001). Recombination rates at loci *outside* telomeric regions, however, have been shown to be equal in ALT, telomerase-positive, and normal cells (Bechter, *et al.*, 2003, 2004; Londoño-Vallejo, *et al.*, 2004).

Likewise, studies have excluded a possible mutator-phenotype (on the nucleotide level) for the induction of mutations in a transgenic *lacI* target gene in *tp53*^{-/-} fibroblasts, thymocytes, and tumor tissue (Sands, *et al.*, 1995) and the frequencies of spontaneous and radiation-induced mutations in the autosomal hypoxanthine phosphoribosyltransferase (*hprt*) gene in *tp53*-knockout versus wild-type *tp53* TK6 lymphoblastoid cells (Chuang, *et al.*, 1999). Shao, *et al.* (2000) observed a significant, approximate three-fold increase in the spontaneous adenine phosphoribosyltransferase (*aprt*) mutant frequency of 2,6-diaminopurine (DAP)-resistant *tp53*^{-/-} fibroblasts, but not in the frequency of DAP-resistant *tp53*^{-/-} T-lymphocytes. Conversely, another report by Liang, *et al.* (2002) demonstrated significantly higher mutation frequencies at the *aprt* and *hprt* loci in stimulated T-lymphocytes obtained from *tp53*^{-/-}/*aprt*^{+/-} mice following whole-body irradiation of 4 Gy of X-rays, due mainly to large interstitial deletion LOH events at these loci.

Clearly, the phenotypes of giant cell formation, nuclear deformation and chromosome rearrangement, telomere fission, and centrosome defects are inter-related, contributing the effects of their deregulation to the overall instability of these cells. Spontaneous or continuous LDR irradiation-induced DNA damage induced every hour in every cell cycle likewise contributes to both the initiation of these cells from their normal diploid cell counterparts and the further generation of unstable clonal descendents once the phenotype has been established. The requisite *secondary* mutation responsible for these phenotypes in the *tp53*^{-/-} MEF clone 6 cells is still unknown at this time and beyond the scope of this work.

The two most prevalent models of cancer are the somatic mutation model, in which cancer cells must accumulate a number of requisite mutations in a step-wise progression (Hanahan and Weinberg, 2000), and the aneuploidy/genomic instability model, in which mutation of a few critical genes (*tp53*, *Rb*, *c-myc*, *etc.*) alone is sufficient to generate an unstable neoplastic cell (Duesberg, *et al.*, 1998, 1999, 2000; Maser and DePinho, 2003; Pihan and Doxsey, 2003). The aneuploidy/genomic instability model traces its roots to the work of Hansemann (1890, 1891) and Boveri (1914) and their microscopic observations of patterns of centrosome and chromosome dysfunction (aberrant mitotic figures, anaphase bridges, chromosome aberrations) in human tumor specimens. Both models consider that certain mutant variants may possess a selective advantage for enhanced growth in culture or in the tissue/tumor micro-environment (recently reviewed in Barcellos-Hoff, *et al.*, 2005). In a fascinating study by Hida, *et al.* (2005), mouse endothelial cells growing adjacent to implanted human tumor xenografts were shown to be genetically unstable, having multiple centrosomes, heterogeneous nuclei, and rearranged aneuploid chromosome complements as visualized by FISH analysis. This implies a “bystander effect”-type mechanism for DNA damage signals transmitted by tumor cells to other cell types in their local micro-environment, similar to the ionizing radiation-induced bystander effect observed in mammalian cell experimental systems (discussed in Chapter 2).

Telomere dysfunction and cycles of bridge-fusion-breakage in mice encourages epithelial carcinogenesis (carcinomas also account for the majority of human cancer types as well, versus sarcomas). These tumors are typified by complex non-reciprocal translocations and amplifications and large deletions at genomic loci syntenic with

human cancer “hotspots” (Artandi, *et al.*, 2000; O’Hagan, *et al.*, 2002; Goytisolo and Blasco, 2002; Albertson, *et al.*, 2003), the occurrence of which is accelerated in a *tp53*-deficient background (Chin, *et al.*, 1999). Likewise, the extensive DNA fragmentation and generation of complex chromosomal abnormalities associated with the centromere and telomere dysfunction-mediated BFB-cycle phenotypes has been found for both human carcinomas *and* sarcomas (Pihan, *et al.*, 1998; Duesberg, *et al.*, 1998, 1999, 2000; Gisselsson, *et al.*, 2001; D’Assoro, *et al.*, 2002; Albertson, *et al.*, 2003), leading to a constant “re-shuffling” of the cancer cell genome (Hanahan and Weinberg, 2000; Maser and DePinho, 2003; Pihan and Doxsey, 2003).

Recent research into the effects of centrosome dysfunction and mitotic spindle defects have indicated that cells may not simply be able to overcome and “adapt” to a *tp53*-dependent “tetraploidy checkpoint” following an improperly executed mitosis (Lanni and Jacks, 1998; Andreassen, *et al.*, 2001, 2003; Borel, *et al.*, 2002; Meraldi, *et al.*, 2002; Margolis, *et al.*, 2003; discussed in Section 2.3.2 of Chapter 2), this tetraploidy checkpoint in mammalian cells may not even exist (Wong and Stearns, 2005; Uetake and Sluder, 2005). Uetake and Sluder (2005) reported that for binucleated normal human fibroblasts (BJ) generated in asynchronous cultures treated with 0.5 μ M cytochalasin D, 94.3–97.5% of these cells underwent DNA synthesis (monitored by BUdR incorporation) within 12 hours after drug removal and 76–100% went through mitosis within 36 hours after drug removal, as revealed by time-lapse video microscopy (the upper ranges reported are for cells grown on fibronectin-coated coverslips rather than bare glass). All these studies arrive at the same conclusion that a tetraploid state may precede more stochastic aneuploid states, typical of tumor cells, as a percentage of the tetraploid cells

suffer recurrent chromosome breakage and rearrangement when attempting mitosis with multipolar spindles inherited from the binucleated parental cells that preceded them.

Two recent papers by Shi and King (2005) and Fujiwara, *et al.* (2005) likewise provide interesting correlation to the current results. Shi and King (2005) showed that hTERT-immortalized N/TERT-1 human keratinocytes and HeLa cervical carcinoma cells have relatively high rates of spontaneous chromosome nondisjunction (0.05–0.10% and 0.24–0.39% per mitosis, respectively) as measured by FISH analysis using centromeric probes specific for human chromosomes 8 and 12. The frequency of chromosome mis-segregation was significantly higher (45–166-fold) in spontaneously occurring binucleated cells. By observing GFP-histone 2B-tagged binucleated cells in culture with time-lapse microscopy, the authors reported that 65–75% became tetraploid by mononucleated cells exiting mitosis after a long delay through nuclear envelope furrow regression, 12–18% arose from grossly abnormal mitoses of mononucleated cells, 12% arose through the division of existing binucleated cells, and 3–6% arose by cell fusion events between descendants of the same parental cell. The majority (60%) of the wild-type *tp53* tetraploid N/TERT-1 cells followed by time-lapse microscopy did not proceed into the following mitosis (suggesting a tetraploidy checkpoint), but of those that did, 65% formed a bipolar spindle and mononucleated daughter cells and 35% formed multipolar mitotic spindles and become further aneuploid. On the other hand, nearly all the *tp53*-deficient binucleated HeLa cells (carrying ~130–160 chromosomes!) formed multipolar spindles and continued to divide to become mononucleated, binucleated, and further aneuploid cells.

The report by Fujiwara, *et al.* (2005) followed the fate of tetraploid *tp53*-deficient mouse mammary epithelial cells (MMEC) generated by a brief exposure to the actin inhibitor dihydrocytochalasin B (DCB) and separated by flow-sorting from the diploid cells of the culture. The tetraploid MMEC cells showed a significant increase in the frequency of chromosome translocations and mitotic non-disjunction during passaging in culture. Although neither the diploid or tetraploid cells were capable of anchorage-independent growth in soft agar, *only* the early-passage, flow-sorted tetraploid cells (not the diploid cells) generated malignant mammary epithelial spindle-cell carcinomas and myoepitheliomas 9–12 weeks following transplantation into nude mice. These MMEC-derived tumor cells likewise contained multiple centrosomes, large numbers of non-reciprocal chromosome translocations, dicentrics and double-minutes by spectral karyotyping (SKY) analysis and genomic amplification of a region containing a cluster of matrix metalloproteinase (MMP) genes by genome-wide array-comparative genomic hybridization (CGH) analysis.

The cytogenetic and immunocytochemical experiments with the unstable *tp53*^{-/-} clone 6 MEF cell line presented in this chapter are testament to the impact severe telomere dysfunction, massive chromosomal rearrangements and the potential lack of a G1 tetraploidy checkpoint (in all or just *tp53*-deficient mammalian cells) may have in tumorigenesis. The ever-growing list of interactions observed among various DNA repair, telomere-binding proteins and *tp53* at the mammalian telomere, centromere, mitotic spindle, and locations of DNA damage further implicate *tp53* deficiency as a critical source of genomic deregulation and tumorigenic potential. The more recent appreciation that tetraploid cells can undergo subsequent rounds of DNA replication and

abnormal mitoses and may be tumorigenic when implanted in nude mice, implies such genome-scale duplication events in normal diploid cells provide the major route to the higher chromosome numbers and extensive BFB-driven rearrangements characteristic of the aneuploid state in most types of human solid tumors.

CHAPTER 7

Final Discussion

7.1. Summary of Experimental Results

The experimental protocols employed in Chapters 3 through 5 were successful in terms of both of the main objectives of this dissertation presented in Chapter 1. These objectives were to first validate the assignment of the moderate and hyper-radiosensitivity phenotypes of the Rb family member fibroblast strains made in Fitzek, *et al.* (2002) using two acute, high dose-rate (HDR) irradiation protocols and validated reference CIMR control fibroblast strains routinely used in *this* laboratory. The second objective was to determine if a continuous low dose-rate (LDR) irradiation protocol (colony formation in low-density asynchronous cultures) could generate a large enough degree of differential survival between the hyper-radiosensitive Rb family member and apparently normal CIMR control fibroblast strains to attempt to correct the radiosensitivity phenotype with a genetic correction and complementation strategy. The key to a successful LDR irradiation selection strategy for radiosensitive human fibroblast strains would be the ability to recover a significant fraction of “rescued” cell clones (carrying a wild-type gene introduced from a transfected cDNA library) at a dose rate where uncorrected clones (“false-positives” with the same degree of parental strain LDR hyper-radiosensitivity) would be recovered at a very low frequency.

In Chapter 3, acute dose (HDR) ^{137}Cs γ -irradiation survival measurements for confluent G0/G1-phase cultures of the 14 Rb family member and 2 radiosensitive apparently normal CIMR control (GM04505 and GM08447) fibroblast strains allowed approximately 24 hours of confluent-holding post-irradiation potentially lethal damage (PLD) repair were presented. All 14 Rb family member and both radiosensitive apparently normal CIMR fibroblast strains examined in this chapter were significantly more radiosensitive (2–4-fold, $P < 0.05$) than this laboratory's range of apparently normal CIMR fibroblast strains using the 24-hour PLD repair protocol. This protocol achieved a better discrimination of the degree of radiosensitivity of the Rb family member fibroblast strains compared to reference apparently normal control strains than the protocol employed by Fitzek, *et al.* (2002). Survival curve ${}_1D_0$ values (the reciprocal of the *initial* slope of the survival curve, α , in the low-dose region) ranged from 133–244 cGy for the 14 Rb family member fibroblast strains and 325–630 cGy for the laboratory range of validated reference apparently normal CIMR fibroblast strains (AG01521, AG01522, GM02149, GM08333, and GM08429). As previously reported for other Rb patient fibroblast strains, the degree of PLD repair recovery, measured as the ratio of the initial slopes of the asynchronous culture and the confluent culture plus PLD repair survival curves (D_0 and ${}_1D_0$, respectively), was reduced (although not significantly, $P = 0.074$) in the bilaterally affected $RbI^{+/-}$ individuals (~ 2.1) compared to their unaffected first-degree relatives (~ 2.6) in these five Rb families.

In Chapter 4, G2 chromosomal radiosensitivity assay data for asynchronous cultures of fibroblast strains derived from the 14 Rb family members, two radiosensitive apparently normal CIMR controls (GM04505A and GM08447), two radioresistant

apparently normal CIMR controls (AG01521 and GM02149), and a radioresistant *RbI*^{+/-} CIMR control (GM06419) were presented. The 14 Rb family member and two radiosensitive CIMR control fibroblast strains had significantly higher (~1.5-fold, $P < 0.05$) chromatid-type aberration frequencies (CAF, chromatid gaps and breaks) in the first post-irradiation mitosis after 50 and 100 cGy doses of G2-phase HDR ¹³⁷Cs γ -irradiation, compared to the three other radioresistant CIMR control strains. The average radiation-induced chromatid break and combined chromatid gap and break frequencies $\pm$ SD for the three radioresistant CIMR strains was 3.15 ± 0.34 (range 2.77–3.43) and 7.39 ± 0.64 (range 6.95–8.12) aberrations per cell, respectively. The average radiation-induced chromatid break and combined chromatid gap and break frequencies $\pm$ SD for the two radiosensitive CIMR strains was 4.88 ± 0.77 (range 4.34–5.43) and 10.11 ± 1.20 (range 9.26–10.96) aberrations per cell, respectively. The average radiation-induced chromatid break and combined chromatid gap and break frequencies $\pm$ SD for the 14 Rb family members strains was 4.45 ± 0.59 (range 3.34–5.33) and 9.47 ± 0.91 (range 7.74–10.83) aberrations per cell, respectively (interestingly, CAF values were slightly higher in the *unaffected* Rb family member strains). Significantly high spontaneous levels of tetraploidy (8–17%) and endoreduplication (2–3%) were observed in some of the Rb family member fibroblast strains but were not observed in the apparently normal CIMR fibroblast strains, and were not correlated with the frequencies of radiation-induced chromosome aberrations.

In Chapter 5, measurements of the continuous LDR (0.5–8.4 cGy/h) colony formation ability of asynchronous cultures of fibroblast strains derived from the 14 Rb family members and 14 age- and sex-matched apparently normal and *RbI*^{+/-} CIMR

controls were presented. Fibroblasts derived from the 14 Rb family members and from 7 of the 14 (50%) apparently normal CIMR controls were significantly more radiosensitive (1.5–2.3-fold, $P < 0.05$) than the remaining radioresistant CIMR controls, using the endpoint of dose rates required to reduce relative survival to 10% and 1%. The average dose rates required to reduce relative survival to 10% and 1% were ~2.6 and 4.6 cGy/h, respectively, for the 7 radioresistant CIMR fibroblast strains; ~1.7 and 3.1 cGy/h, respectively, for the 10 moderately radiosensitive Rb family member and apparently normal CIMR strains; and, ~1.1 and 2.0 cGy/h, respectively for the 11 hyper-radiosensitive Rb family member and apparently normal CIMR strains. These continuous LDR irradiation survival dose rates were highly and significantly correlated ($P < 0.0001$) with the measured plating efficiency (PE) values and the frequencies of radiation-induced chromatid aberrations (breaks and gaps) measured in the G2 chromosomal radiosensitivity assay in Chapter 4.

The continuous LDR irradiation studies described in Chapter 5 were successful in achieving an appreciably larger survival differential of the moderate to hyper-radiosensitive fibroblast strains examined (both Rb family member and some apparently normal CIMR strains) than the acute irradiation survival protocols employed in Fitzek, *et al.* (2002) and Chapter 3. Of the 14 unrelated apparently normal and *Rb1*^{+/-} CIMR strains examined, 4 of them (29%) could be considered hyper-radiosensitive (GM00041, GM04503, GM04505, and GM08447); 3 of them (21%) could be considered moderately radiosensitive (GM00969, GM03440, and GM05756); and the remaining 50% considered typical normal response in terms of the dose rates required to reduce relative survival to 1% (including the radioresistant *Rb1*^{+/-} strain GM06419). By the same criteria, 7 of the

14 (50%) Rb family member fibroblast strains are considered moderately radiosensitive, and the remaining half considered hyper-radiosensitive. These results again illustrate the need for choosing validated apparently normal fibroblast strains in such studies.

For each radiobiological assay performed in Chapters 3–5, the Rb family member fibroblast strains were ranked from least (1) to most (14) radiosensitive for the relevant parameter measured in these experiments (survival and chromosome aberration induction, see Tables 3.1, 4.4, and 5.2). These rankings have been combined into Table 7.1 to compare the degree of relative radiosensitivity for each Rb family member fibroblast strain in each of these assays. These strains have also ranked for their acute irradiation survival D_0 values reported by Fitzek, *et al.* (2002) and Table 1.1. Generally speaking, the same patterns of radiosensitivity are evident among the 14 Rb family member fibroblast strains, although deviations in these trends can be seen for some of the strains and (cell cycle-phase specific) assays. Specific correlations between each of these radiobiological parameters can be found in the final figures of the Results sections of Chapters 3–5. As mentioned above, the most significant correlations were seen between the frequencies of radiation-induced chromatid-type aberrations in the G2 chromosomal radiosensitivity assay (Chapter 4) and the dose rates required to reduce relative survival to 10% and 1% in the continuous LDR colony formation assay (Chapter 5, see Figure 5.23). Clearly, *Rb1* haplotype was not significantly correlated with the degree of radiosensitivity measured in *any* of the assays described in this dissertation.

Finally in Chapter 6, several continuous LDR colony formation experiments were performed with a series of radiosensitive *atm* homozygote and heterozygote mouse embryo fibroblast (MEF) strains and a radioresistant *tp53*^{-/-} MEF cell line to demonstrate

Table 7.1. Summary of radiosensitivity parameter rankings for the 14 Rb family member fibroblast strains reported in Fitzek, *et al.*, 2002 and Chapters 3–5 of this volume. Strains are ranked from least (1) to most (14) radiosensitive for each particular assay.

Fibroblast Strain	Fitzek, <i>et al.</i> (2002) Acute Survival D ₀ Rank ^a	<i>Wilson Measured Parameters</i>		
		Acute Survival + PLDR D ₀ Rank ^b	G2 Chromosomal Radiosensitivity Assay Rank ^c	Continuous LDR Colony Formation Rank ^d
MF-2M Rb Family I	4	1	11	7
MF-3R Rb Family I	6/7	4	5	6
MF-4F Rb Family I	9	3	2	1
MF-6F Rb Family II	8	10	9	14
MF-7R Rb Family II	2	5	3	5
MF-10R Rb Family III	3	11	10	4
MF-11F Rb Family III	5	6	13	3
MF-12M Rb Family III	6/7	2	14	10
MF-13M Rb Family IV	11	14	12	11
MF-14R Rb Family IV	1	7	1	2
MF-15F Rb Family IV	10	8	7	8
MF-16M Rb Family V	14	13	4	9
MF-17R Rb Family V	13	12	6	12
MF-18F Rb Family V	12	9	8	13

^a See Table 1.1.

^b See Table 3.1.

^c See Table 4.4.

^d See Table 5.2.

the probable limits of mutant rodent cell survival responses in this LDR assay. The assay clearly delineated A-T heterozygote strains (10% relative survival at 4–5.5 cGy/h) as intermediate in response to A-T homozygote (10% relative survival at <3 cGy/h) and wild-type control strains (10% relative survival at ~13–14 cGy/h), illustrating its potential in identifying a significant degree of differential LDR radiosensitivity in human A-T heterozygote cells. Continuous LDR irradiation (but not acute HDR irradiation) stimulated a previously observed phenotype of giant, aneuploid cell formation and massive chromosomal aberrations in the *tp53*^{-/-} MEF cell line, which was confirmed to be radioresistant in acute and continuous LDR (10% relative survival at 15 cGy/h) survival measurements. Detailed cytogenetic and immunocytochemical analyses of unirradiated interphase preparations of these *tp53*^{-/-} giant cells demonstrated highly aberrant centrosome morphology accompanied by fragmented micronuclei and multi-lobed macronuclei with large numbers of γ -H2AX foci (a marker of DNA double-strand breaks). Unirradiated metaphase chromosome preparations of these giant cells showed massively rearranged chromosomes (non-reciprocal translocations, complex structural aberrations, and fragmented chromosome material) by multiplex fluorescence *in situ* hybridization (mFISH) analysis, and a novel phenotype of telomere deregulation revealed by telomere-specific FISH involving large numbers of telomeric double minutes and extra-chromosomal telomeric repeats (TDMs/ECTRs). Neither of these spontaneous genomic instability phenotypes (the latter of which I have termed “telomere fission”) have previously been reported for this *tp53*^{-/-} MEF cell line.

7.2. The Benefits of the Continuous LDR Irradiation Colony Formation Assay

While “traditional” *in vitro* low dose-rate exposures are completed with (mostly) density-inhibited G0/G1-phase cultures to reduce intra-experimental variability caused by exposing cells in more radiosensitive phases of the cell cycle (G2 and mitosis), the continuous LDR irradiation exposure strategy *embraces* the potential to record the effects of LDR radiation during all the phases of the cell cycle, each with its distinct regulatory components in terms of DNA damage response, signaling and checkpoint induction. As discussed in Chapter 2, mutations of a number of genes in these various cellular pathways may result in moderate to hyper-radiosensitivity and may contribute to cancer predisposition in an affected individual. Cell cycle phase-dependent responses have been shown to lead to variable rates of radiation-induced mutagenesis and cell killing in the different cell cycle phases (Burki, 1980; Jostes, *et al.*, 1980; Chuang and Liber, 1996). This factor, coupled with the stochastic nature of dose (single electron track) delivery during the long continuous LDR radiation exposures, registers as the inherent clonal variability seen in the surviving colonies observed in these LDR growth experiments (*e.g.*, see Figure 5.17, panel B). The ability to discern LDR radiosensitivity generated from mutations that may affect critical biological processes in any of the cell cycle phases, rather than just the G0 and G1 phases, makes this assay more attractive and potentially more informative for broader-scale genetic complementation and correction testing of radiosensitive cell strains.

Another major benefit of this continuous LDR irradiation strategy is the potential for a greater survival differential of *moderately* radiosensitive cell strains than can be achieved with the traditional acute (HDR) or LDR exposures of density-inhibited

quiescent cultures. Using the data from Nagasawa *et al.* (1992) as a typical example, exposures of density-inhibited, confluent human fibroblast cultures to low dose-rate (2.3 cGy/h or 15.3 cGy/h) ^{137}Cs γ -rays resulted in dose-rate modification factors (DRMF) of 4–7 compared to acute, high dose-rate exposures. However, the magnitude of this effect was similar for the apparently normal control ($n=6$) and moderately radiosensitive bilateral Rb CIMR fibroblast strains examined (AG01880 and GM01142). The largest degree of differential survival between all these strains at high and low dose rates, calculated as the ratio of D_0 values for AG01522 (the most radioresistant normal cell strain examined) and AG01880 (the most radiosensitive Rb cell strain examined), was about the same for the LDR exposures (7.3 Gy/5.4 Gy = 1.35) and the HDR exposures (1.2 Gy/0.85 Gy = 1.41). Alternatively, by examining the relative surviving fractions for each cell strain after a dose of 2 Gy (SF_2), the degree of differential survival for the high dose-rate exposures is 2 ($\text{SF}_{2\text{AG01522}}/\text{SF}_{2\text{AG01880}} = 0.19/0.095$) and even lower for the low dose-rate exposures, with a value ~ 1.2 ($\text{SF}_{2\text{AG01522}}/\text{SF}_{2\text{AG01880}} = 0.8/0.65$).

This concept is shown schematically in Figure 7.1 using the data from Nagasawa, *et al.* (1992; top panel) and the data for the continuous LDR irradiation colony formation experiments summarized by Figure 5.17 (bottom panel). In the top panel of Figure 7.1, the survival differential between the most radioresistant apparently normal CIMR strain, AG01522 (solid line), and the radiosensitive bilateral Rb CIMR strain, AG01880 (dashed line), following acute irradiation doses in the range required to reduce relative survival to $\sim 10\%$ for AG01522 [$\sim 200\text{--}300$ cGy] is indicated by the large black arrows and is only $\sim 2\text{--}3$ -fold. Similarly, the large black arrows in the bottom panel of Figure 7.1 illustrate the survival differential between the 7 radioresistant apparently normal CIMR fibroblast

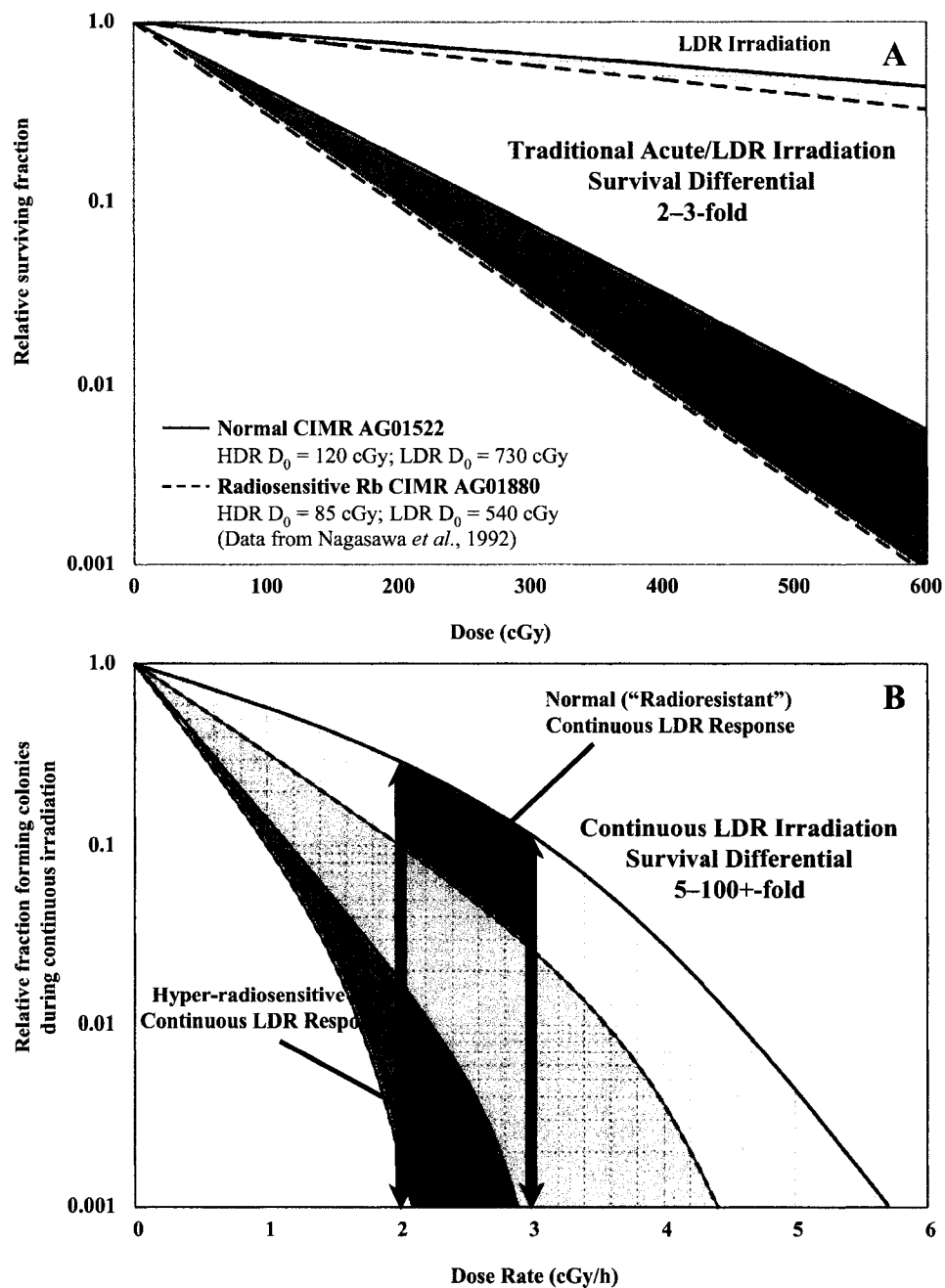


Figure 7.1. Radiation survival differentials between traditional acute/LDR irradiation schemes (**A**) and the continuous LDR irradiation scheme (**B**). Both traditional irradiation schemes in **A** involve exposures of contact-inhibited G0/G1-phase fibroblast cultures and are incapable of producing the much larger survival differential observed in **B** (large black arrows) between asynchronous cultures of normal and radiosensitive Rb fibroblast strains.

strains (green-shaded area) and the 11 hyper-radiosensitive apparently normal CIMR and Rb family member fibroblast strains (red-shaded area) at the dose rates required to reduce relative survival to ~10% for the radioresistant CIMR strains (~2–3 cGy/h). The degree of differential survival in the continuous LDR irradiation colony formation assay is at minimum 5-fold and may approach 100-fold or greater for the more radiosensitive fibroblast strains compared to the radioresistant normal strains.

From Figure 7.1, the traditional use of high or low dose-rate exposures in density-inhibited fibroblast cultures would not generate a sufficient degree of differential survival required for genetic screening purposes. Attempts to screen moderately radiosensitive cell strains transfected with a genome-wide cDNA library using these traditional irradiation protocols would result in a large number of false-positive “corrected” clones, simply because uncorrected colonies (with irrelevant or without any cDNA inserts) would survive at a ~50% frequency of that observed for radioresistant cells (truly apparently normal cells and true-positive cDNA-corrected clones). The potential for a very high degree of differential survival and the ability to recover a significant fraction (5–30+%) of cells of normal radioresistance during continuous LDR exposures compared to radiosensitive strains (relative survival of <0.1–1%), on the other hand, provides a much more powerful system to attempt correction of the hyper-radiosensitivity phenotype by complementation testing with a cDNA library or other candidate sequences (see Section 7.3). The similar degree of hyper-radiosensitivity observed in some of the CIMR control strains implies that this strategy could be extended to “apparently normal” individuals from families not typically associated with familial cancer predisposition.

The use of randomly mutagenized human cell strains (generated by alkylating agent treatment, *etc.*), similar to the technique of Wells and Bedford (1988) and Stackhouse and Bedford (1993), may provide a rich source of genetic variation for future LDR genetic screening and selection studies. This technique is currently being employed by the Ullrich laboratory at CSU to identify EMS-mutagenized mouse embryonic stem (ES) cells hypersensitive to continuous low dose-rate ^{137}Cs irradiation in the MRB012 facility (Dr. Robert Ullrich, personal communication). The original report by Stackhouse and Bedford summarizes the rationale for this randomized mutagenesis/continuous low dose-rate irradiation selection strategy best: “It is unlikely that sufficient numbers of different naturally occurring mutations can be isolated from the human population to provide a complete genetic analysis of the many repair pathways and other systems that regulate cellular radiation sensitivity since many of these defects may not be compatible with the successful development of a fetus.”

7.3. Use of Continuous LDR Irradiation for Genetic Screening Purposes: Potential Insights into the Retinoblastoma Families

The large degree of differential survival between the radioresistant apparently normal CIMR controls and the hyper-radiosensitive Rb family member fibroblast strains in the continuous low dose-rate colony formation assay depicted in Figure 7.1 was judged sufficient for genetic correction and complementation purposes. A schematic for a viral cDNA library transfection strategy based on the Stratagene ViraPort™ pFB cDNA library system and transfectant screening by colony formation during continuous LDR irradiation is shown in Figure 7.2, panel A. This LDR survival selection strategy has all

Figure 7.2. Schematic of the continuous LDR irradiation selection-based genetic complementation/correction strategy for radiosensitive human fibroblast strains.

A. Cultures of radiosensitive fibroblasts are transfected with a viral human cDNA library pool (inserted into the MMLV-based pFB vector, Stratagene), seeded as single cells, and subjected to 2–3 cGy/h for a two week growth period. Corrected (radioresistant) clones (circled in red) are retrieved and expanded in cell culture without irradiation. Individual cells from these colonies are re-seeded, and again subjected to 3 cGy/h (only truly corrected clones will now obey a normal LDR response). Correcting cDNAs in these clones may be recovered with PCR primers flanking the pFB insert, sequenced and identified by BLAST search. Using full-length cDNA constructs in low-passage radiosensitive strains, genetic complementation can be verified by other radiosensitivity assays (*e.g.*, the G2 assay) or alternatively, by siRNA/shRNA knockdown of the gene in normal cell strains.

B. Both pFB-Luc (total activity) and pFB-hrGFP (percent transfected) were used to establish the viral multiplicity-of-infection (MOI) using control NIH3T3 mouse cells and Rb family member fibroblast strains MF-2M and MF-18F (judged promising candidates for the genetic complementation strategy outlined in A). Using the pFB insert-flanking PCR primers, the *hrGFP* sequence was successfully PCR amplified for decreasing dilutions of the viral titer, yielding a properly-sized 830-bp PCR product on a 3% NuSieve 3:1 agarose gel (Cambrex). The frequency of hrGFP-positive cells (cells with green cytoplasm in the two right panels) was accomplished by immunofluorescent detection of live cultures co-stained with the nuclear dye Hoechst 33258 (Sigma, blue nuclei). Total luciferase protein activity in pFB-Luc-transfected NIH3T3, MF-2M, and MF-18F cultures was measured using a Turner Biosystems model TD-20/20 luminometer (courtesy of Dr. Michael Weil, data not shown).

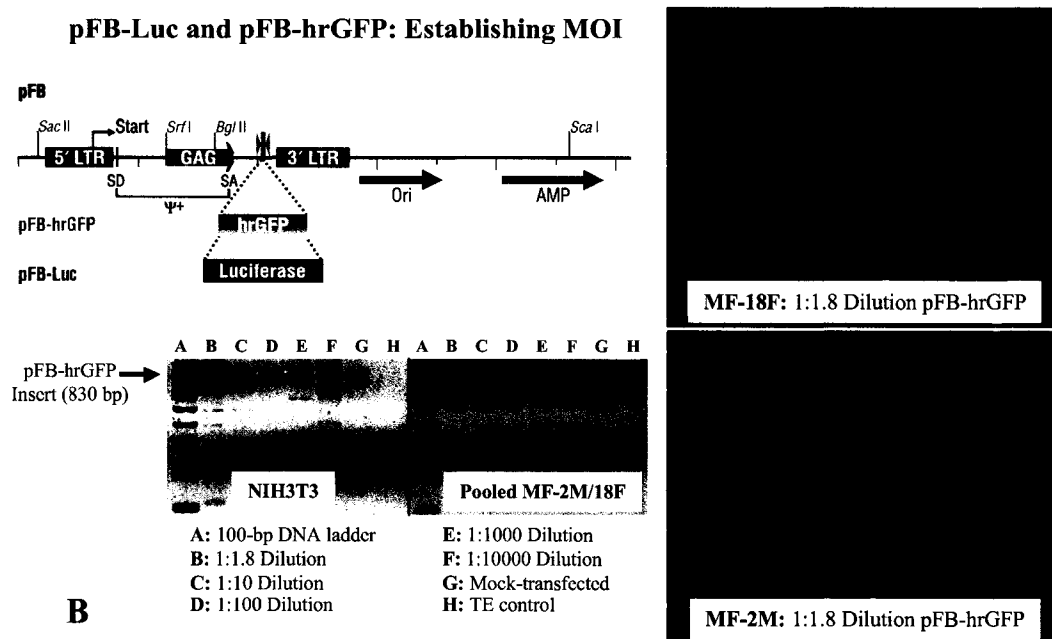
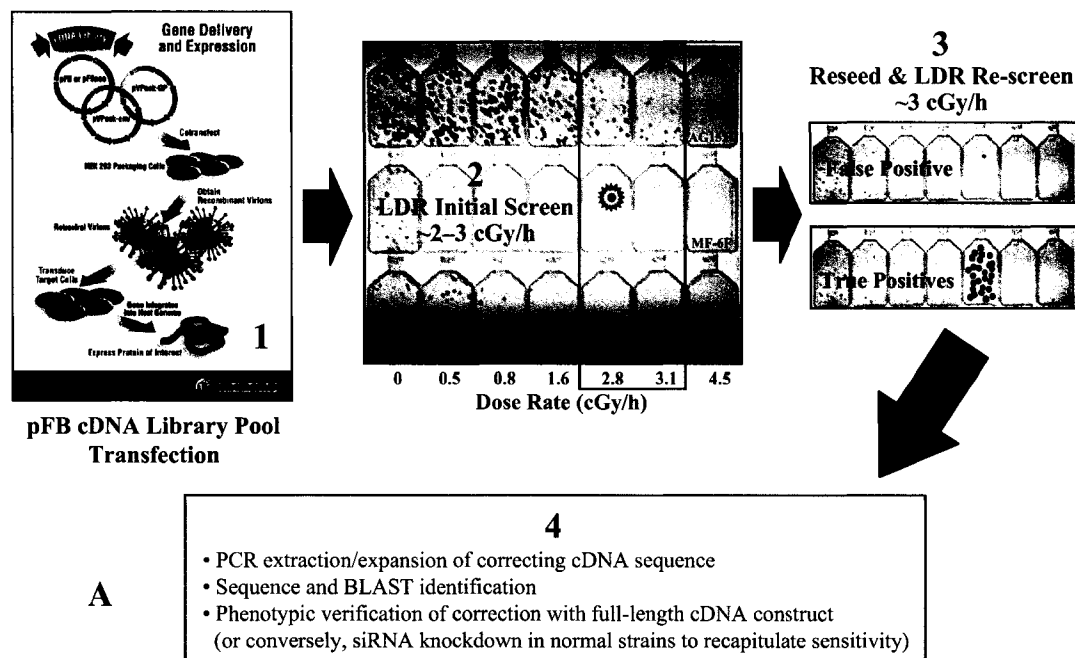


Figure 7.2.

requisite elements for a successful mammalian cell genetic complementation screen (Grimm, 2004), taking precedence from the successful isolation of the *prkdc*-deficient CHO 10B2 mutant *irs*-20 in this laboratory (Stackhouse and Bedford, 1993). A similar approach was applied in the initial isolation and characterization of the first cloned mammalian radiosensitivity gene, *XRCC1*, from human cosmid and pcD2 plasmid-based cDNA libraries transfected into EMS-sensitive and radiosensitive CHO EM9 clones (Thompson, *et al.*, 1990). Transfection of the human *XRCC1* gene from these restored the levels of cell killing and sister chromatid exchange and the kinetics of DNA DSB repair in complemented clones to that of their wild-type CHO AA8 counterparts.

The original cDNA expression library strategy for this project was to be based on a plasmid vector, the Clontech (<http://www.clontech.com>) pEXP1 cytomegalovirus (CMV) promoter-driven construct, containing puromycin and ampicillin genes as selectable expression markers. Based on literature I read from Clontech and Stratagene (<http://www.stratagene.com>), I suggested we try the Stratagene pFB retroviral-based expression vector as an alternative cDNA library delivery vehicle, primarily based on reports of the very high transfection efficiencies (approaching 100% in the manufacturer's claims) that surpassed the transfection efficiencies routinely achieved in this laboratory with lipofectamine-based plasmid transfection protocols (*e.g.*, Peng, *et al.*, 2002; 2005). Also, the Stratagene pFB XR vector allows larger cDNA inserts up to 8 kilobases (kb), while the *pur* and *amp* selectable markers limited the Clontech pEXP1 cDNA inserts to 6 kb. Both vectors had similar average insert sizes of 1.3–1.5 kb, but I hoped the longer maximum insert size of the pFB vector may give a slightly better chance of recovering a full-length cDNA insert from a restored, radioresistant clone.

Per the Stratagene literature, ViraPort™ pFB plasmid libraries containing cDNA inserts reverse-transcribed from human lung mRNA sequences pooled from five unrelated, apparently normal Caucasian male and female individuals (ages 15–40) were packaged in HEK (human embryonic kidney) 293 cells by co-transfection with pVPack-GP (*gag*, *pol*) and pVPack-VSV-G (*env*) CMV-based viral vectors for two days. The ViraPort™ retroviral cDNA library system discussed below is available as transduction-ready retroviral supernatants collected from these HEK 293 cultures. The Stratagene pFB XR Moloney murine leukemia virus (MMLV)-based retroviral vector is designed to be deficient for viral replication and packaging by the lack of the retrovirus *gag*, *env* and *pol* genes. The multiple cloning site (MCS) of the pFB vector is located between two viral long terminal repeats (LTRs) containing the retroviral promoter/enhancer, polyadenylation, and reverse-transcription sequences for enhanced cDNA expression. Target cell division is required for infection, as the retroviral particles integrate into the genome during nuclear envelope breakdown in mitosis.

In Figure 7.2, panel B, multiplicity-of-infection (MOI) experiments with the Stratagene control pFB retroviral vectors containing the luciferase (*Luc*) and humanized recombinant green fluorescent protein (*hrGFP*) genes transfected into asynchronous cultures of Rb family member fibroblast strains MF-2M and MF-18F and reference control NIH3T3 mouse cells (grown in six-well plates) are presented. The pFB-Luc vector was used to establish the total activity of expressed luciferase protein in the infected cultures for various increasing dilutions of the viral supernatant. The pFB-hrGFP vector was used to determine the transfection efficiency of the virus, for the same dilutions of the viral supernatant. Both experiments were performed according to

protocols supplied by Stratagene, which recommended NIH3T3 cells as a positive transfection control. Infection with a 1:10,000 dilution of the viral titer should result in a ~5–10-fold increase in protein expression over background levels in the NIH3T3 cells. Cells transfected with pFB-Luc were assayed with the Stratagene Luciferase Assay Kit using a Turner Biosystems model TD-20/20 luminometer, courtesy of Dr. Michael Weil, CSU (data not shown), according to the manufacturer's directions. Cells transfected with pFB-hrGFP were stained with the vital nuclear dye Hoechst 33258 (Molecular Probes/Invitrogen) in PBS and imaged live in culture with a Zeiss Axiovert inverted fluorescence microscope. Examples of MF-2M and MF-18F Rb family member fibroblast cultures, infected at a 1:1.8 pFB-hrGFP viral supernatant dilution, are shown in panel B of Figure 7.2.

After imaging, cell cultures infected with pFB-hrGFP were collected from the six-well plates, and genomic DNA was extracted using a QIAGEN DNeasy tissue kit (QIAGEN, Inc.). The DNA concentrations of these samples were accurately measured using a NanoDrop[®] ND-1000 UV spectrophotometer (NanoDrop, Inc.). Using a Stratagene TaqPlus[®] Precision PCR kit according to the supplied protocol, the hrGFP insert within the pFB-hrGFP MCS was successfully PCR amplified using MCS insert-flanking PCR primers (3'-pFB and 5'-Retro; concentrations were also accurately measured using the NanoDrop spectrophotometer) for samples of genomic DNA isolated from infected control NIH3T3 cells and pooled from both the MF-2M and MF-18F cells (who were infected equally well by pFB-Luc and pFB-hrGFP). The PCR products and a 100-base pair (bp) DNA ladder (Bio-Rad Laboratories) were then run on a 3% NuSieve 3:1 high-resolution agarose gel (Cambrex) in 1X TAE buffer at 60V for 2 hours. The

gels were stained with ethidium bromide and imaged with a UV gel camera system (Kodak, Inc.); these results are presented in panel B of Figure 7.2 for the increasing dilutions of viral supernatant. PCR amplification of the pFB-hrGFP *hrGFP* insert yielded an expected 830-bp PCR product, verifying the ability to recover the random cDNA inserts from the pFB vector cDNA library pool.

The pFB-Luc and pFB-hrGFP control virus data supported the Stratagene claim that a 1:10,000 dilution of the viral supernatant would result in a ~5–10-fold increase in detectable protein expression in the NIH3T3 cells. A multiplicity-of-infection (MOI) of 0.55 virions/cell gave an ~300-fold increase in luciferase expression over background and ~37% hrGFP-positive cells in the MF-2M and MF-18F fibroblast cultures. From this control virus MOI data, using a MOI of 1.0–2.0 should result in a transfection efficiency approaching 100%, with ~26–60% of transfected cells carrying ≥ 2 cDNA library inserts, respectively (calculated by Poisson statistics). The effects of changing the MOI from 0.5–2.0 on the number of cDNA library inserts and the fraction of transfected clones carrying this number of inserts is plotted in Figure 7.3. Although a MOI of 1.0 implies 37% of transfected clones will not properly carry or express the cDNA library insert, the 37% of transfected clones carrying only one insert (green circle) and only 26% of transfected clones carrying ≥ 2 inserts implies this is the optimal MOI for future pFB cDNA library transfection experiments performed with the hyper-radiosensitive Rb family member fibroblast strains.

Following transfection of the ViraPort pFB cDNA library viral supernatants ($\sim 3 \times 10^6$ virions) into approximately the same number of cells, cultures of transfected radiosensitive human fibroblast strains (the Rb family parent strains in this case) would

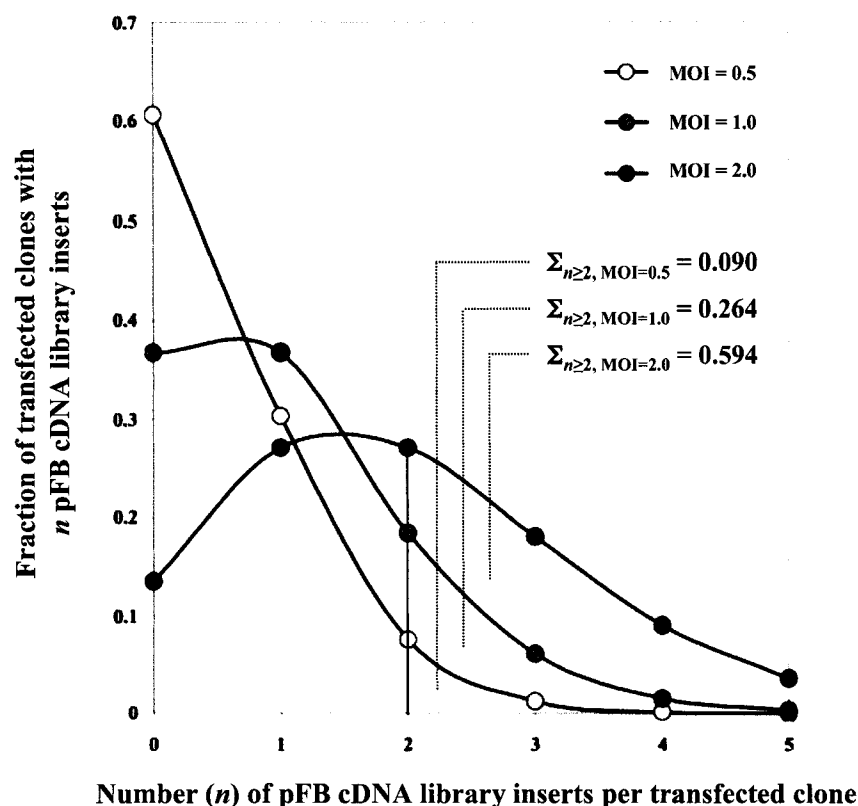


Figure 7.3. Plot illustrating the effects of changing the virus multiplicity-of-infection (MOI) with 0.5 (open circles), 1 (grey circles), and 2 (black circles) virions/cell on the number of Stratagene pFB cDNA library inserts per transfected clone and the fraction of transfected clones carrying this number of cDNA library inserts (including those carrying ≥ 2 inserts) following viral transfection into cultures of radiosensitive human fibroblast strains in the continuous LDR irradiation selection-based genetic complementation/correction strategy outlined in Figure 7.2. The optimal MOI is 1.0, giving the highest percentage of clones carrying one cDNA insert (0.37, green circle) and only ~27% of clones with ≥ 2 cDNA inserts.

be seeded as single cells and subjected to a selection dose rate of 2–3 cGy/h for a two week growth period in the MRB008 or MRB012 LDR facilities, as discussed in Chapter 5. Hopefully, corrected clones of normal radioresistance would successfully grow at this restrictive growth rate, as depicted in panel A of Figure 7.2. These clones would be retrieved from the culture dish, and further expanded in cell culture without irradiation. The majority of these cells would be saved for further analyses, but a small subset would again be seeded as single cells and grown for two weeks at 2–3 cGy/h. Cells expanded from true positive-corrected clones would now obey a LDR survival response of the apparently normal radioresistant CIMR strains at these dose rates (relative survival of 5–30+%) and many colonies should be evident in these flasks. Cells expanded from false positive-corrected clones that survived the initial irradiation period by chance would not be able to form many colonies in the second round of LDR irradiation exposure.

PCR products recovered from the corrected, radioresistant clones following LDR growth selection would be sequenced and the cDNA inserts identified by Internet BLAST searches of human genomic DNA sequence databases. Full-length cDNA constructs of candidate genes could then be integrated into a larger vector (YAC) and transfected into low-passage Rb family member fibroblast cultures. The radiosensitivity of these clones could be tested using the assays described in Fitzek, *et al.* (2002) and Chapters 3–5 of this dissertation for which baseline data for these uncorrected strains are available. Alternatively, short interfering RNA (siRNA) or more stable short hairpin RNA (shRNA) transfections into radioresistant apparently normal fibroblast strains may cause an increase in radiosensitivity, similar to the levels observed for the Rb family member fibroblast strains in these studies (see Peng, *et al.*, 2002, 2005).

Recently, another arm of the Rb family radiosensitivity research study examined differences in gene expression profiles of the Rb family member and apparently normal CIMR control fibroblast strains by RNA microarray analysis, with and without acute HDR irradiation (Chuang, *et al.*, 2006). Significant differences in the relative expression (many-fold up- and down-regulation) of 42 genes were observed, many of which have been previously associated with deregulation in tumor cells. A candidate gene identified by the viral cDNA library/LDR selection strategy discussed above that also appears in the Chuang, *et al.* (2006) list of 42 genes identified by microarray analysis would obviously be considered a strong candidate gene responsible for the radiosensitivity phenotype of (some of) the Rb family member fibroblast strains. However, the possibility certainly exists that the mutated gene responsible for their radiosensitivity phenotype could be expressed at equal levels to its wild-type counterpart in normal cells. It may be that only the expression of genes *downstream* in pathways mediated by the mutant protein may be deregulated and identified by microarray analysis, while the responsible mutant gene evades detection. The benefit of the viral cDNA LDR selection approach is that I believe you are more likely to identify the “true culprit” genetic mutation by introduction of its wild-type counterpart in the pFB cDNA library. It is recognized that certain mutations (*e.g.*, dominant-negative mutations) may not be successfully corrected with introduction of the wild-type gene, and corrected clones would not be recovered using this type of assay as well.

The microarray experiments with the Rb family member fibroblast strains discussed in Chuang, *et al.* (2006) identified a number of interesting cancer-related genes that were significantly up- or down-regulated in the Rb family member fibroblast strains

compared to the apparently normal CIMR controls (the same age- and sex-matched strains I identified in Chapter 5 for the LDR studies). Proteins encoded by some of these genes are involved with a number of critical cellular functions including chromatin, cytoskeletal and extra-cellular matrix structure and maintenance, cell cycle control, DNA replication, signal transduction, and proper centrosome/mitotic spindle function. The main authors thought it was “puzzling and counter-intuitive” that some of these genes associated with cellular proliferation and cancer development were down regulated. Specifically, E2F-1 was shown to be significantly down-regulated, which was unexpected because *Rb1*^{-/-} mutant cells do not express pRb, E2F-1’s primary negative activity and expression regulator, and E2F-1 expression levels in *Rb1*-deficient cells would be expected to increase.

I would like to offer a different explanation why the down-regulation of E2F-1 in the Rb family member fibroblast strains may be a critical finding in this study by discussing evidence of the direct roles E2F-1 may play in DNA repair, in addition to its well-established role in the initiation of DNA replication in S-phase (Stevens and La Thangue, 2004; Chapter 2). E2F-1 protein expression levels increase in cells exposed to UV- or γ -irradiation with kinetics similar to the induction of Tp53, while expression levels of the other E2F family members do not change (Blattner, *et al.*, 1999; Hofferer, *et al.*, 1999). Similarly, E2F-1 is subject to phosphorylation of serine 31 by ATM and ATR and serine 364 by Chk2, and acetylation of lysines 117, 120, and 125 by the p300/CBP acetyltransferases in response to DNA damage and may directly participate alongside pRb in halting cell cycle progression and initiating subsequent DNA repair activities or

apoptosis (Marzio, *et al.*, 2000; Lin, *et al.*, 2001; Stevens, *et al.*, 2003; Galbiati, *et al.*, 2005).

The negative regulator of Tp53, Mdm2, also binds E2F-1 at a similar binding motif (nearby the S364 Chk2 phosphorylation site) and likewise targets E2F-1 for ubiquitin-mediated proteasome degradation (Martin, *et al.*, 1995; Loughran and La Thangue, 2000). Upon DNA damage-induced phosphorylation by Chk2, E2F-1 forms nuclear foci (Stevens, *et al.*, 2003) along with topoisomerase-II binding protein 1 (TopBP1) and BRCA1 (Liu, *et al.*, 2003). The MRN complex has likewise been shown to interact with E2F-1 (through N-terminal interactions of Nbs1) at sites of DNA damage and at origins of DNA replication in S-phase (Maser, *et al.*, 2001). E2F-1 also regulates the expression of ATM, BRCA1, Chk1, Msh2 and Msh6, and PCNA, proteins directly involved in the DNA damage response (Polager, *et al.*, 2002; Ren, *et al.*, 2002). Thus, the down-regulation of E2F-1 may result in a global deactivation of critical DNA DSB response and repair pathways, and provides a *very* attractive candidate gene responsible for the radiosensitivity phenotypes of the Rb family members. With lower E2F-1 expression levels in these cells, the selection for wild-type functionality of the pRb protein may be relaxed and a higher incidence of *Rb1* mutations may result.

7.4. The Effectiveness of Single Electron Tracks: Chromosome Aberration Induction, Cell Survival, and Oncogenic Potential

Assuming a single electron track is alone capable of generating a DNA double-strand break (DSB) and 1 Gy of low-LET ionizing radiation generates approximately 35–40 DSBs (Ward, 1998; Rothkamm and Löbrich, 2003), it is interesting to relate the rate

of generation of these critical lesions at the dose-rates used in these studies to the overall proliferative capacity of cells growing under continuous LDR ionizing radiation exposure. The following “thought experiment” is especially pertinent to the results generated in this dissertation and the growing body of evidence related to the failure of the activation of mammalian DSB repair systems at low radiation doses and dose rates (see Section 2.4.4).

Essential to the following discussion is the size of the biological target being exposed to radiation (Hall, 2000), in this case the nucleus of a human fibroblast growing in a culture flask. Both the exposed nuclear surface area and the nuclear volume traversed by the secondary electrons liberated from γ -ray interactions (photoelectric effect and Compton scattering of the 662-keV ^{137}Cs γ -ray photons) are the critical determinants of the amount of energy and dose deposited by the incident radiation. To address this need, I conducted a series of experiments to accurately measure the nuclear dimensions of live and methanol-fixed normal human fibroblasts (apparently normal CIMR strain AG01521) grown in culture by epi-fluorescent and confocal laser-scanning microscopy (LSM).

Previous work by Cornforth, *et al.* (1989) measured the three spatial dimensions of AG01521 fibroblast nuclei for similar microdosimetry purposes (in this case, ultrasoft X-ray exposures). However, there is one major difference between the current study and the Cornforth study, *i.e.*, the number of cellular fixation (ethanol/methanol, acetone paraformaldehyde, glutaraldehyde, osmium tetroxide, uranyl acetate) and embedding (resin or metal vapor-coating) steps required to process the samples for scanning or transmission electron microscopy (SEM or TEM) in the latter report. It is well accepted

in the field of microscopy that the steps of fixation required for preparing biological specimens for electron microscopy results in significant dehydration and shrinkage of these specimens (Lee, *et al.*, 1980; Kozubek, *et al.*, 2000) on the order of 20–30%, more so than the 5–10% suggested in Cornforth, *et al.* (1989). Thus, morphometric data obtained from the Cornforth report may not be representative of the actual dimensions of *live* contact-inhibited G0/G1-phase AG01521 human fibroblast nuclei.

The results of my measurements of the nuclear dimensions of live and methanol-only fixed contact-inhibited and serum-starved (0.1% FBS) G0/G1-phase AG01521 human fibroblasts grown on microscope chamber slides are shown in Figures 7.4 and 7.5 and reported in Table 7.2. Comparison of the live culture data set ($n = 680$ cells) with the methanol-fixed culture data set ($n = 580$ cells) revealed only a 3–4% change in the average nuclear sizes measured from the methanol treatment so the results of both series of experiments were combined together. Interestingly, this change was larger for the surface area measurements (major and minor axes) and smaller for the nuclear depth measurements, apparently due to the increased attachment and spreading of the cells to the slides after fixation (Kozubek, *et al.*, 2000). The details of the nuclear morphometry protocols are discussed below in section 7.4.1.

From Table 7.2, the average major axis ($2a$) of these nuclei was $23.1 \pm 2.7 \mu\text{m}$, the average minor axis ($2b$) of these nuclei was $11.2 \pm 1.9 \mu\text{m}$, and the average nuclear depth ($2c$) was $3.5 \pm 0.3 \mu\text{m}$. The calculated average nuclear surface area (πab) and volume ($4/3\pi abc$) of an ellipsoid with these dimensions is $\sim 200 \mu\text{m}^2$ and $\sim 400 \mu\text{m}^3$, respectively. Cornforth *et al.* (1989) reports a mean nuclear surface area of $144 \pm 45 \mu\text{m}^2$ and a mean nuclear depth of $1.2 \mu\text{m}$; these data equate to a volume of $4/3\pi abc = 4/3(144$

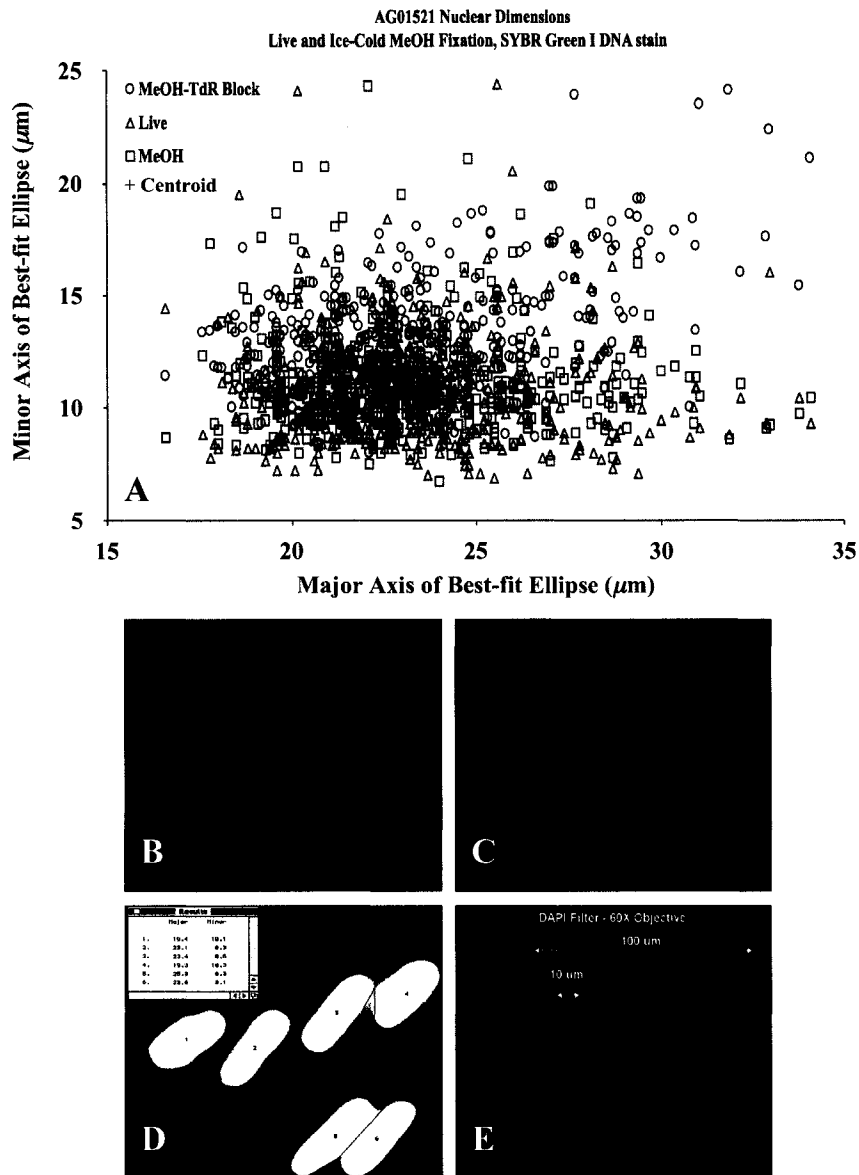


Figure 7.4. Distribution of ellipse major and minor axes (in μm , panel A) for confluent G0/G1-phase AG01521 human fibroblast nuclei (live and methanol-fixed) stained with SYBR Green-I DNA stain (Molecular Probes/Invitrogen, panel B). The NIH Image software masked (panel C) and fitted ellipses of the photographed nuclei and provided measurements of the major and minor axes (panel D, overall average indicated by the centroid in panel A) following proper spatial calibration of the microscope system and the NIH Image software. Spatial calibration with a stage micrometer (panel E, 10- μm and 100- μm etched lines shown in blue with DAPI) and the software yields calibration values of 4.40 ± 0.05 pixels/ μm for the 60X objective and 7.35 ± 0.05 pixels/ μm for the 100X objective of the Olympus AX-70 microscope.

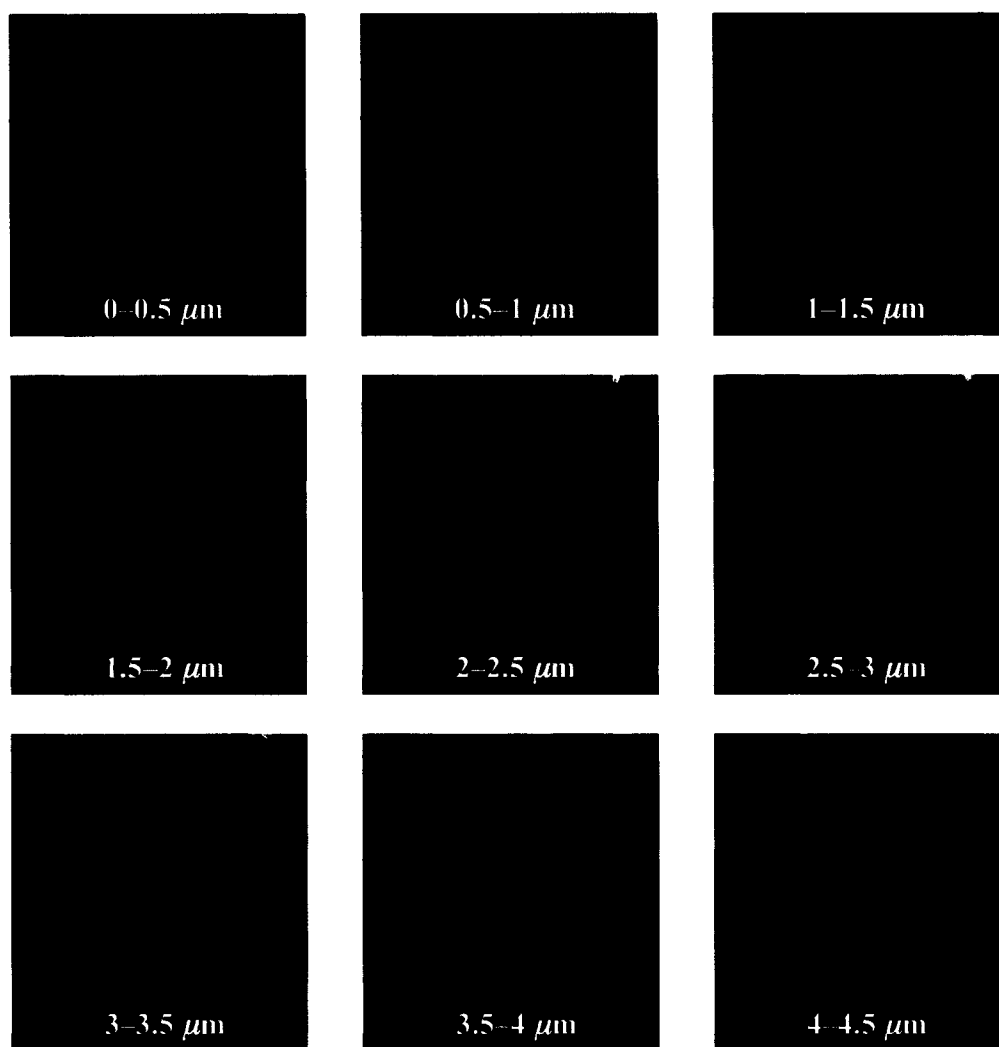


Figure 7.5. Representative z-stack image series of confluent G0/G1-phase AG01521 normal human fibroblasts imaged with a confocal laser-scanning microscope at 0.5- μm depth intervals. Cytoplasmic staining is accomplished with 10 μM Vybrant CFDA-SE (Molecular Probes, Invitrogen) and nuclear staining with 0.5 $\mu\text{g/ml}$ propidium iodide (Sigma). Multiple series of confocal XZ scans yield an average nuclear depth of $3.5 \pm 0.3 \mu\text{m}$ for confluent G0/G1-phase AG01521 normal human fibroblasts.

Table 7.2. Average dimensions of G0/G1-phase AG01521 human fibroblast nuclei

•Average major axis of best-fit ellipse ($2a$)	$23.1 \pm 2.7 \mu\text{m}$
•Average minor axis of best-fit ellipse ($2b$)	$11.2 \pm 1.9 \mu\text{m}$
•Average nuclear depth ($2c$) of fitted ellipsoid	$3.5 \pm 0.3 \mu\text{m}$
•Average nuclear surface area (πab) of fitted ellipsoid	$\sim 200 \mu\text{m}^2$
•Average nuclear volume ($4/3\pi abc$) of fitted ellipsoid	$\sim 400 \mu\text{m}^3$

$\mu\text{m}^2)(0.6 \mu\text{m}) = 115 \mu\text{m}^3$. The Cornforth measurements are ~30% smaller in surface area, equivalent to a 58% reduction in both the major and minor axes (assuming the same relative ratio of major to minor axes as my data, *i.e.*, they are the same shape), and over 65% reduction in the nuclear depth. The data presented in Table 7.2 closely agree with recent similar measurements made of the mean nuclear surface area of contact-inhibited AG01522 apparently normal CIMR fibroblasts ($235 \mu\text{m}^2$; Jakob, *et al.*, 2003), and the average volume of a spherical human lymphocyte nucleus of ~8–9 μm in diameter ($270\text{--}380 \mu\text{m}^3$) with an identical amount of DNA content (ignoring differences in chromatin organization between the two cell types; Fidorra, *et al.*, 1981).

From microdosimetric data of Goodhead (1992), 1 mGy corresponds approximately to 1 electron track per cell for human fibroblast cells of $\sim 200 \mu\text{m}^2$ surface area (Table 7.2). Thus, 1 cGy/h corresponds to 10 electron tracks per hour and assuming 35–40 DSBs/Gy, the generation of 0.4 DSBs/cell per hour (*i.e.*, 1 of every 25 electrons tracks, on average, generates a DSB per hour). Assuming ~10% of DSBs are converted to chromosome breaks, this dose rates would generate 0.04 chromosome breaks/cell per hour (1 out of every 250 electron tracks). With cell cycle times at 1 cGy/h of ~24 hours, identical to that of unirradiated controls (Figure 5.7), roughly 10 radiation-induced DSBs and 1 radiation-induced chromosome break would be generated per cell cycle, a level apparently below “the radar” of the DNA damage surveillance systems in mammalian cells (see Section 2.4.4 in Chapter 2).

Per Figure 7.1 and Chapter 5, this dose rate is sufficient to drop the relative survival of the normal, radioresistant fibroblast strains to 30–60%, and the relative survival of the hyper-radiosensitive strains to only 10–15% of unirradiated control levels.

From Figure 5.18, exposure of these strains to 1 mGy/h (an order of magnitude less) still results in a 10% drop in relative survival for the radioresistant CIMR strains AG01521 and GM02149, and a statistically significant 35–60% drop in the relative survival of the radiosensitive CIMR strains GM00041C, GM03440C, GM04503B, and GM04505A. This reduction in survival is consistent with an even lower threshold for DNA damage response in the more radiosensitive fibroblast strains.

For illustrative purposes, Figure 7.6 has been included to demonstrate the relative scale of 50 and 5 keV electron tracks compared to the dimensions of a G0/G1-phase GM06419 human fibroblast nucleus “painted” by 24-color multiplex fluorescence *in situ* hybridization (mFISH). The electron track data was provided by Dr. Vladimir Semenenko for a project examining the effectiveness of different portions (track-length versus track-end) of cell nucleus-incorporated ^3H -TdR and ^{14}C -TdR β -particle tracks in generating chromosome aberrations (see Semenenko, 2002). The track ends of low-energy electrons deposit ~50% of their entire dose in a nanometer-scale volume at the very end of their trajectories prior to capture in an atomic orbital of the incident material (Goodhead, 1992).

Exposures to the track-end electron segments of nuclear-incorporated tritium β -particles (average energy ~5 keV) or ultrasoft carbon K-shell (C_K), aluminum K-shell (Al_K) and titanium K-shell (Ti_K) X-rays (energies of 0.28 keV, 1.5 keV, and 4.5 keV, respectively) result in greater radiobiological effect, with RBE values of 1.2–4.1 for the endpoints of survival, mutation, chromosome aberrations, DSB induction and rejoining, and transformation (Cox, *et al.*, 1977; Goodhead, *et al.*, 1979; Frankenberg, *et al.*, 1986, 1995; Raju, *et al.*, 1987; Cornforth, *et al.*, 1989; Goodhead and Nikjoo, 1989; Goodhead,

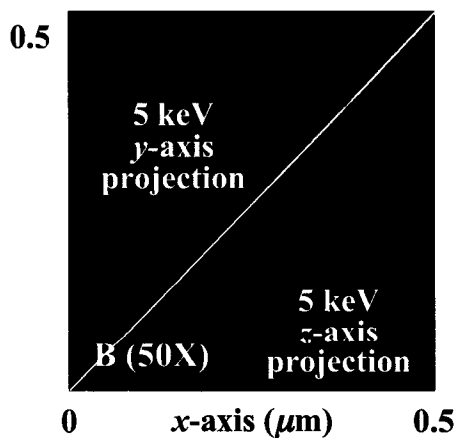
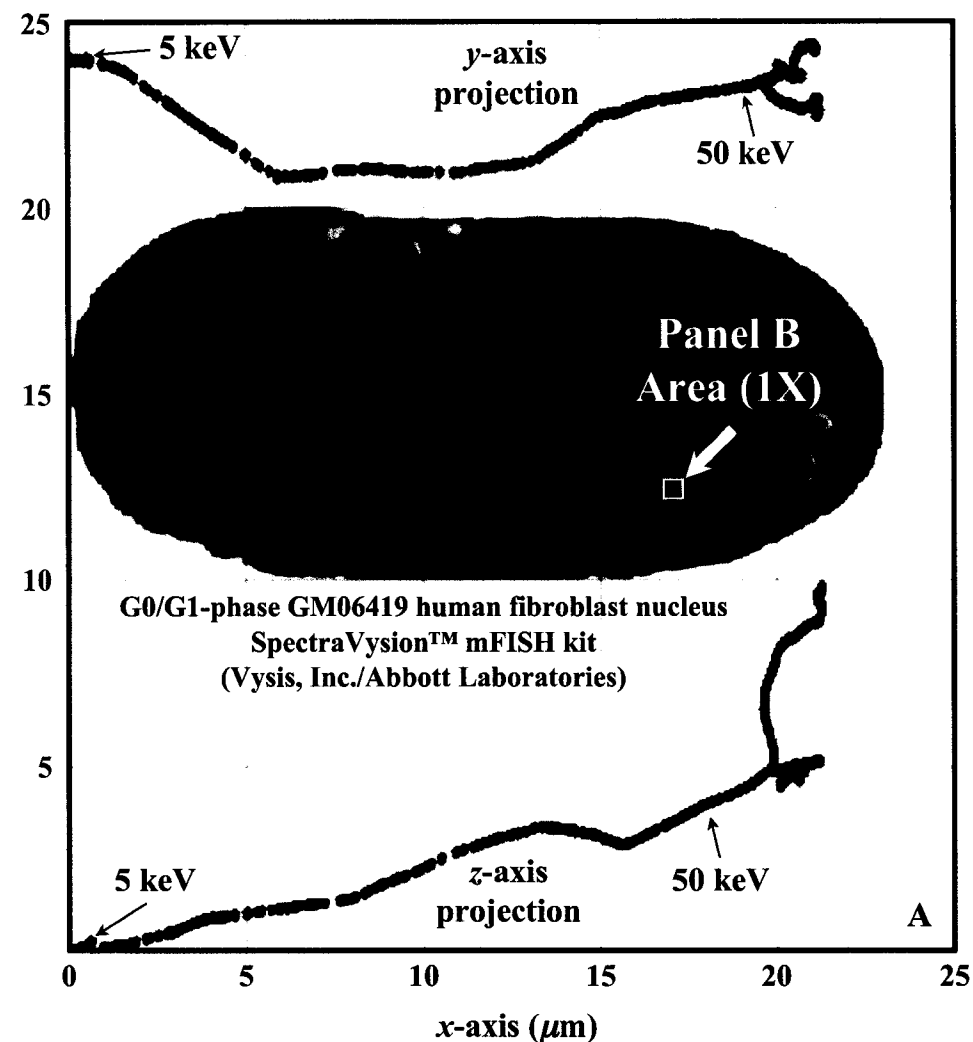


Figure 7.6. Illustrative example of the scale of a G0/G1 GM06419 human fibroblast nucleus traversed by y-axis and z-axis scalar projections of modeled 50-keV and 5-keV electron tracks (Semenenko, 2002). Individual chromosome domains are visualized by mFISH staining, which demonstrates that tracks of *both* energies are capable of crossing multiple chromosome domains. Panel **B** is expanded 50X from the white box in Panel A to visualize the capability for the 5-keV track of traversing four different chromosome domains.

1992; Griffin, *et al.*, 1996, 1998; Yang, *et al.*, 2000; de Lara, *et al.*, 2001; Hill, *et al.*, 2002; Semenenko, 2002; Bryant, *et al.*, 2003).

The mFISH-painted G0/G1-phase GM06419 human fibroblast nucleus is shown to scale based on the dimensions in Table 7.2. The mFISH staining was performed myself according to the Vysis SpectraVysion™ protocol supplied by the manufacturer (Vysis, Inc./Abbott Laboratories) and described in Dugan and Bedford (2003) with small modifications (see MetaSystems™ GmbH 21XMouse mFISH protocol in Chapter 6 for further details). The electron track projections and cell nucleus dimensions (see Figure 7.4 and Table 7.2) are all to scale. The 50-keV electron track represents a computer-modeled β -particle released by ^{14}C with the same approximate average energy (Semenenko, 2002). With its ~800+ track ionization events (assuming an average on 60-eV deposited per ionization event; Johns and Cunningham, 1983; Ward, 1998), the 50-keV electron track is clearly capable of traversing the entire volume of the nucleus [the continuous slowing-down approximation (CSDA) range for an electron of this energy is approximately 40 μm]. The 5-keV track represents a computer-modeled β -particle released by ^3H with the same approximate average energy, and is significantly shorter (CSDA range of ~1 μm , ~100 ionization events) than the 50-keV electron track.

From calculations based on Semenenko (2002) of 50,000 Monte-Carlo modeled decays for each radionuclide, the average energy and dose deposited per decay from all primary and secondary electrons liberated in a nucleus (following thymidine analog labeling techniques) of a confluent G0/G1-phase apparently normal human fibroblast of dimensions listed in Table 7.2 is: for ^3H ($\bar{E} = 5.7$ keV), 4.4 keV and 1.5 mGy deposited per decay; for ^{14}C ($\bar{E} = 49.2$ keV), 3.1 keV and 1.0 mGy per decay; and for ^{125}I ($\bar{E} = 19.3$

keV), 11.6 keV and 3.9 mGy deposited per decay. The smaller amount of energy and dose deposited for the ^{14}C β -particles, as compared to the ^3H β -particles, is due to the exit of the ^{14}C β -particles and their high-LET-like track-ends, in the large majority of cases, completely out of the cell nucleus. The greater amount of energy and dose deposited per ^{125}I decay is reflective of the high-LET-like nature of the Auger and Coster-Kronig electron cascades, which release an average of 19 electrons (range 1–56), with mean total energies of ~62 keV per decay, in nanometer-scale volumes closely localized to the DNA molecule and nearby chromatin (Charlton, *et al.*, 1994; Geselowitz, *et al.*, 1995). Visual examination of electron tracks generated from the decay of all three radionuclides with greater spatial resolution (ten to hundreds of nanometers) demonstrated the existence of smaller high-LET-like clusters in the initial track segments as well (evident in both panels of Figure 7.6), indicating the electron track ends certainly do not fully account for the energy deposition in the target material following low-LET irradiation (for further discussion, see Goodhead, 1992 and Semenenko, 2002).

The purpose of the panel B of Figure 7.6, magnified 50 times in scale from the small white box in panel A, is to better visualize this short 5-keV electron track and to demonstrate that such a fairly low energy electron is capable of traversing a number of chromosome domains (4 differently colored domains in this example). If multiple chromosome breaks are induced from ionization events in this localized region, and assuming an approximate 0.2- μm chromosome break diffusion range of such breaks (Nikiforova, *et al.*, 2000; Chubb, *et al.*, 2002; Aten, *et al.*, 2004), it is quite conceivable that an interchange or even a complex chromosome aberration involving the multiple

chromosomes could be induced (similar to discussions in Cornforth, *et al.*, 2002 and Loucas, *et al.*, 2004).

Figure 7.6 could be considered a visual “snapshot” of electron tracks traversing a human fibroblast in the first femtosecond (10^{-15} seconds) after radiation exposure; the work of Aten, *et al.* (2004) demonstrates the spatial and temporal dynamics of radiation-induced DSB and repair protein clustering and diffusion (visualized through the use of fluorescently-tagged γ -H2AX, Rad51, and Mre11 antibodies, time-lapse video microscopy, and computer modeling) within minutes to hours following irradiation; and, the above-mentioned FISH and mFISH-based cytogenetic studies of Bedford, Cornforth and Loucas illustrates the arrival of the repaired (properly or improperly) and/or unrepaired chromosomes at the first post-irradiation mitosis following irradiation.

These studies, together with the results of this dissertation, imply that a single electron track traversing the cell nucleus is quite capable of generating chromosome rearrangements responsible for cell killing and carcinogenesis. The application of cutting-edge molecular biology techniques, from FISH, CGH, and fluorescent-based microarray technologies to individual cell “interrogation” techniques (in the words of Dr. Joe Gray, LBNL) based on the recently completed and continually updating databases born from the Human Genome Project, will undoubtedly continue to shed new insight into more of the now almost-century old questions in radiation biology. These tools will better delineate the effects of low dose/dose-rate radiation exposures on individuals with varying degrees of radiation sensitivity and cancer predisposition, in an attempt to tailor individual radiotherapy regimens and appropriate radiation risk estimates.

7.4.1. G0/G1-Phase Human Fibroblast Nucleus Morphometry Measurements

These experiments were completed as a portion of the radioisotope project briefly described above (see Semenenko, 2002) for which the nuclear size of confluent G0/G1-phase AG01521 human fibroblasts was measured for accurate microdosimetric and nuclear dose deposition calculations for nuclear-bound ^3H , ^{14}C (β -particles) and ^{125}I (Auger/Coster-Kronig electron cascade) radioisotope decays. AG01521 normal human fibroblasts (passages 5–8) were grown in complete medium in Chapters 3–5 in Lab-Tek™ chamber slides (Nalge Nunc International). G0/G1-phase cultures were obtained by long-term density-inhibited contact inhibition, low serum (0.1–1%) FBS treatment, and double-thymidine blocking procedures, with each treatment yielding continuous (24-hour) BUdR labeling indices of $\leq 5\%$.

One hour prior to analysis, the cell medium was aspirated from chamber slides destined for confocal “z-axis” microscopy and 10 μM of Vybrant® CFDA-SE live-cell cytoplasmic stain (Molecular Probes/Invitrogen) suspended in PBS was added. The chamber slides were returned to the 37°C incubator for 15 minutes for the stain to enter the cells, after which the Vybrant® solution was aspirated and replaced with fresh complete cell culture medium. Intracellular esterases in the cytoplasm then cleave the CFDA-SE molecule, exposing the previously quenched FITC signal. Following an additional hour in the 37°C incubator for completion of this reaction, the chamber slides were processed as below (50% live and 50% fixed samples were prepared). Nuclear counterstaining for these samples was achieved with 0.5- $\mu\text{g}/\text{ml}$ propidium iodide (a red fluorescent dye; Sigma), as seen in Figure 7.5.

Cells grown on the chamber slides were either imaged live (in PBS) or fixed by 100% ice-cold methanol (4 minutes directly on the chamber slide with the growth chamber well removed). Nuclear counterstaining in the “xy-axis” series of chamber slides (as opposed to the “z-axis” chamber slides prepared above for confocal microscopy) was accomplished by 1X SYBR Green-I DNA stain (Molecular Probes/Invitrogen) suspended in PBS for the live cell samples or the SlowFade™ antifade solution (Molecular Probes/Invitrogen) used to mount the methanol-fixed samples. The live cell samples were stained by aspirating the cell culture medium and incubating the cells for 15 minutes with the 1X SYBR Green-I/PBS solution in the 37°C incubator. This solution was aspirated and replaced by PBS alone until imaging.

Multiple “xy-series” chambers slides were imaged in three separate experiments using live and methanol-fixed G0/G1-phase AG01521 normal human fibroblasts by an Olympus AX-70 microscope equipped with a 100X objective and 488-nm excitation light (see Figure 7.20). Many thousands of SYBR Green-I-stained cell nuclei were imaged and, following proper spatial calibration with a stage micrometer (see Figure 7.4 panel E), the dimensions of the cell nuclei were measured using NIH Image software (Figure 7.4 panel D). The “z-series” chamber slides were imaged in a single experiment using live and methanol-fixed G0/G1-phase AG01521 normal human fibroblasts with an Olympus FVX-IHRT Fluoview confocal laser-scanning microscope (CSU Pathology Department) equipped with 40X and 100X objectives and 488-nm or 543-nm excitation wavelengths (Figure 7.5). Multiple “z-stack” image series were captured with both sets of chamber slides (live/fixed) at successive 0.25–0.5- μ m steps along the z-axis of the microscope under computer control.

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