DISSERTATION

TCFAP2C REGULATION OF PRIMORDIAL GERM CELL DEVELOPMENT

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ABSTRACT

TCFAP2C REGULATION OF GERM CELL DEVELOPMENT

The development of germ cells during embryonic development is driven by a complex expression pattern of genes. The transcription factor *Tcfap2c* is expressed in germ cells throughout development from specification to adult sperm and oocytes. *Tcfap2c* expression is first seen in primordial germ cells around embryonic day (E)6.75 and has been classified as a germ cell specification gene. This study implicates *Tcfap2c* as a potential key factor in germ cells during specification, proliferation, migration and differentiation. In order to investigate the role of *Tcfap2c* in germ cells, we utilized the Cre/loxP conditional gene mutation strategy. Cre/loxP allows us to overcome the early embryonic lethality that arises from loss of *Tcfap2c* in traditional knock-out mice by creating *Tcfap2c* null mutation in specifically-targeted tissues. We created *Tcfap2c* mutant mice using the epiblast-specific *Sox2*-Cre model. Mutant ovaries from this model failed to express both germ cell specific markers and meiotic markers at E12.5. Immunohistochemistry at E18.5 failed to detect the germ cell specific marker NOBOX or the meiotic protein SYCP3, which confirmed that *Sox2*-Cre, *Tcfap2c* mutant mice lacked germ cells at late embryonic stages. However, *Sox2*-Cre, *Tcfap2c* mutant mice die prior to or at birth preventing us

from studying adult gonads from these mice. To this end we used tamoxifen inducible ERTM-Cre mice to create *Tcfap2c* mutation in adult animals. We assessed ERTM-Cre, *Tcfap2c* mutant animals for fertility and gametogenesis; surprisingly, fertility, spermatogenesis and oogenesis were not affected in *Tcfap2c* mutant gonads. These results show that *Tcfap2c* is not necessary for adult maturation of gonocytes to produce mature sperm and oocytes. However, *Sox2*-Cre, *Tcfap2c* mutants lack germ cells indicating that *Tcfap2c* is necessary during fetal germ cell differentiation. The *Sox2*-Cre model was limited because *Tcfap2c* was deleted in the entire embryo and the mutants died at birth. *Prdm1*-Cre was used to produce a mouse where *Tcfap2c* is only deleted in germ cells beginning around specification. *Prdm1*-Cre, *Tcfap2c* mutants initially specified germ cell-like cells at E7.5; however, by E8.5 the germ cell numbers were decreased and they had not initiated migration towards the genital ridges. By E9.5 few if any germ cells were observed in *Prdm1*-Cre, *Tcfap2c* mutants. At E12.5 no germ cells were seen in *Prdm1*-Cre, *Tcfap2c* mutant XX or XY gonads. Adult ovaries and testes from *Prdm1*-Cre, *Tcfap2c* mutant mice were noticeably smaller than littermate controls and showed no oogenesis or spermatogenesis. The *Prdm1*-Cre model showed that mutation of *Tcfap2c* results in loss of germ cells in embryos by E9.5 suggesting that *Tcfap2c* plays a role during germ cell specification, proliferation and migration. We identified *Tcfap2c* as an important factor during early germ cell development; however, *Tcfap2c* expression is observed in germ cells well past specification. We believe that *Tcfap2c* is present in germ cells during during fetal gonad differentiation because it plays a role in

regulating the gene expression pathways necessary for this event. We show that *Tcfap2c* is expressed in germ cells during the period of fetal gonad differentiation. Gene expression analysis of gonads from E11.5-13.5 reveals *Tcfap2c* as the most highly expressed member of the *Tcfap2* family member. *Tcfap2c* is a member of a transcription factor family that regulates gene expression by binding consensus sequences within target gene promoters. TCFAP2 binding sites are present in promoter regions of germ cell specific genes Cadherin1 *(Cdh1)* and Kit oncogene (*Kit)*, as well as in the promoter regions of genes involved in regulating pluripotency High mobility group AT-hook 2 (*Hmga2),* Nanog homeobox *(Nanog)* and *Lin28*. Using chromatin immunoprecipitation we demonstrate that TCFAP2C binds the promoter regions of *Cdh1, Kit, Hmga2, Nanog and Lin28*. The interaction between TCFAP2C and the promoter regions of *Cdh1, Kit, Hmga2, Nanog* and *Lin28* indicates that *Tcfap2c* likely plays a functional role in the regulation of these genes. These genes are necessary for germ cell survival, migration and pluripotency. In conclusion, our results provide a new understanding of the role of *Tcfap2c* during different stages of germ cell development from specification to differentiation.

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Jillian Guttormsen

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Introduction

Perpetuation of a species is fundamentally dependent on the passage of genetic material from one generation to the next. The germ cell lineage produces gametes that will deliver genetic information from an individual to the next generation; these gametes will carry epigenetic marks and any genetic mutations that are present in the genome to the next generation.

Germ cells differentiate into gametes, which ultimately produce mature sperm and oocytes in XY and XX individuals respectively. The first cells identified that give rise to germ cells are referred to as primordial germ cells (PGCs). These PGCs will mitotically divide and establish a founding population of gametes.

Germ cell determination occurs in one of two pathways, termed preformation and epigenesis. In some organisms such as Ascarids, Anura, and Diptera, germ cells are specified in early embryogenesis by the localization of maternally derived determinants (germ plasm) being unequally divided during the first few zygotic divisions (Williamson and Lehmann; 1996; Extavour and Akam 2003). Germ cells specified through epigenesis arise much later during development; these cells arise as a result of inductive signals from surrounding

cells. In mice, and probably all mammals, germ cells are specified through epigenesis; germ cells differentiate from the primitive ectoderm of the epiblast (Saitou and Yamaji 2010). Notably, both specification pathways involve repression of the somatic program and upregulation of similar pluripotency and PGC specific markers (Seydouz and Braun 2006).

In mice, all the pluripotent cells of the primitive ectoderm in the epiblast are capable of differentiating into germ cells; however, inductive signals from the surrounding extraembryonic cells will determine the population of cells that will be specified into germ cells (Surani et al., 2007).

Germ cells ultimately fuse with another germ cell to generate a totipotent zygote able of producing all cell lineages. In order to confer pluripotency, germ cells must possess genetic programs responsible for genomic pluripotency, suppress somatic differentiation and undergo epigenetic reprogramming. Some of these events depend on interactions of the germ cells with surrounding, while others are programmed cell autonomously into germ cells (Ewen and Koopman 2010).

This chapter will describe the events and genes involved in germ cell specification, proliferation, migration, gonad colonization, differentiation, as well as germ cell epigenetic and gene expression reprogramming in mice.

Primordial Germ Cell Specification

Early Embryo Formation and Structures

To understand the specification of germ cells, the basic anatomy of the pre and post-implantation embryo must be understood. By embryonic day 3.5 (E3.5) in the mouse, the blastocyst consists of two distinct cell types: the trophectoderm that will form the placental tissues and the inner cell mass (ICM). The ICM will give rise to all germ layers of the developing embryo forming all the cells in the adult body including the germ cells (Gardner et al., 1979). At E4.5, the cells on the surface of the ICM differentiate into the primitive endoderm (Figure 1). The remainder of the ICM maintains pluripotency and is now referred to as the primitive ectoderm (Fossat et al., 2007). When the embryo implants into the uterine wall, the trophectoderm cells that are in direct contact with the primitive ectoderm proliferate and thicken to form the extraembryonic ectoderm cells. The primitive ectoderm cells begin to form a cavity, producing a cup-like structure called the epiblast (Figure 1; Coucouvanis et al., 1995). The posterior end of the embryo will develop into a structure termed the allantois. This mesoderm-derived structure is important as a landmark during PGC development, and eventually gives rise to placental blood vessels and umbilical cord. The epiblast will differentiate into all the somatic cells of the body and the germ cells (Figure 1).

In mice, germ cells are derived from the multipotent cells of the proximal epiblast at E6.0 (Figure 1). More specifically, germ cells are specified from primitive endoderm cells covering the epiblast that receive specification signals from the extraembryonic ectoderm (Figure 1; Fujiwara et al., 2001). At E5.5, *Bmp4* and *Bmp8b* are initially expressed in the extraembryonic ectoderm that contacts the proximal epiblast. Germ cell specification and localization is

dependent on *Bmp4* and *Bmp8b* signaling from the extraembryonic ectoderm (Fujiwara et al., 2001; Ying et al., 2000). *Bmp2*, expressed in the visceral endoderm, also plays a role in signaling the proximal epiblast for PGC specification and location (Ying and Zhao, 2001). Bmp ligands bind cell surface receptors on PGC precursors. Binding of the Bmp ligands initiates a phosporylation cascade that results in the activation of *Smad5/8*. These *Smad* proteins then relocate to the nucleus where they function as transcriptional regulators, initiating a PGC specific transcriptional pattern different from the surrounding somatic tissues (Shi and Massague, 2003; Massague et al., 2005). The exact mechanism of BMP and SMAD proteins is still not fully understood, but it appears that these factors are key in the initial specification and localization of PGCs. Early studies demonstrated that primordial germ cells can be visualized in the epiblast at the base of the allantois by increased levels of tissue non-specific alkaline phosphatase (*TNAP*; Figure 2; Snow, 1981; Ginsburg et al., 1990; McLaren, 2003). *TNAP* does not appear to be necessary for PGC specification, but has been a useful marker for PGCs (MacGregor et al., 1995). During gastrulation, cells capable of giving rise to germ cells become localized to the proximal posterior region of the allantois located at the base of the allantoic bud (Figure 2; Saitou et al., 2002; Ohinata et al., 2005).

Lineage tracing experiments verified that a founding population of approximately 45 germ cells originates from the area of the proximal epiblast next to the extraembryonic ectoderm (Figure 2; Lawson and Hage, 1994). Interestingly, cells from other regions of the distal epiblast can give rise to germ

cells when transplanted into the proximal epiblast, indicating that additional cells in the epiblast have the potential to give rise to germ cells if given the appropriate signals (Tam and Zhou, 1996). Germ cell specification is driven by extracellular signals, *Bmp4*, *Bmp8b*, *Smad 5* and *8,* which emanate from the extraembryonic and visceral ectoderm (Lawson et al., 1999; Ying et al., 2001; Ying and Zhao, 2001; Zhao and Garbers, 2002; de Sousa Lopes et al., 2007; Itman et al., 2006). Specification of PGCs from somatic neighbors is characterized by drastic changes in gene expression patterns. Studies employing single-cell gene expression analysis have identified some of these genes (Saitou et al., 2002). Founder PGCs show high levels of *Ifitm3* and *Dppa3* that repress the somatic genes *Hoxa1* and *Hoxb1* (Saitou et al., 2002; Tanaka et al., 2002; Sato et al., 2002). *Lin28* expression is observed during PGC specification. This known pluripotency factor has been shown to process *let7* microRNAs, allowing them to upregulate the transcription factor *Prdm1* at E6.25 (West et al., 2009). *Prdm1* is considered to be a 'master regulator' during PGC specification (Ohinata et al., 2005; West et al., 2009; Kurimoto et al., 2008). *Prdm1* positive cells are lineage-restricted to the PGC precursor population (Ohinata et al., 2005). *Prdm1* functions to repress *Hox* genes and activate PGC specific genes such as *Dppa3* (Ohinata et al., 2005). Similar to *Prdm1*, *Prdm14* functions to upregulate pluripotency genes within the PGC population such as *Sox2* (Yamaji et al., 2008). *Prdm14* is seen in PGC precursors at E6.5. During specification, PGCs upregulate expression of the pluripotency markers *Sox2*, *Nanog* and *Pou5f1* (Avilion et al., 2003; Matsui et al., 2007). The signals from the surrounding

tissues and transcriptional regulators upregulated within PGCs will initiate major reprogramming of gene expression and alteration of epigenetic states (discussed later in this section).

Migration

By E7.5, PGCs have formed a tight cluster at the base of the allantois. Between E7.5 – 8.5 the hindgut invaginates, passively sweeping the PGCs along and relocating them into the hindgut (Figure 2; Lawson and Hage, 1994). Once in the hindgut, PGCs are highly motile along the midline of the embryo and exit into the mesentery after E9.0. From E10.0-10.5, PGCs actively migrate forward along the dorsal body wall in two separate cluster pathways to populate the genital ridges (Figure 2; Tam et al., 1981; Gomperts et al., 1994; Anderson et al., 2000; Molyneaux et al., 2001).

Prior to migration, PGCs proliferate rapidly to around 100 cells by E8.0. While PGCs are in the hindgut from E8.0-9.0, approximately 60% are arrested at the G2-phase of the cell cycle. Once PGCs have exited the hindgut, they are released from cell cycle arrest and begin rapid proliferation once again (Seki et al., 2007).

During migration, PGCs are receptive and respond to a number of signals that are necessary for mobility and directionality (Wylie, 1999). A number of chemoattractant signals produced by surrounding tissues, surface proteins on mesenteric cells and PGC-PGC contact signals are believed to foster successful migration of PGCs to the genital ridges (Ewen and Koopman 2010). *Kit* and *Cdh1*

are two genes necessary for PGC migration and cell-cell contact during proliferation and migration. *Kit* is expressed on the cell surface of PGCs where it acts as a receptor to *Kitl*, which is secreted by surrounding somatic cells during proliferation and migration (Sakuma et al., 2003). This signaling is necessary for proliferation, cell survival and motility (Goddard et al., 2007). *Cdh1* mediates cell-cell interaction by communication between CDH1 proteins on other PGCs. CDH1-CDH1 signaling between PGCs allows close contact enabling instructive signaling from surrounding tissues (Bendel-Stenzel et al., 2000). *Nanos3* also is expressed in migrating germ cells. *Nanos3* functions to maintain PGC survival by suppressing the apoptotic pathway.

Colonization

Germ cells migrate from the hindgut and enter the developing genital ridge at ~E9.5-10.5. Upon colonization, PGCs are now referred to as gonocytes or simply as germ cells (Figure 3; McLaren 2003). Up until E12.5, the germ cells in the genital ridges remain sexually bi-potential (Adams and McLaren, 2002). Initially, the changes germ cells undergo are independent of chromosome content. First, germ cells change cell morphology and gene expression as they change from pluripotent PGCs into differentiated gonocytes (Ewen and Koopman, 2010). Germ cells become large and round, forming tight clusters, making them morphologically distinguishable from their somatic neighbors (Donovan et al., 1986; De Felici et al., 1992). Following colonization, germ cells undergo gene expression changes, sex specific differentiation and epigenetic remodeling.

The events that establish either the spermatogenic or the oogenic pathways are based on cell autonomous and somatic cues within the genital ridges (Adams and McLaren, 2002; McLaren, 2003; Bowles et al., 2006; Koubova et al., 2006). Both XX and XY germ cells continue to undergo epigenetic changes, during which imprinted loci undergo widespread DNA de-methylation (Hajkova et al., 2002; Lane et al., 2003; Sato et al., 2003). Epigenetic reprogramming during this phase is thought to be necessary to re-set the germ cells to an epigenetic blank slate for the production of competent gametes. After fertilization, gametes must be able to produce a totipotent zygote capable of giving rise to all embryonic and extraembryonic tissues to produce a viable embryo. Some genomic imprints are re-established in order to pass the unique imprints of the parent onto the next generation. As the gonadal germ cells undergo epigenetic reprogramming, they lose the capability to produce germ cells in culture. This likely is due to the epigenetic changes as well as the onset of germ cell sex determination (Western 2009).

Once in the genital ridge, the germ cells will upregulate a new set of germ cell specific genes, including germ cell nuclear antigen 1 (*Gcna1*), mouse vasa homologue (*Mvh*), deleted in azoospermia like (*Dazl*), estrogen related receptor b (*Erb*), *Nobox* and germ cell-less (*Gmcl1*; Enders and May, 1994; Kimura et al., 1999; Toyooka et al., 2000; Noce et al., 2001; Saunders et al., 2003; Mitsunaga et al., 2004; Lechowska et al., 2011).

Meiosis

The major divergence of sexual differentiation is entry into meiosis in XX germ cells and mitotic arrest in XY germ cells (McLaren, 1984; Adams and McLaren, 2002). Sexual identity of germ cells in mice is determined by a combination of germ cell sex chromosome constitution and somatic cell signals. The Sry gene is expressed from the sex-determining region of the Y chromosome in somatic cells of XY gonads. Presence of SRY activates *Sox9* expression leading to Sertoli cell formation and activation of testis development (Sekido and Lovell-Badge 2008; Defalco and Capel 2009).

Germ cells rely on the somatic environment to initiate sex-specific cell fates. Around E12.5, germ cells stop mitotic divisions and both XY and XX germ cells enter a pre-meiotic stage and up-regulate genes necessary for meiosis such as *Sycp3* and *Dmc1* (Di Carlo et al., 2000). Meiosis proceeds no further in XY germ cells, *Sycp3* is downregulated, and the germ cells enter G0/G1 arrest (Figure 3; McLaren, 1984).

XY germ cells do not enter meiosis until puberty. Around E12.5, germ cells are organized into cords in XY gonads and germ cells begin to enter mitotic arrest (Figure 3; Hilscher et al., 1974; Western et al., 2008). After birth, XY germ cells will re-enter mitosis (Hilscher et al., 1974).

XX germ cells enter meiosis and proceed through prophase, leptotene, zygotene, pachytene and finally arrest in diplotene around the time of birth (Figure 3; McLaren, 1995; McLaren and Southee, 1997). By E15.5, most of the XX germ cells have entered meiosis. XX germ cells begin to arrest in diplotene at

E17.5 and finish around 5 days after birth (Figure 3; Borum, 1961). Each oocyte will be surrounded with a single layer of somatic cells, forming a primordial follicle (McLaren, 1984).

Induction of meiosis is initiated by diffusion of retinoic acid (RA) from the mesonephros into the gonad (Figure 3). RA induces the expression of *Stra8* in germ cells, a gene necessary for premeiotic DNA replication (Koubova et al., 2006; Bowles et al., 2006). In the ovary, meiosis progresses in an anterior to posterior wave. RA originates from mesonephric tubules that lie adjacent to the anterior end of the gonad. These tubules connect with the mesonephric duct at E11.5 allowing the influx of RA into the anterior end of the ovary and testes (Bowles et al., 2006). However, germ cells in the testes do not enter into meiosis due to the presence of the cytochrome P450 enzyme, *Cyp26b1* (Figure 3; Fujisawa et al., 1997; Koubova et al., 2006). CYP26B1 effectively degrades RA, preventing *Stra8* expression and entrance of the XY germ cells into meiosis. *Cyp26b1* is expressed in somatic cells of the gonad, but most specifically in Sertoli cells. *Cyp26b1* expression occurs in both ovaries and testes, but is downregulated by E11.5 in the ovary while it continues to increase expression to peak in the XY gonad at E13.5. Early expression of Cyp26b1 may function to prevent premature meiotic entry in both sexes, but continued expression in testes prevents meiotic entry until puberty (Bowles et al., 2006; Koubova et al., 2006). Gene ablation studies have shown that XX embryos lacking *Stra8* do not initiate meiosis and lack of *Cyp26b1* in XY embryos leads to upregulation of *Stra8* and initiation of meiosis in germ cells (Baltus et al., 2006; Bowles et al., 2006).

R-spondin1 (*Rspo1*) has recently been identified as a key factor in determining female gonad fate. *Rspo1*, a secreted factor is a potent modulator of *Wnt* signaling and activates the β-catenin pathway (Nef and Cassalli 2009). *Rspo1* is upregulated in XX somatic cells at E11.5, *Rspo1* upregulates *Wnt4* and functions to stabilize *β-catenin*, which promotes ovarian fate and blocks testis development (Chassot et al., 2008). *Rspo1*, *Wnt4* and *β-catenin* are three factors of only one pathway that promotes ovarian development and suppresses testis cord formation. *Foxl2*, a transcription factor, functions in a different pathway to regulate granulosa cell differentiation and ovarian maintenance. Loss of either *Foxl2* or *Rspo1* results in mascularization of the XX gonad (Nef and Cassalli 2009).

Sexual differentiation of germ cells within the gonad heralds massive gene expression changes that will eventually give rise to mature gametes competent for fertilization and zygote production. During the initiation of meiosis in XX germ cells and mitotic arrest in XY germ cells, many of the genes that were highly expressed in PGCs are down-regulated, such as *Cdh1, Kit, Lin28* and *Nanog* (Bendel-Stenzel et al., 2000; Manova et al., 1991; Zheng et al., 2009; Yamaguchi et al., 2009).

Molecular Regulation

The understanding of the molecular regulation of germ cell specification has been advanced by recent studies. Gene knock-out studies have vastly improved the comprehension of the signaling molecules and genes necessary for

germ cell specification (Zhao et al., 2002; Chang et al., 2002). This review will touch on a few of the genes and factors that are the most relevant to the work in this dissertation.

Bone Morphogenetic Proteins

Specification of PGCs from the proximal epiblast is dependent on extracellular signals (*Bmp4* and *Bmp8b*) originating from extraembryonic ectoderm as well as *Bmp2*, which originates from the visceral endoderm. BMPs regulate germ cell specification, localization and survival (Ying et al., 2000; Ying et al., 2001; Dudley et al., 2007; Ross et al., 2007). For example, *Bmp4* is initially expressed in the extraembryonic ectoderm at E5.5 (Lawson et al., 1999). In *Bmp4* mutants, the alkaline phosphatase positive germ cell founder population is not formed. *Bmp4* expression is necessary for the survival and localization of PGCs (Fujiwara et al., 2001). *Bmp4* was the first factor to be identified that proved that germ cell specification in mice is dependent on a secreted signal from the trophectoderm. BMPs activate *Smad5*/8, which are relocated to the nucleus and act to induce expression of *Ifitm3* (Saitou et al., 2002).

BMPs also play a role in PGC migration. BMP signals within the somatic cells upregulate *Kitl* expression (discussed later), among other genes, in cells lining the PGC migratory path and in the genital ridges. These signals are believed to guide the PGCs towards the genital ridge (Dudley et al., 2007).

Genetic Reprogramming Factors

Germ cell specification requires the establishment of the correct genetic programs and a unique epigenetic state (Saitou et al., 2002; Ohinata et al., 2005;

Seki et al., 2005; Seki et al., 2007; Surani et al., 2007). Nascent germ cells must silence somatic differentiation genes; this process is believed to be necessary to reacquire germ cell pluripotency (Seydoux and Braun, 2006; Strome and Lehmann, 2007). Indeed, upon specification PGCs upregulate ~300 genes believed to be involved in pluripotency and PGC specification, and downregulate ~500 genes involved in somatic cell differentiation (Kurimoto et al., 2008; Saitou and Yamaji 2010). Many studies over the past 20 years have sought to elucidate the complex network involved in PGC production.

Unlike other stem cell populations that can give rise to multiple cell fates, germ cells only follow a singular fate to become a gamete; however, in producing a gamete they also must have the ability to become all the tissues in a zygote. The genetic programming required to produce a totipotent offspring involves a very specialized differentiation process. This program is three-fold: germ cells must suppress somatic differentiation program, re-activate underlying genomic pluripotency and activate specialized gene expression to drive germ cell differentiation. Germ cell differentiation is a unique event that requires exoressuib of genes that will regulate cell migration, erasure of epigenetic markers, initiation of meiosis, induction of spermatogenesis or oogenesis and finally production of a mature and competent gamete. These processes will be achieved by the initiation of the appropriate genetic program at various stages during germ cell development.

Recently, gene expression screens have identified novel genes that are differentially expressed in PGCs at specification compared to neighboring

somatic cells. Saitou et al., (2002) performed a set of experiments in which tissue containing PGCs and the surrounding somatic cells was dissected from E7.5 embryos. Single cells were collected and cDNA libraries were prepared from each cell. cDNA libraries that expressed high levels of the somatic marker *Hoxb1* were designated as somatic cells, while libraries exhibiting high levels of *TNAP* were designated as germ cells. Comparison between the two cell cDNA populations identified many novel genes that are preferentially expressed in newly specified PGCs and not in neighboring cells; including a unique set of genes involved in specification, *Prdm1*, *Prdm14*, *Tcfap2c, Ifitm3, Dppa3 Elf3, Elk1, Isl2, Mycn, Klf2, Fiat, Sp8, Smad3, Sox2* and *Nanog*.

The novel genes identified provide a more comprehensive understanding of the events necessary for PGC specification. The following sections will summarize the roles of key genes that play important roles in PGC development.

Pr Domain Containing 1

Pr domain containing 1 (*Prdm1*, also known as *Blimp1*) plays an integral role in suppressing somatic differentiation genes. *Prdm1* contains an N-terminal proline rich region, a Suv39 Enhancer, and a Trithorax domain and functions as a transcription factor in PGCs. *Prdm1* was identified as a gene specifically expressed in PGCs in a single-cell study comparing gene expression in *TNAP* positive PGC precursors with somatic neighbors (Ohinata et al 2005). *Prdm1* was initially identified as a gene upregulated during terminal differentiation of B-cells into plasma cells. *Prdm1* is first identified in about 6 cells of the proximal epiblast at E6.25. The number of *Prdm1*-positive cells increases to 25 cells in the

proximal epiblast at E6.75, and at E7.25 *Prdm1* is seen throughout the *TNAP* positive germ cell founder population (Turner et al., 1994; Ohinata et al., 2005). Lineage tracing experiments showed that all the *Prdm1*-positive cells contribute to the germ cell lineage (Saitou et al., 2005). The exact function of *Prdm1* is not well understood, but loss of *Prdm1* results in loss of the majority of the founder population of germ cells; the remaining *TNAP*-positive germ cells do not proliferate or migrate (Ohinata et al., 2005; Vincent et al., 2005). The PGCs that are specified in the absence of *Prdm1* fail to suppress somatic Hox genes and do not upregulate PGC genes such as *Dppa3*. Shortly after *Prdm1* expression begins in PGC precursor cells, somatic cell markers such as *T, Fgf8* and *Snail* are repressed while pluripotency and PGC marker genes *Sox2*, *Nanog*, *Dppa3* and *Nanos3* are upregulated (Ohinata et al., 2005; Kurimoto et al., 2008). These studies demonstrate that *Prdm1* suppresses the default somatic differentiation program that other surrounding cells in the epiblast follow.

PRDM1 forms a complex with PRMT5, a protein that catalyzes the methylation of histones and spliceosome proteins. PRDM1 and PRMT5 co-localize in germ cells from E8.5 to 10.5, and their interaction plays a role in continued suppression of somatic gene expression programs and germ cell survival (Ancelin et al., 2006).

The progression from a *Prdm1* positive PGC precursor to a more advanced migrating PGC involves a orchestration of gene transcription that will restore pluripotency, activate PGC-specific genes, repress somatic fate and re-structure epigenetic organization.

Pr Domain Containing 14

It was originally believed that *Prdm1* might act to maintain pluripotency genes and initiate PGC specification as well; however, *Prdm1* has been shown to be dispensable for these processes (Kurimoto et al., 2008). Recent studies have identified expression of another PR domain-containing transcription factor, related to *Prdm1* in PGCs and ES cells (Yamaji et al., 2008). Pr domain containing 14 (*Prdm14)* functions independently of *Prdm1* and serves to maintain and reacquire expression of pluripotency genes in PGCs (Yamaji et al., 2008).

PRDM14 expression first begins in the morula and continues to be expressed in the ICM of the blastycyst, but expression is downregulated and disappears around E5.0. *Prdm14* expression is re-initiated in PGC precusors at E6.5 and remains in this population until E13.5 (Yamaji et al., 2008). *Prdm14* ablated embryos form and repress Hox genes, but they fail to upregulate *Sox2* expression. PGCs in these mutants fail to proliferate and are eventually lost (Yamaji et al., 2008). It appears that *Prdm1* and *Prdm14* both function independently as two major transcriptional regulators of PGC specification; one represses somatic cell fate and the other upregulates expression of pluripotency genes (Saitou and Yamaji 2010).

At E7.0, specified PGCs begin expressing PGC-specific genes such as *Dppa3*, *Ifitm3* and *TNAP* (Tam and Zhou, 1996; Fox et al., 1981; Sato et al., 2002). Germ cell proliferation and survival are two processes regulated by genetic factors shortly after specification and during migration.

Developmental Pluripotency-Associated 3

Developmental pluripotency-associated 3 *(Dppa3,* also known as *Pgc7* or *Stella*) encodes a protein that has a purported SAP domain, which may function to regulate DNA methylation (Saitou et al., 2002; Sato et al., 2002). *Dppa3* is a maternal effect gene expressed in pre-implantation embryos, embryonic stem cells and in germ cells starting around E7.0. Ablation of *Dppa3* does not have an effect on germ cell formation, but *Dppa3* is required during pre-implantation development, where it regulates DNA methylation patterns (Payer et al., 2003; Bortvin et al., 2004; Nakamura et al., 2007). In early embryos, *Dppa3* prevents DNA demethylation from maternal alleles and imprinted genes on the paternal allele (Nakamura et al., 2007). *Dppa3* is hypothesized to play a similar role in PGCs (Saitou et al., 2009).

Interferon Induced Transmembrane Protein 3

Interferon induced transmembrane protein 3 *(Ifitm3*, also known as *Mil1* or *Fragilis*) encodes an interferon inducible transmembrane protein needed for cell-cell adhesion of germ cell progenitors. *Ifitm3* is first detected in the proximal epiblast at about E6.0. *Ifitm3* expression is upregulated until it occupies about 150 cells at the proximal end of the epiblast at the base of the allantois (Saitou et al., 2002). Not all *Ifitm3*-positive cells will become PGCs, but all PGCs will express *Ifitm3*. *Ifitm3* also represses Hoxb1 and Hoxa1 and thus plays a supporting role to suppress a somatic cell fate; however, loss of *Ifitm3* does not affect germ cell specification or numbers (Saitou et al., 2002; Tanaka et al., 2002; Lange et al., 2003; Tanaka et al., 2004; Tanaka et al., 2005).

Cadherin 1

Cadherin 1 (*Cdh1)* is a cell surface receptor that mediates cell-cell interaction by binding *CDH1* proteins on neighboring cells (Bendel-Stenzel et al., 2000; Okamura et al., 2003; Di Carlo and De Felici, 2000). *Cdh1* is expressed in PGCs and mediates adhesion between PGCs allowing for close contact, and enabling instructive signals to reach the same population of cells. Expression of *Cdh1* occurs in a proximal region of the extraembryonic mesoderm adjacent to the epiblast at E6.75, but does not occur in the distal area that is developing into the allantois. *Cdh1* expression in the proximal extra-embryonic mesoderm might protect PGCs precursor cells from differentiation into the allantois. *Cdh1* is hypothesized to function to anchor precursor cells within the region, allowing them to receive PGC specification signals, such as *BMP4*, from surrounding tissues. (Okamura et al., 2003). By mediating PGC interactions, *Cdh1* helps to regulate PGC fate, transmits instructive signals and facilitates PGC progenitor clustering.

At E8.5 *Cdh1* expression is seen both in the PGCs and surrounding tissue; however, between E9.5 and 10.5 *Cdh1* is observed in the sites of PGC-PGC contact during migration suggesting a role for *Cdh1* during migration. By E12.5, *Cdh1* is expressed solely in germ cells where it plays a role in PGC clustering within the gonad (Bendel-Stenzel et al., 2000). Blocking *Cdh1* at various stages of development leads to the failure of PGCs to develop in vitro, or failure of the PGCs to fully colonize the gonad (Bendel-Stenzel et al., 2000; Okamura et al., 2003). Initial studies have shown that *Cdh1* functions to regulate

PGC cell-cell interactions that are necessary throughout germ cell determination, specification, migration, and colonization.

Kit Oncogene and Kit Ligand

Kit ligand (*Kitl*, also known as stem cell factor, mast-cell growth factor or steel factor) is a molecule secreted by surrounding somatic cells that is necessary for PGC migration from the allantois to the hindgut and into the genital ridges (Runyan et al., 2006; Gu et al., 2009). During migration, loss of *Kitl* results in loss of PGC motility, but directionality is not altered (Gu et al., 2009). To stimulate motility, *Kitl* binds a tyrosine kinase receptor expressed on the surface of PGCs called *Kit* (McCoshen et al., 1975). Interaction between *Kitl* and *Kit* starts the PI3K/AkT cellular signaling cascade within PGCs that activates migration (Buehr et al., 1993; Runyan et al., 2006).

Mice mutant for *Kit* display normal numbers of PGCs at specification, but they fail to proliferate after E8.5. The PGCs form clumps in the hindgut and some fail to leave the epiblast at the base of the allantois. The few germ cells that travel into the hindgut fail to migrate properly and many end up in ectopic locations as they are passively moved during embryo turning and formation (Gu et al., 2009). These germ cells ultimately undergo apoptosis, leaving *Kit* mutants devoid of germ cells. These studies suggest that *Kitl* and its associated receptor *Kit* are involved in cell surface properties that confer survival and motility to PGCs (Reith et al., 1990; Besmer et al., 1993; Buehr et al., 1993; Dolci et al., 1991; Matsui et al., 1991; Manova et al., 1991). *Kit* and *Kitl* signaling are required for germ cell proliferation, survival and migration. Aberrant signaling in this

pathway leads to abnormal proliferation and transformation of germ cells into somatic tissues or germ cell tumors (Dolci et al., 1991; Godin and Wylie, 1991; Matsui et al., 1991; Dolci et al., 1993; Pesce et al., 1993; Donovan and de Miguiel, 2003; Sakuma et al., 2003; McIntyre et al., 2005; Goddard et al., 2007).

Nanos Proteins

Nanos3 is an evolutionarily conserved gene plays a critical role in germ cell development in Drosophila (Wang et al., 1991). In mice, *Nanos3* expression in PGCs begins at E7.25 and continues through migration. PGCs are specified normally in mice deficient for *Nanos3*; however, PGC numbers begin to decline at E8.0 and are all eventually lost (Tsuda et al., 2003). *Nanos3* is expressed in migrating germ cells, and functions to maintain survival by suppressing the apoptotic pathway (Suzuki et al., 2007).

Nanos2 is activated in early PGCs, but expression becomes restricted to XY germ cells and is necessary for survival and differentiation of germ cells in the XY gonad after colonization (Suzuki et al., 2007; Saga 2008). *Nanos2* encodes an RNA binding protein and has been shown to be necessary for sexual differentiation in XY germ cells, suppression of XX fate in XY gonads and maintenance of spermatogonial stem cells after birth (Saga 2010). *Nanos2* works in a similar manner to *Cyp26b1*, it is required to suppress Stra8 expression and suppress meiosis in XY germ cells at E13.5 (Saga et al., 2008). *Nanos2* also upregulates male germ cell-specific genes such as *Dnmt3L* and *Tdrd1* (Sakai et al., 2004). The Nanos genes are key factors in regulating germ cells during embryonic and adult stages.

Stromal Cell Derived Factor 1

SDF1 (stromal cell derived factor 1) is required for guiding PGC migration and promote survival in the migrating population until gonad entry (Stebler et al., 2004). When germ cells take the wrong path and lose contact with SDF germ cells and undergo cell death (Stebler et al., 2004).

Pluripotency genes

One feature that makes germ cells unique is the expression of genes that are necessary for pluripotency. These genes are often seen in PGCs and stem cells.

POU Domain, Class 5, Transcription Factor 1

POU domain, class 5, transcription factor 1 (*Pou5f1;* also known as Oct4), is a POU domain containing transcription factor that is necessary to maintain pluripotency in pre-implantation embryos and embryonic stem cell populations (Nichols et al., 1998; Niwa et al., 2000). *Pou5f1* is detected shortly after fertilization during embryo cleavage and becomes restricted to the pluripotent inner cell mass. *Pou5f1* is downregulated in all cells and becomes restricted to the primordial germ cells around E8.5. Expression of this pluripotency marker is maintained in germ cells continuously through specification, migration and gonad colonization. *Pou5f1* can be detected throughout the life cycle of a germ cell until the primitive type A spermatogonium and fertilized oocytes (Yoshimizu et al., 1999). *Pou5f1* deficiency in pre-implantation embryos leads to loss of pluripotency in the ICM and these cells instead differentiate towards the

trophectoderm lineage (Nichols et al., 1998). Ablation of *Pou5f1* expression in germ cells results in apoptosis, indicating that *Pou5f1* functions to maintain germ cell survival (Kehler et al., 2004).

Lin28

Recent studies have identified the RNA-binding protein LIN28 as a putative upstream regulator for *Prdm1*. *Lin28* expression is seen in PGCs starting at E7.5 and persists until E12.5 (West et al., 2009). LIN28 also is observed in adult undifferentiated spermatogonia (Zheng et al., 2009). Knock-down of *Lin28* in PGCs led to a reduction in AP positive cells and a reduction of *Prdm1* expression, while overexpression of *Lin28* produced increased numbers of PGCs (West et al., 2009). *Lin28* inhibits the processing and maturation of the *let7* microRNA family. *Let7s* effectively suppress *PRDM1* in vitro, and it is believed that in PGCs *Lin28* represses *let7* allowing upregulation of *Prdm1* (Bussing et al., 2008; West et al., 2009). In accordance with this hypothesis, West et al., showed that the loss of the *let7* binding region on *Prdm1* rescued the loss of PGC formation seen with *Lin28* ablation (2009). Thus, *Lin28* is a major regulator in PGC specification.

Interestingly, *Lin28*, *Pou5f1*, *Sox2* and *Nanog* are four genes that have been shown to reprogram human fibroblasts into induced pluripotent stem cells (Yu et al., 2007).

Sox2

Sox2 first is upregulated in a small number of the PGCs at E6.75, prior to the initiation of PGC marker gene *Dppa3* expression, and most of the PGCs are

Sox2 positive by E8.5. *Sox2* expression is maintained until germ cells have populated the genital ridge but decreases shortly after sex-specific differentiation into XX or XY fates (Yabuta et al., 2006)

Nanog Homeobox

The pluripotency factor *Nanog* is also upregulated in PGCs shortly after specification and shows a similar expression pattern as *Sox2* (Yamaguchi et al., 2005; Yabuta et al., 2006). Loss of *Nanog* in the germ cells leads to a decreased number of specified germ cells and to a total loss of germ cells shortly after E10.5 due to apoptosis, suggesting that *Nanog* is necessary for PGC proliferation and survival (Yamaguchi et al., 2009).

Nanog and *Sox2* play important roles in embryonic stem cell pluripotency and self-renewal within the pre-implantation embryo inner cell mass (Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). Based on the expression of these two factors in PGCs and their functions in ES cells, it is likely that *Nanog* and *Sox2* function to establish pluripotency and self-renewal in the germ cell population.

High Mobility Group AT-Hook 2

Hmga2 is a member of the high mobility group, the most abundant non-histone, DNA-binding chromatin factors (Bianchi and Agresti 2005). *Hmga2* is observed in most embryonic tissues between E10.5 and 15.5, especially in the head, gut mesenchyme and limb buds (Zhou et al., 1995). *Hmga2* is a regulator of body height, adipocyte development and stem cell proliferation (Zhou et al., 1995; Fedele et al., 2006). Due to its high expression in embryonic stem cells,

Hmga2 has become the focus of many recent studies (Li et al., 2006; Li et al., 2007). Mice lacking *Hmga2* are approximately 50% smaller due to a lack of proliferation (Zhou et al., 1996). Male mice lacking *Hmga2* are sterile due to disrupted spermatogenesis (di Agostino et al., 2004). Expression of *Hmga2* has been seen in *Xenopus laevis* oocytes and spermatogonia as well as in mouse meiotic sperm (di Agostino et al., 2004; Hock et al., 2006). Despite this phenotype and expression pattern, *Hmga2* has not been studied in germ cell lineages, but its role in embryonic stem cells suggests that *Hmga2* may regulate pluripotency in PGCs.

Epigenetic Reprogramming

During development, a zygote will generate a large variety of cell types, each with a unique gene expression pattern. This pattern is influenced in part through epigenetic modifications of the genome. Epigenetic regulation is achieved through DNA methylation, histone modifications and specific nuclear architecture. These epigenetic modifications are mitotically heritable from one cell generation to the next. However, PGCs must be capable of epigenetic reprogramming in order to produce a zygote (Bird 2002; Peters and Schubeler 2005; Bernstein et al., 2007; Saitou 2009).

During migration to the developing gonads, germ cells undergo extensive epigenetic reprogramming. Initially, newly specified germ cells and the somatic neighbors are epigenetically similar, both have Histone H3 Lysine 9 dimethylation (H3K9me2), a repressive histone mark, both contain comparable DNA

methylation patterns, and both have X-chromosome inactivation (Tam et al., 1994; Seki et al., 2007; Sugimoto and Abe, 2007).

The PGCs that are specified from the primitive endoderm of the epiblast already have received some epigenetic reprogramming towards a somatic fate. PGCs must be epigenetically reprogrammed to confer a zygote capable of producing all tissue types. In order to achieve this, PGCs will undergo extensive de-methylation, erasure of DNA imprints and re-activation of the X-chromosome during development (Kafri et al., 1992; Tam et al., 1994; Surani et al., 2001; Hajkova et al., 2002).

From E8.0 onward, the migratory PGCs begin to show a reduction of the two major repressive epigenetic modifications; DNA methylation and H3K9me2 (Seki et al., 2007; Seki et al., 2005). During epigenetic remodeling from E8.0 to 9.5, germ cells enter transient G2 cell cycle arrest, repress RNA polymerase transcription and repress the histone methyl-transferase EHMT1, which is required for the establishment of H3K9me2 methylation (Tachibana et al., 2005; Seki et al., 2007). Just as H3K9me2 decreases, at E8.0 there is an increase in H3K27me3 in germ cells, but not in the somatic cells of the proximal epiblast. While H3K9me2 is a repressive histone mark, H3K27me3 has been shown to be a prominent histone mark associated with activation of pluripotent gene markers in embryonic stem cells. Ezh2, a histone methyltransferase necessary for modifications of H3K27me3 is also strongly expressed in migrating germ cells. This data suggests that Ezh2 modifies H3K27me3 allowing the histone to establish pluripotency in germ cells (Yabuta et al., 2006).

DNA methylation

DNA methylation patterns decrease in germ cells due to the down regulation of DNA methyltransferases DNMT1, DNMT3b and DNMT3a (Seki et al., 2005; Yabuta et al., 2006; Seki et al., 2007). After the germ cells have colonized the gonad, DNMT1 expression is seen in the nuclei of the germ cells (Hajkova et al., 2002). Re-establishment of important genetic imprints is achieved by DNMT3L and DNMT3A (Hata et al, 2002; Kaneda et al., 2004; Webster et al., 2005; Kato et al., 2007). Mutations of DNMT genes result in global genome demethylation and embryonic lethality (Li et al., 1992).

Between E9.5 and 12.5, parental DNA methylation imprints are erased in germ cells (Hajkova et al., 2002). By the time germ cells have entered the genital ridge, genome-wide methylation at CpG sites already is low due to the decrease in methylation genes previously discussed. Upon colonization, the parentally imprinted genes must be erased in the germ line in order to be re-established according to the chromosomal configuration of the germ cell. Erasure of maternally or paternally imprinted genes is complete around E12.5 (Szabo and Mann, 1995). During oogenesis and spermatogenesis maternal and paternal imprints will be re-acquired respectively (Reik and Walter, 2001). Re-methylation occurs in a very specific manner, the majority of imprinted genes are found in XX gametes (Ueda et al., 2000). XX germ cells establish a maternal imprint pattern after birth, while the only three known paternally imprinted genes are established in XY germ cells from E14.5 until birth (Ueda et al., 2000).

X Inactivation

Sugimoto and Abe showed some genes are expressed from both X alleles in germ cells that are normally repressed following X-chromosome inactivation at E7.75 (Sugimoto and Abe, 2007). In XX PGCs, a major factor in X-inactivation, *Xist*, is downregulated in 5% of primordial germ cells and Xist expression is lost in all germ cells by E10.5 (Sugimoto and Abe, 2007). The silent X-chromosome in XX germ cells has been reactivated upon entry to the genital ridge (Monk and McLaren 1981).

The reprogramming of PGCs ensures that previously repressed genes can be upregulatead when differentiation is necessary for zygotic formation. Notably, the epigenetic pattern established in early PGCs resembles embryonic stemm cell genome organization. The extensive epigenetic reprogramming that takes place during germ cell development and migration ensures the maintenance and establishment of genomic pluripotency within this lineage (Seki et al., 2007; Surani et al., 2007).

Germline Apoptosis

Programmed cell death, known as apoptosis, is a key event in germ cell development. Apoptosis in germ cells ensures that integrity of the germ line is maintained by preventing germ cell tumors from arising from germ cells that fail to reach the gonad.

Germ cell apoptosis generally occurs at three time points: during migration to the genital ridges, after genital ridge colonization and after birth (Molyneaux et

al., 2001; Wang et al., 1998; Runyan et al., 2006). PGCs that follow the correct migration pathway rely on a series of survival and proliferation signals. Loss of these signals such as *Kitl*, can lead to initiation of apoptosis. During migration, any PGCs that deviate or fall behind during migration are left outside of the range of the signals that promote survival. The PGCs that remain in the hindgut or surrounding areas eventually undergo cell death (Stallock et al., 2003; Runyan et al., 2006). Errantly located PGCs lose contact with signals that promote survival and are exposed to retinoic acid within the hind gut causing them to first enter meiosis and eventually undergo apoptosis. The destruction of these cells is necessary to reduce the chance for germ cell tumor formation outside of the gonad (McLaren, 1983; Molyneaux et al, 2001).

Apoptosis also occurs during normal development of germ cells after they have reached the gonad (Coucouvanis et al., 1993; Wang et al., 1998). XY germ cells undergo two waves of apoptosis, the first between E13 and 17, the second shortly after birth (Wang et al., 2008). XX germ cells show a similar patten, the first wave of apoptosis occurs at E13.5 and the second occurs from E15 to birth (Coucouvanis et al., 1993; Ratts et al., 1995). These rounds of apoptosis leave the gonads with around 25% of the initial germ cell numbers at birth in both XY and XX gonads (Wang et al., 1998; Pepling and Spradling, 2001). A notable difference between the sexes is that while the male have a limitless sperm production potential, the remaining germ cells in the female will constitute the entirety of the XX oocyte pool. However, recent studies have proposed that the ovary may have some postnatal germ cell renewal from a purported renewable

germ cell pool, but these claims have yet to be validated (Johnson et al., 2004; Johnson et al., 2005). Apoptosis is believed to ensure the quality of the genetic information id germ cells that will give rise to the next generation (Morita et al., 1999; Ewen and Koopman, 2010). Further studies elucidating the underlying mechanisms controlling PGC specification and development will provide a foundation to improve reproductive and regenerative mechanisms.

The AP-2 Family of Transcription Factors

Mammalian development is achieved by a complex expression pattern of genes encoding for proteins necessary to form embryonic tissues. The stem cells of nascent embryos undergo gene expression changes that direct the differentiation of cell fates to produce various tissues. Gene expression programs are unique for each cell type, for example the genes expressed in germ cells will be vastly different than the expression pattern seen in epidermal keratinocytes. Transcription factors function to regulate genes by either upregulating or repressing expression of targeted genes, producing the gene expression patterns needed to form proper cell types. Essentially, transcription factors work as the “traffic lights” of gene expression. One such family of transcription factors is the five-membered AP-2 transcription factor (TF) family. The AP-2 TF family consists of 52 kDa proteins TCFAP2A, TCFAP2B, TCFAP2C, TCFAP2D, TCFAP2E. AP-2 TF family orthologs are found in frogs, chickens, rats, mice, fish and humans and homologs are seen in invertebrates (Hilger-Eversheim et al., 2000; Eckert et

al., 2005). In general, TF proteins follow a modular pattern (helix-loop-helix), containing different domains for protein dimerization, DNA binding, and transcriptional activation or repression. In mammals, all AP-2 proteins except *Tcfap2d* are encoded by seven exons and share characteristic domain structure (Eckert et al., 2005). In mice, *Tcfap2a, Tcfap2b* and *Tcfap2c* are all encoded by separate genes located on chromosomes 13A5-B1, 1 A2-A4, and 2 H3-H4 respectively. *Tcfap2d* and *Tcfap2e,* the less conserved members of the family, are on 1 A3 and4 D2.2 respectively (Gaynor et al., 1991; Williamson et al., 1996; Zhao et al., 2001; Feng and Williams 2003).

All the members in the AP-2 TF family share a conserved helix-span-helix DNA binding dimerization motif at the carboxyl terminus of the proteins. This region consists of two amphipathic α-helices separated by an ~80 amino acid span that plays a role in DNA binding and in protein:protein dimerization. The AP-2 transcription factors are able to form protein hetero- and homodimers using this region. Similar helix-span-helix (HSH) motifs can be found in other DNA binding proteins myoD, E12, TFE2 and achaete scute daughterless (*Ascl1*). The AP-2 HSH region is sufficient for protein dimerization but a downstream basic region of the protein is necessary to act with this region to complete DNA binding (Williams and Tijian, 1991). The highly conserved C-terminus HSH and centrally located basic region enable AP-2 proteins to recognize a range of G/C rich sequences in DNA to bind to, including the pallindromic consensus sequence 5'-$GCCN_3GGC$-3' which can be found in various promoter regions genes involved in cell differentiation (Hilger-Eversheim et. al., 2000). Transactivation of genes is

achieved by a less conserved proline and glutamine rich domain at the amino terminus in AP-2 proteins (Eckert et al., 2005). This region yields the variability seen in the AP-2 family members when binding target genes and activating processes such as cell growth and differentiation. TCFAP2D shows a different transactivation motif than is seen in the other AP-2 family members suggesting that it functions using a different activating mechanism than the other AP-2 members. The similarity in the other four AP-2 family members suggests that these proteins could be playing redundant roles as well as playing individual roles. Indeed, the central basic region responsible for DNA binding and dimerization of the AP-2 proteins has been highly evolutionarily conserved (Hilger-Eversheim et al., 2000). TCFAP2A, TCFAP2B and TCFAP2C are all capable of binding the promoter region of *c-erbB-2* as either homo- or heterodimers; however, TCFAP2A and TCFAP2C binding of this promoter region results in a greater increase of transcript than TCFAP2B binding the same promoter, suggesting that some AP-2 isoforms will display greater affinity for certain promoter regions over others (Bosher et al., 1996). Some AP-2 members have similar expression patterns, and often more than one AP-2 protein can be identified in a cell population. While AP-2 members may be able to bind to the same promoter sites within a cell, it is likely that one member will show greater affinity for a promoter region and be responsible for target gene expression (Eckert et al., 2005; Kuckenberg et al., 2010). The AP-2 genes have been implicated in cell fate determination and each plays a role in regulating cell proliferation, differentiation, apoptosis and carcinogenesis in different tissues

(Imagawa et al., 1987; Luscher et al. 1997; Hilger-Eversheim et al., 2000; Eckert et al., 2000).

AP-2 transcription factors regulate genes that contain AP-2 binding sites within their promoter sequences. AP-2 regulation of these targets control important cellular processes (Eckert et al., 2005; Hilger-Eversheim et al., 2000; Pfisterer et al., 2002). In general, AP-2 proteins suppress genes inducing terminal differentiation, apoptosis and growth retardation in order to promote cell proliferation. AP-2 genes have been demonstrated to directly activate the promoter of several genes related with growth and differentiation, such as *p21,* transforming growth factor-α, estrogen receptor-α, Kit and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (Zeng et al., 1997; McPherson et al., 1997 and Bosher and Williams 1995; Wang et al., 1997; Leask et al., 1991; Huang et al., 1998). AP-2 proteins also act as suppressors and negatively regulate melanoma cell adhesion molecule, CCAAT/enhancer binding protein *(C/EBP),* alpha and myelocytomatosis oncogene (Jean et al., 1998; Jiang et al., 1998; Gaubatz et al., 1995). The AP-2 family of transcription factors acts in a complex network of genes, and are also regulated by other TFs. AP-2 interacting molecules modify the transcriptional activity of AP-2. Some of these molecules are DNA binding factors that act as cofactors with the AP-2 family members and either stimulate or suppress AP-2 activity, such as Yin Yang 1, Y-box binding protein 1, *p53, Sp1, Myc, PAX-6*, retinoblastoma protein, CCAAT displacement protein and SV40 large T antigen (Allouche et al., 2008; Wu and Lee 2001; Mertens et al., 1998; McPherson et al., 2002; Pena et al., 1999; Batsche et al.,

1998; Sivak et al., 2004; Wu and Lee 2002; Mitchell et al., 1987). Other factors that alter the activity of AP-2 family members do not interact with target DNA sequences and instead modify activity by direct interaction, phosphorylation or sumoylation. WW domain containing oxireductase, glioma-amplified sequence 41, polyADP-ribose polymerase, adenomatosis polyposis coli, p300/cAMP response element binding protein, positive cofactor 4, DEK oncoprotein and yes-associated protein all have shown to act as cofactors that directly interact with AP-2 proteins (Aqeilian et al., 2004; Ding et al., 2006; Kannan et al., 1999; Li and Dashwood 2004; Braganaca et al., 2002; Kannan and Tainsky 1999; Campillos et al., 2003; Yagi et al., 1999; Garcia et al., 1999; Eloranta and Hurst 2002). AP-2 members also can be regulated by non-genetic factors. *Tcfap2a* expression is induced by various agents such as retinoic acid, cyclic amp, phorbol ester, UV light and singlet oxygen (Williams et al., 1988; Luscher et al., 1989; Grether-Beck et al., 1996; Huang and Domann 1998). In recent years, *Tcfap2a* has been extensively studied. Binding partners, co-activators, repressors and target genes have been identified for *Tcfap2a*, but similar studies are still being undertaken for the other four AP-2 genes.

During embryogenesis AP-2 transcription factors are expressed in a variety of neural crest derivatives, neural, epidermal and urogenetial tissues (Moser et al., 1997; Hilger-Eversheim et al. 2000). In developing embryos, each AP-2 family member shows distinct spatial and temporal expression patterns. AP-2 family member expression in flies through to humans all share a similar expression patterns during embryonic development in facial structures, central

nervous system, epidermis and neural crest (Bauer et al., 1998; Epperlein et al., 2000; Shen et al., 1997). The conserved pattern of gene expression for the AP-2 family shows that AP-2 proteins are important evolutionarily for proper embryonic development (Hilger-Eversheim et al., 2000). *Tcfap2a* transcripts are found in limb buds, dorsal root ganglia and tooth germs, while *Tcfap2b* is expressed in the midbrain, sympathetic ganglia, adrenal medulla and cornea (Moser et al., 1997). Both *Tcfap2a* and *Tcfap2b* show expression in skin, facial mesenchyme, spinal cord, cerebellum and renal tubular epithelia (Moser et al., 1997).

Tcfap2a is necessary for proper limb, eye, craniofacial, cardiovascular, skeletal and body wall development. Mice lacking *Tcfap2a* show malformed outflow tracts in developing hearts and double limb formation (Brewer et al., 2002; Notolli et al., 1998). The *Tcfap2a* mutant phenotype results in a perinatal lethality resulting from the failure of the body cavity and neural tube to close (Notolli et al., 1998; Schorle et al., 1996; Zhang et al. 1996). *Tcfap2a* has been studied extensively due to their important role in both embryonic development and in neoplasms. Recently, TCFAP2A has been shown to upregulate *Kit* expression in malanocytes, promoting melanocyte differentiation and survival (Van Otterloo et al., 2010). *Tcfap2a* has also been implicated in the human disease Fredreich's Ataxia. TCFAP2A is able to directly bind the Frataxin (*Fxn*) gene promoter upregulating expression. Fredreich's Ataxia is characterized by increased *Fxn* expression and *Tcfap2a* is theorized to play an important role in this disease (Li et al., 2010).

Null mutation of *Tcfap2b* results in embryos with polycystic kidney disease, which again results in a perinatal lethality caused by enhanced apoptotic death of renal epithelial cells (Moser et al., 2003; Zhang et al., 1996). Char syndrome in HUMANS characterized by patients who exhibit patent ductus arteriosis, facial feature abnormalities and malformation of the fifth finger, is caused by a missense mutation in TCFAP2B (Hilger-Eversheim et al., 2000; Satoda et al., 2000; Zhao et al., 2001). Recently, it has been shown that *Tcfap2b* levels are increased in the brainstem of rats prone to alcoholism. *Tcfap2b* plays an important role in regulating genes involved in alcoholism (Oreland et al., 2010).

The gene coding for TCFAP2D appears to have separated from the rest of the family early in vertebrate evolution and only was recently identified as a member of the AP-2 TF family (Eckert et al., 2005; Zhao et al., 2001). TCFAP2D has a unique protein structure that lacks several highly conserved residues found in the other four member's transactivation domains (Eckert et al., 2005; Zhao et al., 2001b; Zhao et al., 2003). The change in TCFAP2D structure suggests that it may transactivate genes in a different manner than the other family members. *Tcfap2d* also has a unique expression pattern; during development transcripts are seen in the central nervous system, retina and heart (Zhao et al., 2003). Research yet has to elucidate the role of *Tcfap2d* during development because a null mutation of the gene has yet to be generated.

The fifth AP-2 family member is *Tcfap2e*, which recently was identified as a member of the family. The TCFAP2E protein shares all the structures seen in

TCFAP2A, TCFAP2B and TCFAP2C; however, unlike these three proteins, TCFAP2E is not found in neural crest cells or their derivatives (Feng and Williams, 2003). In fact *Tcfap2e* shows a unique expression pattern during development, the transcript can be found in the developing olfactory bulb, midbrain, hindbrain, melanocytes and chondrocytes (Feng and Williams, 2003; Wang et al., 2004; Wenke et al., 2006). *Tcfap2e* binds the promoter regions of integrin alpha 10 (*Itga10*) and chemokin ligand 1 (*Cxcl1)*, both genes necessary for cartilage development (Wenke et al., 2006; Wenke et al., 2010). In fact, *Tcfap2e* is a regulator of hypertrophy in cartilage and increased levels of TCFAP2E leads to osteoarthritis (Wenke et al., 2010).

AP-2 Proteins in Neoplasia

AP-2 proteins play regulatory roles in cellular processes such as apoptosis, differentiation and proliferation. Because of these roles, AP-2 members are essential for maintaining normal growth; an aberrant expression pattern of AP2 proteins disrupts cellular homeostasis leading to neoplasias (Eckert et al., 2005; Bosher et al., 1996; Hilger-Eversheim et al., 2000; Pellikainen and Kosma 2007). Multiple studies have correlated altered AP-2 activity with malignant transformation. Recent studies have elucidated roles of *Tcfap2a* and *Tcfap2c* in melanoma, prostate cancer, ovarian cancer as well as glioma (Bar-Eli 2001; Ruiz et al., 2004; Lipponen et al., 2000; Williams et al., 2004; Sumigama et al., 2004; Anttila et al., 2000; Odegaard et al., 2005). Loss of *Tcfap2a* expression has been associated with several invasion and metastasis promoting events such as over expression of melanoma cell adhesion molecule

and protease-activated receptor 1 as well as down-regulation of *Kit* and Cdkn1a (Bar-Eli 2001; Huang et al., 1998; Jean et al., 1998 and Tellez et al., 2003; Scibetta et al., 2010). Indeed, loss of *Tcfap2a* expression has been referred to as a key event in progression of melanoma (Bar-Eli 2001).

The literature provides a number of conflicting reports on the specific roles of *Tcfap2a* and *Tcfap2c* in neoplasias; however, the consensus is that *Tcfap2a* functions as a tumor suppressor and loss of *Tcfap2a* leads to increased cell proliferation and tumor malignancy (Richardson et al., 2001; Gee et al., 1999). It has been hypothesized that *Tcfap2a* functions as a tumor suppressor by inducing expression of the cell cycle inhibitor p21 and by directly interacting with p53 (Zeng et al., 1997). In contrast, *Tcfap2c* expression is typically increased in malignancies and functions to impair cellular differentiation and stimulate cell proliferation (Jager et al., 2003). AP-2 members play important roles in the strictly controlled and multidimensional regulation network of the genes and cofactors necessary for various cellular processes; failure at any point during this process may lead to carcinogenesis. Further understanding of the AP-2 members during embryogenesis may lead to better comprehension and treatment of cancer (Pellikainen and Kosma 2007; Eckert et al., 2005).

These transcription factors help to regulate the formation of vital structures needed for survival. Each family member plays an important role in the development of various tissues; however, due to high sequence homology and the ability of AP-2 transcription factors to form heterodimers it is likely that members of the AP-2 transcription family work in redundant roles. The combined

loss of *Tcfap2a* and *Tcfap2c* accelerated the embryonic lethality seen with either individual knock out suggesting the two transcription factors play redundant roles in the extraembryonic tissues (Winger et al., 2006). Similarly, TCFAP2E is able to induce the expression of Kit in melanocytes lacking *Tcfap2a.* Mouse embryos deficient in both *Tcfap2a* and *Tcfap2e* show a more severe melanocyte phenotype than either single mutation, indicating redundant roles for the two AP-2 members (Van Otterloo et al., 2010). In studying these transcription factors the possibility of redundant roles must be acknowledged.

Tcfap2c: An Important Regulator of Cell Development

In recent years, it has become apparent how important the five members of the *Tcfap2* TF family are during embryogenesis. These transcription factors help to regulate the formation of vital structures needed for survival. The members of the *Tcfap2* family organize development by driving the regulatory network of genes necessary for proliferation, differentiation and cell survival (Eckert et al., 2005; Jager et al., 2003). This study focused on third member of the *Tcfap2* family, *Tcfap2c*, and the role that it plays in germ cell development. *Tcfap2c* first was detected in P19 embryonal carcinoma cells (Oulad-Abdelghani et al., 1996). TCFAP2C is 449 amino acids long and shows approximately 65% overall identity to TCFAP2A and TCFAP2A. Expression of *Tcfap2c* during embryonic development is similar to that of its family members *Tcfap2a* and *Tcfap2b*; it is present in extraembryonic tissues, central and peripheral nervous system, ectoderm, limbs, face, epithelium and mammary glands (Chazaud et al.,

1996; Mitchell et al., 1991; Auman et al., 2002; Li et al., 2006). *Tcfap2a* and *Tcfap2c* show similar expression patterns during early embryognesis (E8.0-8.5) but their expression patterns diverge during later stages (Chazaud et al., 1996). At E16.5, we identified *Tcfap2c* in the brain, heart, kidney, lung, skin and gonads (Guttormsen et al., 2008). Each family member plays a unique role in the development of various tissues; however, due to high sequence homology and the ability of AP-2 transcription factors to form heterodimers it is likely that some members of the family work in redundant roles (Moser et al., 1997b; Wang et al., 2006). In studying *Tcfap2c*, the possibility of other *Tcfap2* family members playing redundant roles must be acknowledged. Interaction between TCFAP2 members and other cofactors may direct TCFAP2 members to different target genes (McPherson et al., 2002; Eckert et al., 2005). These cofactors may change in different cell types altering the effects of *Tcfap2* members in each tissue. In some tissues, interaction between cofactors and TCFAP2C may act to repress target gene expression, while in another tissue with different coactivators TCFAP2C can act to upregulate expression of a target gene. Similarly, epigenetic modifications of target genes can also may alter the availability of *Tcfap2* target sequences (Woodfield et al., 2009). The broad expression pattern of *Tcfap2c* suggests that it plays an important role during mouse embryogenesis.

The study of *Tcfap2*c has been hindered due to an early embryonic lethal phenotype seen in embryos lacking *Tcfap2c*. *Tcfap2c* mutants fail between E7.5 and 8.5 from failure of the embryos to implant caused by defects in extraembryonic tissues (Auman et al., 2002; Winger et al., 2006). Tetraploid

rescues of *Tcfap2c* null embryos suggested that this transcription factor was not necessary for development of the embryo proper (Auman et al., 2002). However, conditional ablation of *Tcfap2c* in the embryo also results in embryonic lethality between E18.5 and birth. *Tcfap2c* conditional mutants were smaller than littermate controls and showed epidermal and skeletal defects (Guttormsen et al., 2008). Mutants embryos died prior to birth, indicating that despite the results reported in tetraploid study, *Tcfap2c* indeed is necessary for embryo development.

Tcfap2c was identified in P19 embryonal carcinoma cells as a gene that increased in expression when cells were induced to differentiate with retinoic acid (RA; Oulad-Abdelghani et al., 1996). Increased *Tcfap2c* was not observed when cell differentiation was induced using other methods like 1% dimethylsufoxide (Philipp et al., 1994). This suggested that *Tcfap2c* is regulated by the RA pathway, which regulates differentiation of multiple systems within developing embryos. RA is a physiologic metabolite of vitamin A that is produced and released by cells in a gradient to induce cell fate determination. RA alters cell transcriptional networks by binding the nuclear receptors RAR or RXR (Chambon, 1994; Hofmann and Eichele 1994; Linney and La Mantia, 1994). These receptors bind response elements as heterodimers and modulate the transcriptional activity of target genes (Chambon 1994; Kastner et al., 1994). RA signaling induces differentiation in chondrogenesis, skeletogenesis, adipogenesis, gonadogenesis and neuronal cell differentiation among other processes. RA either increases or decreases *Tcfap2c* levels. For instance,

Tcfap2a levels are decreased in skin cell cultures when exposed to high RA levels (Wang et al., 2002; Wanner et al., 1996). During chondrogenesis, RA acts to increase *Tcfap2* levels, which then decrease the transcription of cartilage-derived retinoic acid-sensitive protein (Xie et al., 1998). RA signaling drives skeletal formation. Since the AP-2 TF family is linked to RA, it is likely that *Tcfap2c* plays a role as an intermediate of the RA pathway during skeletogenesis (Martin et al., 1995; Weston et al., 2003).

Tcfap2c first was identified as a RA target gene in P19 embryonal carcinoma cells (Oulad-Abdelghani et al., 1995; Chazaud et al., 1996). RA signaling plays an important role in fetal gonad development and it is possible that *Tcfap2c* is involved in this developmental pathway.

Tcfap2c and Neoplasias

Outside of embryonic development *Tcfap2c* has been implicated as playing a role in tumorigenesis. Since *Tcfap2c* was identified in P19 carcinoma cells, multiple other types of cancer cells have shown *Tcfap2c* expression suggesting that misexpression of *Tcfap2c* may lead to abnormal cell states by aberrantly inducing proliferation or differentiation (Friedrichs et al., 2005; Hoei-Hansen et al., 2004; Hong et al., 2005; Jager et al., 2005).

During normal mammary development, *Tcfap2c* is expressed in cap cell layer and body cells in terminal end buds. Loss of *Tcfap2c* leads to decreased brancing, cell proliferation and morphogenesis (Jager et al., 2010). Overexpression of *Tcfap2a* and *Tcfap2c* results in hyperproliferation and apoptosis, which results in lactation failure (Jager et al., 2003; Zhang et al.,

2003). Both *Tcfap2a* and *Tcfap2c* are important for mammary cell proliferation, establishment of colonies in soft agar, cell migration and zenograft outgrowth (Orso et al., 2008). These studies indicate that *Tcfap2c* controls cell proliferation and differentiation during mammary development. Therefore, aberrant overexpression of *Tcfap2c* may lead to tumorigenesis.

Elevated levels of both TCFAP2A and TCFAP2C have been reported in 25-30% of breast cancers that exhibit elevated levels of the critical regulatory proto-oncogene c-erbB-2 (Bosher et al., 1996). TCFAP2A and TCFAP2C factors have been implicated in the regulation of both ESR1 and ERBB2. TCFAP2C binds the ESR1 promoter (deConinck et al., 1995; McPherson et al., 1997; Schuur et al., 2001). Loss of *Tcfap2c* reduces expression of *Esr1* and estrogen response (Woodfield et al., 2007). Research shows that *Tcfap2a*, *Tcfap2b* and *Tcfap2c* are capable of binding the *Erbb2* promoter as hetero or homodimers and upregulate expression of *Erbb2* (Bosher et al., 1996). The expression patterns of *Esr1* and *Erbb2* are predictive of clinical response to antihormonal therapy like Tamoxifen and Herceptin (Sorlie et al., 2001). Since *Tcfap2c* regulates hormonal response, increased *Tcfap2c* expression correlates with reduced survival and tamoxifen resistance (Guler et al., 2007; Gee et al., 2009). TCFAP2C binds AP2 recognition sequences in the promoter of *Erbb2* and TCFAP2C is capable of inducing expression (Yang et al., 2006). Aside from regulating hormonal response genes, TCFAP2C binds a different site in *Cdkn1a* promoter from TCFAP2A to repress expression of *Cdkn1a* (Scibetta et al., 2010). TCFAP2C was found to regulate pathways related to retinoic acid response and MYC

regulation (Woodfield et al., 2010). TCFAP2C binds the *Myc* promoter to repress transcription (Yu et al., 2009). Apparent discrepancies in the role of *Tcfap2c* in breast cancer suggests that genes targeted by *Tcfap2c* regulation are dependent on the specific breast cancer phenotype (Woodfield et al., 2010). Therefore, *Tcfap2c* and potentially all *Tcfap2* factors will have different activities in different breast cancer phenotypes.

The *Tcfap2c* transcript also has been identified in testicular germ cell tumors (Hoei-Hansen et al., 2004). Within testicular carcinomas as well during development, *Tcfap2c* is regulated by RA, suggesting this pathway could be the trigger to upregulate expression of the various *Tcfap2* genes (Hansen et al., 2004; Notolli et al., 1998). *Tcfap2c* expression is regulated by RA in testicular carcinomas as well, further indicating the importance of RA in regulating expression of *Tcfap2c* (Hoei-Hansen et al., 2004; Kopan and Fuchs, 1989). The presence of *Tcfap2c* in tumors suggests that it plays a vital role in regulating cell proliferation.

Tcfap2c plays a key role in Trophoblast Stem Cell Differentiation

The trophoblast is the first cell lineage to emerge during mammalian development. Trophoblast stem (TS) cells from this lineage are capable of self-renewal or differentiating into placental tissues. TS self renewal and multi-potency requires many transcription factors, including *Tcfap2c* (Auman et al., 2002). *Tcfap2c* plays a role in regulating TS self-renewal and placenta development (Auman et al., 2002; Werling and Schorle et al., 2002). *Tcfap2c* is expressed in the trophectoderm and loss of *Tcfap2c* results in altered gene

expression and failure of the trophectoderm to form. *Tcfap2c* null embryos fail to implant into the uterine wall and die around E8.5 (Auman et al., 2002; Werling and Schorle, 2002). *Tcfap2c* deficient mice die between E7.5 and 8.5 due to defects in the ectoplacental cone. *Tcfap2c* mutants display reduced cell proliferation in the ectoplacental cone and decreased numbers of differentiated trophoblast giant cells (Auman et al., 2002; Werling and Schorle 2002). *Tcfap2c* mutants fail to implant due to failure of trophoblast cells to express eomesodermin and *Cdx2*, two genes that are vital for FGF responsive trophoblast stem cell maintenance (Auman et al., 2002). Chromatin immunoprecipitation analysis identified two targets of TCFAP2C in the TS lineage; TCFAP2C induces *Cdx2* and represses *Nanog* expression in TS lineage (Auman et al., 2002; Kuckenberg et al., 2010). Recent works show that the combined loss of *Tcfap2a* and *Tcfap2c* accelerated embryonic lethality compared to either individual knock out phenotype, suggesting the two transcription factors play redundant roles in the extraembryonic tissues (Winger et al., 2006). Without *Tcfap2c* expression in the trophoblast cells the embryo fails to implant and does not form the vital maternal-embryonic interface, but the embryonic tissues appear to develop normally. However, recent studies have shown that *Tcfap2c* is necessary for multiple systems within the developing embryo.

Tcfap2c Regulates Epidermal Differentiation

Tcfap2c is observed in the basal, spinous and granular layers of the developing skin; this would suggest that the transcription factor is actively functioning in the cells to drive gene expression during the differentiation of

keratinocytes (Oyama et al., 2002). Balance between epidermal growth and differentiation is controlled by basal cells departing from their proliferative state and altering gene expression profile to induce differentiation and finally being sloughed from the skin surface (Blanpain and Fuchs, 2006). Basal and suprabasal genes posess *Tcfap2* binding sites, and *Tcfap2a* and *Tcfap2c* both play a positive and negative regulatory roles (Byrne et al., 1994; Sinha et al., 2000; Dai and Segre, 2004; Koster et al., 2006; Nagarajan et al., 2008). The mechanism underlying basal cell proliferation still is poorly understood. Ablation of *Tcfap2c* in the epiblast results in developmental delay in epidermal stratification, but by birth the epidermis appears morphologically and biochemically normal (Guttormsen et al., 2008). Loss of *Tcfap2c* results in perturbed terminal differentiation and suppression of additional target transcription factors and structural genes involved (Guttormsen et al., 2008; Wang et al., 2008; Koster et al., 2006). Functional redundancies between *Tcfap2a* and *Tcfap2c* were observed during epidermal differentiation (Wang et al., 2008). *Tcfap2c* orchestrates the transition from basal proliferation to suprabasal differentiation (Wang et al., 2008).

Tcfap2c in Germ Cells

A major role of *Tcfap2c* in tissues is to reorganize transcriptional regulation during differentiation. Germ cells undergo massive reorganizational events in order to eventually produce functional gametes. *Tcfap2c* was identified in gonocytes and type A spermatogonia primate testes (Mitchell et al., 2008; Albert et al., 2010). In humans, *Tcfap2c* expression is seen in fetal germ cells

from Week 12 through 37 of gestation and then is down regulated. *Tcfap2c* also is detected in ovulated mouse oocytes (Hoi-Hansen et al., 2004; Pauls et al., 2005; Winger et al., 2006). During primordial germ cell specification, *Tcfap2c* is expressed shortly after PGCs are identified from surrounding somatic tissues and has been classified as a potential "specification" gene (Kurimoto et al., 2008). In mice, *Tcfap2c* is detected from E6.75 until E13.5 in germ cells during embryogenesis (Kurimoto et al., 2008; Weber et al., 2010). The presence of *Tcfap2c* during germ cell development suggests that *Tcfap2c* plays a role during specification and potentially during fetal germ cell differentiation. Loss of *Tcfap2c* in the epiblast by conditional gene knock-out leads to reduction of PGC numbers at E8.5 and adult *Tcfap2c* mutants lack mature gametes (Weber et al., 2010).

Cre Recombinase, A Conditional Gene Knock-out System

In the last 20 years new conditional mutation technologies have been developed in order to bypass embryonic lethal phenotypes like the one seen in traditional *Tcfap2c* knock-out models. Cre recombinase, from the P1 bacteriophage, has been shown to mediate genetic recombination in mammalian cells (Hoess and Abramski, 1984; Bockamp et al., 2002). The 38 kDa Cre protein works to create site specific recombination by removing the DNA contained between two inserted 34 bp sequences called loxP sites (Hoess and Abramski, 1984). A target or floxed allele is produced when two loxP sites are inserted flanking the region of the sequence to be removed. The Cre enzyme recognizes the loxP sites and excises the flanked DNA leaving a single loxP site (Figure 4).

The temporal and spatial expression of the Cre protein can be regulated using tissue-specific promoters (Agah et al., 1997; Brocard et al., 1997; Orban et al., 1992). The Cre protein is fused to a tissue-specific promoter or an inducible ligand ensuring that Cre is only transcribed where this promoter is active. This enables tissue or temporal restricted deletion of gene sequences, while allowing the rest of the tissues to develop normally. In this study, *Sox2*-Cre, *ERTM*-Cre and *Prdm1*-Cre transgenic mouse strains were employed to create conditional mouse mutations of *Tcfap2c*. In *Sox2*-Cre transgenic mice the bacterial Cre recombinase gene sequence is regulated by a 12.5 kb upstream regulatory sequence from the *Sox2* gene. Wherever there is expression of the *Sox2* gene, Cre recombinase also will be expressed restricting the deletion of a targeted gene to these cells. *Sox2*-Cre is expressed in epiblast cells starting at E6.5; this means that recombination of a target gene only will take place in the embryonic portion of the embryo allowing the placental tissues to develop normally (Hayashi et al., 2002). To study gene deletion at a specific time point, tamoxifen *ERTM*-Cre can be used. This transgenic mouse contains a fusion protein consisting of Cre and the mutated form of the ligand-binding domain of the estrogen receptor. Cre is ubiquitously expressed in the animal, but is not active until it is exposed to the drug tamoxifen (Danielian et al., 1998). This allows an animal containing both the *ERTM*-Cre transgene and the floxed allele to develop with normally until injected with tamoxifen. In the *Prdm1*-Cre model, Cre recombinase transgene is inserted behind the *Prdm1* promoter, restricting the expression of Cre to the PGC population at E7.8 (Ohinata et al. 2005). This allows all the other tissues, except

the PGC population, to develop without floxed allele disruption. Other Cre transgenic mice have been created to have heart-, brain- or kidney-specific expression preventing deletion in any other tissues and allowing for a tissue-specific characterization of gene function.

This system is especially helpful in the study of genes that result in embryonic lethal phenotypes from complete embryo gene knock-down models. Cre recombinase allows tissue-specific deletion of genes allowing for focused study on a specific tissue while allowing the other tissues to develop normally. *Tcfap2c* knock-outs have previously been shown to die around E7.5 due to extraembryonic defects (Auman et al., 2002). In this study, a floxed allele of *Tcfap2c* was used to create *Tcfap2c* knock-out homozygous mice. LoxP sites were inserted to flank exon 6 that was removed by Cre recombinase creating a null mutation of *Tcfap2c* (Figure 4). To avoid extraembryonic defects due to the loss of *Tcfap2c* in the placental tissues, Cre transgenes were used to induce *Tcfap2c* deletion in specifically targeted tissues. Cre recombinase has provided an invaluable technology to study gene function by using targeted deletions.

Research Project Rationale

The objective of this project was to identify the role of *Tcfap2c* during germ cell differentiation. The role of *Tcfap2c* as a regulator of cell differentiation has been explored and verified in other systems (Auman et al., 2002; Guttormsen et al., 2008). In this study, I used *Tcfap2c* conditional gene mutation models in mice to elucidate the role of *Tcfap2c* during major stages of germ cell production,

namely specification, proliferation, migration and fetal gonad differentiation and adult maturation.

In chapter one, I explore the loss of *Tcfap2c* using three unique Cre recombinase models. Using ERTM-Cre mice, *Tcfap2c* was deleted after puberty to determine if *Tcfap2c* is necessary for spermatogenesis and oogenesis. *Sox2*-Cre deletes *Tcfap2c* specifically in the epiblast producing embryos devoid of functional *Tcfap2c* providing a model to study *Tcfap2c* during fetal development. Because this model appeared not to be the most appropriate to study *Tcfap2c* in germ cells alone, we used the Prdm1-Cre model to specifically delete *Tcfap2c* in the germ cell population. Markers of germ cell development such as *Pou5f1*, alkaline phosphatase, *Sycp3, Ifitm3, Ddx4* and *Nobox*, discussed in the literature review, were used to observe the progression of germ cells through the various stages of differentiation and development. Using these different Cre models, I evaluated how loss of *Tcfap2c* affected PGC specification, proliferation, migration, differentiation and germ cell maturation.

Chapter two focuses on the role of *Tcfap2c* during fetal gonad differentiation. Chapter one revealed that loss of *Tcfap2c* during embryonic development results in germ cells deficiency. However, *Tcfap2c* expression is observed in germ cells throughout fetal development and into adult gonocytes (Hoei-Hansen et al., 2004; Pauls et al., 2005; Winger et al., 2006). These data indicates that *Tcfap2c* plays a role throughout germ cell development and not just during specification, proliferation and migration. This study sought to further investigate the role *Tcfap2c* during fetal gonad differentiation. This stage of

development includes many important changes within germ. Between E10.5 and 14.5 germ cells will adopt a sex-specific fate, initiate meiosis or enter cell-cycle arrest. As a transcription factor, TCFAP2C functions to regulate gene expression in germ cells during fetal gonad differentiation. In order to identify potential regulatory targets, TCFAP2C recognition sites were identified in the promoter regions of *Cdh1, Kit, Lin28, Nanog* and *Hmga2*. Chromatin immunoprecipitation was performed to determine if TCFAP2C interacts with the promoter regions of these genes. *Cdh1, Kit* and *Nanog* have established roles during germ cell development; however, *Lin28* and *Hmga2* have not been previously studied during fetal gonad differentiation. Like *Nanog*, *Lin28* and *Hmga2* have been shown to regulate pluripotency in embryonic stem cells (Eda et al., 2009). *Lin28* encodes a protein that processes *let7* microRNAs, which in turn regulate the expression levels of genes such as *Hmga2* and *Prdm1* (Ohinata et al., 2008). This is a regulatory pathway that has been studied in embryonic stem cells, cancer as well as early germ cell specification (Eda et al., 2009; Ohinata et al., 2008; Viswanathan et al., 2009). We sought to determine if TCFAP2C interacts with the promoter regions of *Hmga2* and *Lin28*. This interaction would implicate TCFAP2C as an important factor in pluripotency and provide evidence that the *Lin28-let7-Hmga2* pathway exists during fetal gonad differentiation. *Cdh1, Nanog* and *Kit* are genes involved in cell-cell interaction and germ cell clustering during fetal gonad differentiation (Manova et al., 1990; Okamura et al., 2003). Interaction of TCFAP2C with the promoter of these two genes would indicate that TCFAP2C plays a role regulating germ cell-cell interaction within the fetal gonad.

TCFAP2C has previously been shown to regulate *Cdh1* and *Nanog* in other tissues, it is likely TCFAP2C regulates these two genes during germ cell development as well (Schwartz et al., 2007; Kuckenberg et al., 2010). This study sought to identify potential regulatory targets of TCFAP2C in germ cells during fetal gonad differentiation. These experiments will elucidate the role of *Tcfap2c* during germ cell development in mice. Overall, the research in this dissertation indicates that not only does *Tcfap2c* play a necessary role during germ cell specification, proliferation and migration, but required during fetal gonad differentiation.

Research Relevance

Tcfap2c has been indentified in multiple different tissues as an important regulator of gene expression. This transcription factor is especially interesting given the current research and emphasis to understand the pathways that regulate pluripotent stem cell (PSC) differentiation. PSCs, which include embryonic stem cells and induced pluripotent stem cells, are theoretically capable of generating any cell type from the three germ layers. These cells will be an indispensible tool for disease modeling and regenerative medicine in the future. Understanding how PSCs maintain a pluripotent state and how to differentiate them into specific cell fates will greatly increase quality of human life through repairing ageing and diseased tissues. Using induced pluripotent stem cells derived from a patients' own cell population will greatly reduce the chance of autoimmune rejection. This means that there will not be a need for

immunosuppression upon transplantation of the cells. Research that helps develop PSC reprogramming techniques will greatly advance the future clinical applications of PSCs. *Tcfap2c* will be a good candidate for such studies. *Tcfap2c* is observed many differentiating embryonic tissues as well as in some adult stem cell populations suggesting that this transcription factor plays an important role in differentiation and potentially stemness. This current research will help elucidate the role of *Tcfap2c* in germ cells, a unique cell line itself, and the role that it plays during the specification and differentiation of germ cells. As a transcription factor, *Tcfap2c* regulates the expression of its target genes. It is very likely that *Tcfap2c* plays a key role in regulating important genes necessary for differentiation. Indeed, *Tcfap2c* is seen in many cancers that have an less differentiated phenotype. These cancer cells have reverted to a less differentiated genetic state where genes that had been turned off are now expressed and other genes that were expressed have been suppressed. The reappearance of *Tcfap2c* in these less differentiated cells indicates that it plays a role in less differentiated cells rather than the terminally differentiated. *Tcfap2c* has been shown to play a role in regulating differentiation in the epidermis, mammary gland and now the germ cells. This transcription factor could indeed be a key in understanding the reprogramming network necessary to push PSCs from an undifferentiated state into one of these tissue fates.

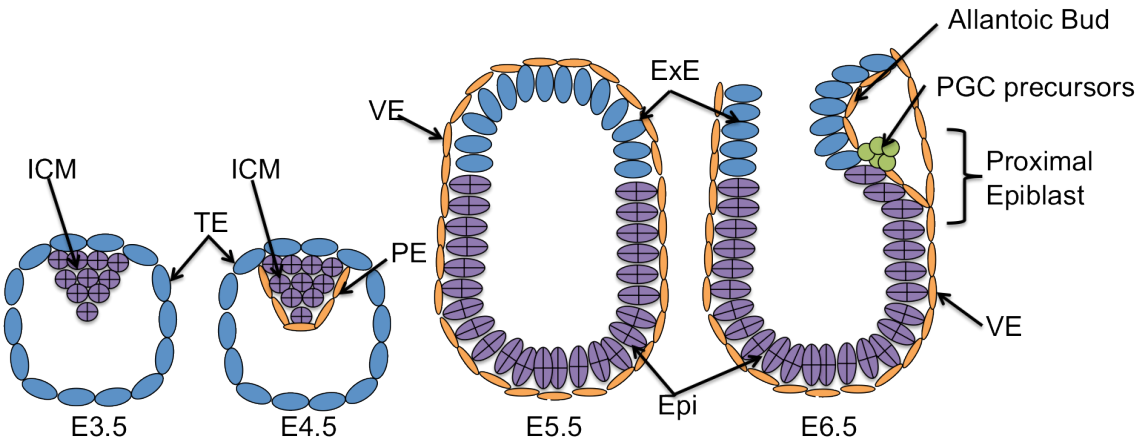


Figure 1 – Early Embryo Morphogenesis. Prior to specification at embryonic day (E)3.5, the blastocyst consists of the pluripotent inner cell mass (ICM; purple) and the trophectoderm (TE; blue). At E4.5, the primitive endoderm (PE; orange) lines the surface of the ICM. The extraembryonic ectoderm (ExE; blue) arises from the TE and surrounds the proamniotic cavity, while the primitive ectoderm begins to cavitate into a cup-like shape forming the epiblast (Epi; purple). The visceral endoderm surrounding the Epi and ExE arises from the PE and will provide differentiation signals to the tissues it contacts initiating PGC precursor formation (green).

TE – Trophectoderm

ICM – Inner Cell Mass

PE – Primitive Endoderm

Epi – Epiblast

VE – Visceral Endoderm

ExE – Extraembryonic Ectoderm

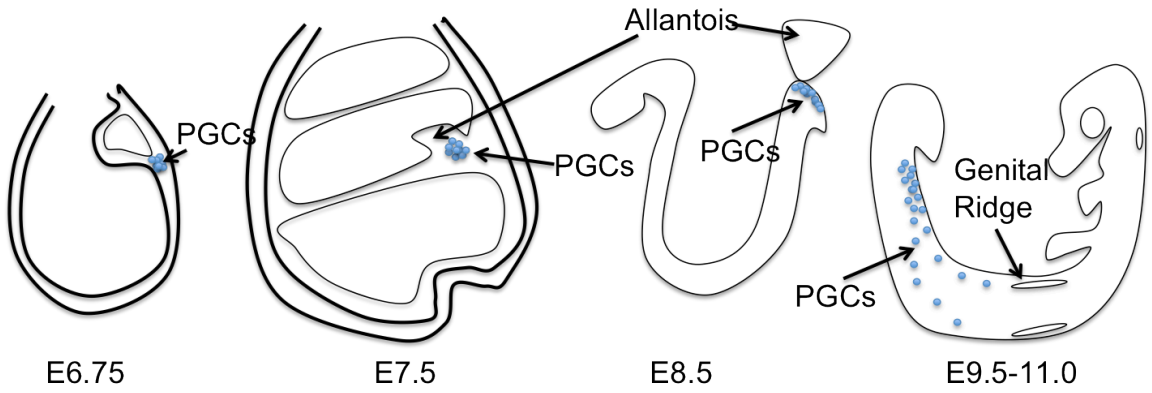


Figure 2 – Germ Cell Specification. Primordial germ cells (PGCs; blue) can first be identified as a few Prdm1-positive cells at the proximal end of the epiblast near the allantoic bud (E6.75). These cells proliferate and can now be indentified by tissue non-specific alkaline phosphatase (*TNAP*) expression (E7.5). Before the embryo turns, the PGCs continue to proliferate and begin to migrate away from the base of the allantois towards the hindgut (E8.5). From E9.5-11.5, the PGCs are passively moved during embryo turning and they actively migrate along the hindgut towards the genital ridges.

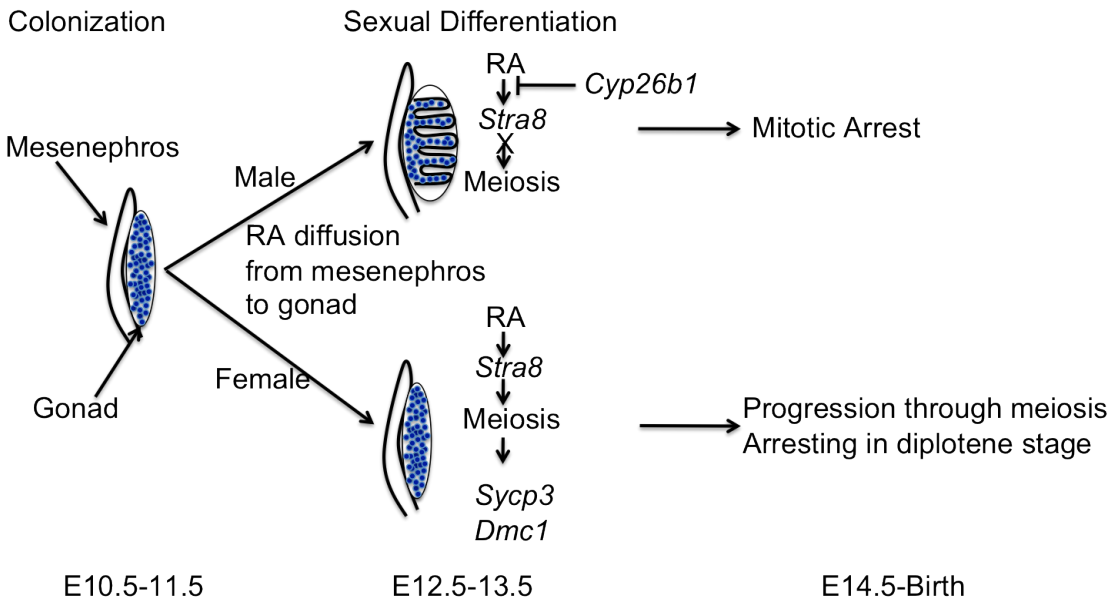


Figure 3 – Fetal Gonad Differentiation. After colonization, retinoic acid (RA) begins to diffuse into the gonad from the mesonephros. RA induces expression of *Stra8* and meiosis in PGCs in XX gonads, while *Cyp26b1* expressed in Sertoli cells of XY gonads degrade RA preventing meiosis in XY gonads. XY germ cells then undergo mitotic arrest, while XX germ cells will proceed into meiosis and arrest at the diplotene stage.

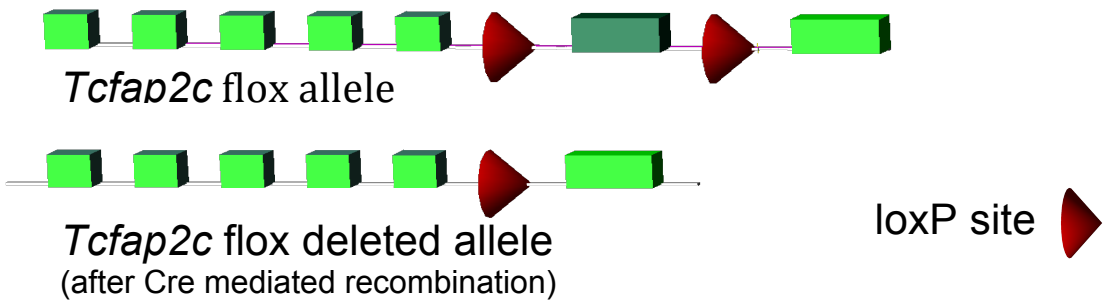


Figure 4 - Loss of function mutation of *Tcfap2c* achieved by Cre recombinase.

CHAPTER ONE

Introduction

Germ cell formation requires a unique set of events that are vital in producing the cell lineage responsible for continuation of the genome (Ohinata et al., 2005). In mice, primordial germ cells (PGCs) are first specified from surrounding somatic tissues, then proliferate and migrate to the fetal gonad and finally undergo differentiation during fetal and adult stages into mature gametes. These processes are each important hallmarks necessary for proper gamete production. Failure of any of these processes will result in infertility and failure of an individual to reproduce. The goal of this study is to determine the role of *Tcfap2c* during key stages of germ cell formation using conditional gene knock-out mouse models.

Identifying the role of *Tcfap2c* in germ cells may further elucidate the mechanisms controlling germ cell specification and differentiation. AP-2 transcription factors play critical roles during embryonic development by regulating proliferation and differentiation in many tissues (Iagawa et al., 1987; Hilger-Eversheim et al., 2000; Guttormsen et al., 2008). Within the murine germ line, *Tcfap2c* is first detected at E6.75 in primordial germ cells shortly after specification and has been classified as a potential “specification” gene

(Kurimoto et al., 2008). *Tcfap2c* continues to be detected in the germ cell population until E13.5 during embryogenesis (Kurimoto et al., 2008; Weber et al., 2010). Expression also is detected in ovulated mouse oocytes and in human germ cells within developing testes from weeks 12-37 of gestation (Hoei-Hansen et al., 2004; Pauls et al., 2005; Winger et al., 2006). These studies show that *Tcfap2c* is detected during all the key stages of germ cell development, namely specification, proliferation, migration, differentiation and spermatogenesis and oogenesis. Presence of *Tcfap2c* suggests it plays a regulatory role during these important development events. Therefore, we investigated the loss of *Tcfap2c* at each of these stages. The *Tcfap2c* knock-out mouse dies at E7.5 due to extraembryonic lineage defects (Auman et al., 2002). To avoid the embryonic lethal phenotype in *Tcfap2c* knock out mice and study the role of *Tcfap2c* in PGCs, we used conditional ablation of *Tcfap2c* specifically in the epiblast, adult mice or germ cells. These conditional mutations of *Tcfap2c* allowed us to determine if loss of *Tcfap2c* affects specification, proliferation, migration, initiation of meiosis in fetal gonads as well as affects spematogenesis and oogenesis. By characterizing the loss of *Tcfap2c* during these events, we show that *Tcfap2c* is necessary for the specification, proliferation and migration of germ cells during development.

Materials and Methods

Production of *Tcfap2c* deficient mice

Mice containing the *Tcfap2c* null allele have been previously described (Auman et al. 2002). Mice containing the *Tcfap2c* flox allele provide a conditional *Tcfap2c* allele containing loxP sites which flank exon 6, and allow for a gene specific deletion of *Tcfap2c* in the presence of Cre recombinase. Exon 6 of *Tcfap2c* was specifically targeted because it is a key component of the DNA binding and transcription factor dimerization motif. Loss of exon 6 prevents TCFAP2C from binding DNA and affecting target gene transcription (Winger et al., 2006; Guttormsen et al., 2008). The Cre recombinase will recognize the loxP sites in the *Tcfap2c* flox allele creating a *Tcfap2c* flox-deleted allele missing exon 6. In order to produce offspring which contained one of the Cre strains, *Tcfap2c* null and *Tcfap2c* flox, male mice were generated that contained both a Cre transgene as well as the *Tcfap2c* null allele (*Tcfap2c*$^{-}$). These mice were mated to female mice that were homozygous for *Tcfap2c* flox. Embryos inheriting the *Tcfap2c* null allele and the Cre transgene needed only one Cre-mediated recombination event per cell of the *Tcfap2c* flox allele to produce cells lacking functional *Tcfap2c* (*Tcfap2c*$^{-/-}$). Control littermates contained only the *Tcfap2c* flox allele (*Tcfap2c*$^{+/+}$). For timed pregnancies, specific matings were set up in the afternoon, and the mice were checked for vaginal plugs the following mornings. Noon of the day of the vaginal plug was considered embryonic day (E)0.5.

To examine the role of *Tcfap2c* in PGCs, *Tcfap2c* flox was deleted using one of three Cre recombinase transgenic mouse strains, *Sox2*-Cre, *ERTM*-Cre or *Prdm1*-Cre. Sox2-Cre mice were purchased from Jackson Laboratories (Strain Name: Stock Tg(Sox2-Cre)1Amc/J; Bar Harbor, ME). The Sox2-Cre mouse strain expresses Cre recombinase under the control of the Sox2 (SRY-box containing gene 2) promoter. Sox2, and therefore Cre recombinase in this transgenic line, is expressed in the epiblast cells at E6.5 with little to no activity in the extraembryonic tissues (Hayashi et al., 2002). ERTM-Cre mice were purchased from Jackson Laboratories (Strain Name Tg(UBC-cre/ESR1)1Ejb/J; Bar Harbor, ME). ERTM-Cre mice ubiquitously express Cre recombinase, but this recombinase is part of a Cre-Estrogen Receptor (ER) fusion protein that remains inactive until the receptor is bound by its ligand, 4-hydroxytamoxifen. The ER is a triple mutant form of the estrogen receptor, which does not bind the natural estrogen ligand, but instead binds the synthetic estrogen ligand 4-hydroxytamoxifen (OHT). Cre recombinase remains restricted to the cytoplasm until ER is bound by OHT at which time Cre recombinase is released and able to access the nucleus where Cre recombinase is able to remove floxed regions of *Tcfap2c*.

Prdm1-Cre mice were generously donated by Michel Nussenzweig from Rockefeller University. *Prdm1* (PR domain containing 1, with ZNF domain also known as B-lymphocyte-induced maturation protein-1, *Blimp1*) expression is detected at E6.25 in the proximal layer of the epiblast and *PRDM1* positive cells are lineage-restricted to PGC by E7.8. In the *Prdm1*-Cre model, Cre

recombinase transgene is inserted behind the *Prdm1* promoter restricting the expression of Cre to the PGC population at E7.8 (Ohinata et al. 2005). This allows all the other tissues that express *Tcfap2c* to develop without disruption of *Tcfap2c*.

For all experiments, negative or *Tcfap2c*$^{+/+}$ control mice were selected from littermates that only carried the *Tcfap2c* flox allele, but did not carry *Tcfap2c* null or a Cre transgene. These mice do not have any disrupted *Tcfap2c* alleles meaning they had normal expression of *Tcfap2c*.

Genotyping of mice was performed using PCR analysis of either placental yolk sac samples or tail DNA. PCR reactions were carried out for 35 cycles (94°C, 40 sec; 67°C, 40 sec; 72°C, 40 sec) in a reaction buffer containing, 200mM dNTPs, 1.5mM $MgCl_2$ and GoTaq polymerase (Promega, WI). Primer sets Cre1 and Cre3 were used to detect the Cre recombinase gene in all Cre mouse models. Neo3KO, XGamma3 and GammaWT5 were used to detect *Tcfap2c* wild-type (WT) and *Tcfap2c* null alleles. Gamma Flox 4, Gamma SQS3 and Gamma XBSQ were used to detect *Tcfap2c* flox and *Tcfap2c* flox-deleted alleles.

Genotyping

All reactions were run using the parameters described above.

Cre1:GCTGGTTAGCACCGCAGGTGTAGAG

Cre3:CGCCATCCTCCAGCAGGCGCACC

Cre allele gives a 430bp product

Neo3'KO: AACGCACGGGTGTTGGGTCGTTTGTTCG

XGamma3: CCTTCTGCTCTCTGGCCTCCTTGCAGCC

GammaWT5: TCATGGCTTTGGCAGCCAGGCCATC

Tcfap2c null allele gives a 144bp product

Tcfap2c WT product gives a 191bp product

Gamma Flox 4: GGACGGAATGAGATGGCCACGCGG

Gamma SQS3: CTTGGAACTCACTTTGTAGCTGAGG

Gamma XBSQ: GAGTTAGAGAGCAAGTTACAGGCTG

Tcfap2c flox gives a 500bp product

Tcfap2c WT gives a 430bp product

Tcfap2c flox-deleted gives a 250bp product

Detection *Tcfap2c* mRNA transcripts

To determine the expression of *Tcfap2c* during development, gonads were collected from E12.5, 13.5, 14.5, 15.5 and 18.5 embryos; embryo ages were assessed by tail somite numbers as described in “Manipulating the Mouse embryo” (2003). At least three embryos from each time point were assessed. Total RNA was collected from ovaries and testes in order to determine if *Tcfap2c* is expressed in these tissues. RNA was extracted using the RNeasy isolation kit according to manufacturer’s specifications (Qiagen, Santa Clarita, Ca). RNA samples were quantified using a ND-1000 nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was prepared from 1µg of total RNA using cDNA synthesis kit according to manufacturer’s instructions (Quanta Biosciences, Gaithersburg, MD). Genotyping of embryos to detect Cre and *Tcfap2c* null was performed using PCR analysis of either yolk sac samples

or tail DNA. RT-PCR reactions to detect *Tcfap2c* were carried out for 35 cycles (94°C, 40 sec; 67°C, 40 sec; 72°C, 40 sec) in a buffer containing 25mM $MgCl_2$. RT-PCR primer sequences were designed to anneal in exons 6 (*Tcfap2c* P1) and 7 (*Tcfap2c* P2) of the *Tcfap2c* gene, producing a 206 bp band for the WT allele. This primer set also was used to confirm complete deletion of *Tcfap2c* as exon 6 is excised in the *Tcfap2c* flox-deleted allele and exon 7 is truncated in the *Tcfap2c* null allele. These primers would not amplify *Tcfap2c* null or *Tcfap2c* flox-deleted alleles but would amplify WT and *Tcfap2c* flox alleles. These primers were used to ensure that the *Tcfap2c* mutant animals we created did not show the presence of functional *Tcfap2c* transcript.

Tcfap2c P1: AAGCGGTGGCTGACTATTTAAC

Tcfap2c P2: CAGGCTGAAATGAGACAAACAG

Detection of Germ Cell and Meiotic marker transcripts

To determine the presence of germ cell and meiotic markers during development, gonads were collected from E12.5 embryos. Genotyping of embryos to detect Cre and *Tcfap2c* null was performed using PCR analysis of tail DNA as described above. Five embryos of each genotype, $Tcfap2c^{+/+}$ and *Sox2*-Cre, $Tcfap2c^{-/-}$, meaning 10 gonads total were analyzed for mRNA expression. Total RNA was collected from ovaries and testes in order to determine if *Ifitm3, Pou5f1, Ddx4, Sycp3, Dmc1* and *Stra8* are present. RNA was extracted using the RNeasy isolation kit according to manufacturer's specifications (Qiagen, Santa Clarita, Ca). RNA samples were then quantified using a ND-1000 nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA

was prepared from 1µg of total RNA as described above. RT-PCR reactions to detect germ cell and meiotic markers were carried out for 35 cycles (94°C, 40 sec; 67°C, 40 sec; 72°C, 40 sec) in a buffer containing 25mM $MgCl_2$. Primers were designed to span intronic sequences ensuring that no genomic DNA was amplified. β-actin expression was used as a positive control.

Ifitim3 F:TGCCTTTGCTCCGCACCAT R:GGGTGAAGCACTTCAGGACC

Pou5f1 F:CTCGAACCACATCCTTCTCT R:GGCGTTCTCTTTGGAAAGGTGTTC

Ddx4 F:GCTCAAACAGGGTCTGGGAAG R:GGTTGATCAGTTCTCGAG

Sycp3 F: GAGCCGCTGAGCAAACATCTA R:ATATCCAGTTCCCACTGCTGC

Dmc1 F: GAAGGAGGATCAAGTTGTGC R:GCTTCATTTTCAGGCATCTC

Stra8 F:GTTTCCTGCGTGTTCCACAAG R:CACCCGAGGCTCAAGCTTC

β-actin F:GATGACGATATCGCTGCGCTG R:GTACGACCAGAGGCATACAGG

Histology and Immunohistochemistry

Gonads from E12.5 and 18.5 embryos and 30dpp (days post partum) mice were collected. Five embryos of each genotype, *Tcfap2c*$^{+/+}$ and *Sox2*-Cre, *Tcfap2c*$^{-/-}$, meaning 10 gonads were assessed. Gonads were washed in PBS and fixed in 4% paraformaldehyde (PFA) for one hour at 4°C. Samples were prepared using standard paraffin processing, embedded and sectioned in 5µm thick sections and mounted on glass slides. For routine histological analysis, slides were stained with hematoxylin and eosin using standard procedures. For immunohistochemistry and immunofluorescence, slides were deparaffinized and thoroughly washed in Tris-buffered saline with 1% tween 20 (TBST). Slides were then boiled in 10 mM sodium citrate for 5 minutes and washed in TBST. Addition

of the primary monoclonal IgG antibody for 12 hours (1:100; goat anti-NOBOX, a gift from Alexander Rajkovic from University of Pittsburgh; rabbit anti-SYCP3 from Novus NB300-231A1; rat anti-PECAM from BD pharmingen 550274; goat anti-GATA4 from Santa Cruz Biotechnology sc-1237) was followed by incubation with an appropriate HRP or fluorophore-conjugated polyclonal IgG secondary antibody for 1 hour (1:500). Slides were then rinsed dried and visualized at 20X using an Olympus BX40 microscope or LSM 510 Meta 405 Leica microscope (Leica Corporation) equipped with a Nikon Coolpix camera (Nikon Corp, Japan).

5-bromo-2'-deoxyuridine Detection

Three male *ERTM*-Cre,*Tcfap2c*$^{-/-}$ and control littermate mice were injected with .05mg/g of body weight with BrdU (Sigma) diluted in 0.9% sterile saline solution. Testes were collected 24 hours later and fixed in 4% PFA. Testes were then embedded and BrdU was detected using immunoprecipitation as described above (BrdU monoclonal IgG BD Pharmingen 555627).

Alkaline Phosphatase Staining of PGCs

Embryos were collected at E8.5, Reichert's membrane and the anterior half of the embryo were collected for genotyping. Five embryos of each genotype, *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ and *Tcfap2c*$^{+/+}$, were fixed in 4% PFA for 2 hours, washed three times in PBS and placed in 70% ethanol for 2 hours. The embryos were then stained in the following solution for 15 minutes; 25ml Tris Maleate buffer (3.6g Trizma base, 1L diH2O, pH 9.0), 200ml 10% $MgCl_2$, 10mg of Naphthol AS-MX phosphate, 25mg Fast Red TR salt (Sigma-Aldrich, MO).

Embryos were transferred to 70% glycerol, visualized on a Nikon Eclipse TS100 microscope, and photographed with a Nikon Digital Sight camera (Nikon, Japan).

Identifying Germ Cells using *Pou5f1* EGFP in *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice

The *Pou5f1*-EGFP (B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J; Jackson Laboratory, MA) transgene was bred into female *Tcfap2c* flox/flox mice, which were mated to *Prdm1*-Cre;*Tcfap2c*$^{-}$ males. These mice contain the EGFP coding sequence inserted behind the distal region of the *Pou5f1* promoter, and express EGFP specifically in the germ cell population starting at E7.75.Embryos were collected at E7.5, 9.5 and 12.5. Embryos and gonad-mesonephric complexes were dissected and placed into PBS. Placental tissues, Reichert's membrane or tail samples from the embryos were collected for genotyping. Five embryos and ten gonad-mesonephric complexes of each gender and genotype, *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ and *Tcfap2c*$^{+/+}$, were visualized for EGFP expression in 1,4-Diazabicyclo[2.2.2]octane using an Olympus SZX12 microscope (Olympus America Inc., PA), and photographed with an 18-2 Color Mosaic Camera from Diagnostic Instruments Inc (Diagnostic Instruments Inc., MI). E9.5 embryos were visualized using a LSM 510 Meta 405 Leica microscope (Leica Corporation) equipped with a Nikon Coolpix camera (Nikon Corp, Japan).

Results

Production of *Tcfap2c*$^{-/-}$ Mice

In order to produce mice mutant for *Tcfap2c*, males with a *Tcfap2c* null allele and a Cre transgene were bred to females homozygous for *Tcfap2c* flox. Cells of offspring inheriting both the *Tcfap2c* null allele and a Cre transgene from the male underwent a single recombinase event, mediated by Cre, of *Tcfap2c* flox allele inherited from the female. Cre recombinase causes deletion of exon 6 on the *Tcfap2c* flox allele producing the *Tcfap2c* flox-deleted allele and effectively removing any functional *Tcfap2c* (Figure 5A). Three different Cre recombinase transgenic mouse strains were employed, *Sox2*-Cre, *ERTM*-Cre and *Prdm1*-Cre. *Sox2*-Cre mice express Cre recombinase under the control of the *Sox2* promoter, which is expressed in the epiblast at E6.5 (Hayashi et al., 2002). *ERTM*-Cre mice have a ubiquitously expressed inducible form of Cre recombinase, gene recombination occurs after treatment of the mice with 4-hydroxytamoxifen (OHT). *Prdm1*-Cre mice express Cre recombinase under the control of the *Prdm1* promoter, which is expressed specifically in PGCs at E6.25 (Ohinata et al., 2005).

Primers were designed to specifically anneal in exon 6 and the latter half of exon 7. In mice that contain a *Tcfap2c* null allele and have undergone a recombination event producing *Tcfap2c* flox-deleted, no PCR product is made.

Loss of *Tcfap2c* was verified using RT-PCR in *Sox2*-Cre, *Tcfap2c*$^{-/-}$ E18.5 testes and E14.5 ovaries did not produce PCR product indicating that *Tcfap2c* had been completely deleted in these tissues (Figure 5B).

Tcfap2c expression was detected in E12.5 through 15.5 ovaries and testes in wild-type animals (Figure 5C). At E14.5, *Sox2*-Cre,*Tcfap2c*$^{-/-}$ ovaries had reduced expression of germ cell markers *Ifitm3*, *Ddx4* and *Pou5f1* compared to littermate controls, and expression of the meiotic markers *Sycp3, Dmc1* and *Stra8* was not detected (Figure 6). Furthermore, immunohistochemical analysis using NOBOX antibody failed to detect any germ cells in E18.5 *Sox2*-Cre,*Tcfap2c*$^{-/-}$ ovaries (Figure 7A). The meiotic marker SYCP3 was also not detected at E18.5 in *Sox2*-Cre,*Tcfap2c*$^{-/-}$ ovaries (Figure 7B). Further analysis of gonad development was not possible using the *Sox2*-Cre transgenic model because *Sox2*-Cre,*Tcfap2c*$^{-/-}$ animals die around the time of birth.

Tcfap2c Deletion Results in Loss of Germ Cells in Adult Gonads

To determine the effect of the *Tcfap2c* deletion during adult germ cell development, mice containing a *ERTM*-Cre allele were used to delete *Tcfap2c* flox alleles. Mice were injected intraperitoneally with OHT at 60 dpp and ovaries and testes were analyzed for occurrence of folliculogenesis and spermatogenesis 30 and 190 days post injection. Histological analysis of *ERTM*-Cre, *Tcfap2c*$^{-/-}$ ovaries did not show any differences compared to *Tcfap2c*$^{+/+}$ ovaries (Figure 8A). *ERTM*-Cre, *Tcfap2c*$^{-/-}$ testes did not show any difference in spermatogenesis and contained proliferative BrdU positive cells similar to *Tcfap2c*$^{+/+}$ testes (Figure 8B). Deletion of *Tcfap2c* after birth did not affect germ

cell maturation and *ERTM*-Cre, *Tcfap2c*$^{-/-}$ female and male mice were fertile and produced gametes containing the *Tcfap2c* flox-deleted allele several months after tamoxifen injections.

Tcfap2c Plays a Role in Early PGC Development

To further examine the role of *Tcfap2c* in PGCs, *Tcfap2c* flox was deleted specifically in germ cells using the *Prdm1*-Cre transgene. Unlike other models previously used to study the role of *Tcfap2c,* the *Prdm1*-Cre model expresses Cre recombinase solely in germ cells upon their specification and therefore deletes *Tcfap2c* flox only in this small population of cells (Ohinata et al., 2005). This allows all the other tissues that express *Tcfap2c* to develop without interruption of *Tcfap2c*. EGFP expression driven by a *Pou5f1* promoter was employed to identify germ cells from E7.5 until E14.5 (Yoshimizu et al., 1999). *Pou5f1*-EGFP expression was used to identify PGCs at E7.5 in *Prdm1-Cre*; *Tcfap2c*$^{-/-}$ and littermate control embryos (Figure 9). At E7.5, *Prdm1-Cre*, *Tcfap2c*$^{-/-}$ and wild-type littermates both display similar clusters of *Pou5f1*-EGFP positive PGCs. The presence of *Pou5f1*-EGFP positive cells in *Prdm1-Cre*; *Tcfap2c*$^{-/-}$ mutant embryos indicates that PGCs are specified at E7.5 similar to wild-type littermates.

At E8.5, presence of PGCs was examined by alkaline phosphatase staining shortly after specification in *Prdm1-Cre*; *Tcfap2c*$^{-/-}$ embryos. A small pool of ~10 alkaline phosphatase positive germ cells was detected at the base of the allantois in *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ embryos compared to over 100 in control littermates at E8.5 (6-8 somites; Figure 10). PGC-like cells in *Prdm1*-Cre,

Tcfap2c$^{-/-}$ embryos were all in a tight cluster without any cells migrating out as seen in control littermates (Figure 10). By E9.5, germ cells in *Pou5f1*-EGFP, *Tcfap2c*$^{+/+}$ embryos have proliferated and begun migration towards the fetal gonad (Figure 11). At E9.5, *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ embryos showed very few or no *Pou5f1*-EGFP PGC-like cells at all. The two *Pou5f1*-EGFP cells identified in the *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ embryo pictured had not begun migrating away from the base of the allantois (Figure 11).

In order to determine if the PGCs specified reach the gonad, E12.5 ovaries and testis were collected from *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ and *Tcfap2c*$^{+/+}$ embryos. Germ cells were identified by *Pou5f1*-EGFP expression in E12.5 gonads littermate controls, but no EGFP positive cells were detected in *Prdm1-Cre*, *Tcfap2c*$^{-/-}$ ovaries and testes (Figure 12). This result indicates that germ cells are not present in E12.5 gonads and were lost prior to E12.5.

In order to determine if germ cells were completely lost in *Prdm1-Cre*, *Tcfap2c*$^{-/-}$ mice, E12.5 gonads were analyzed for the presence of PECAM. PECAM expression is seen in germ cells and endothelial cells in gonads. PECAM positive germ cells can be seen in littermate controls, but while PECAM positive endothelial cells are seen in *Prdm1-Cre*, *Tcfap2c*$^{-/-}$ E12.5 ovaries, no PECAM positive germ cells are seen (Figure 13). GATA4, a somatic cell marker, was used to identify somatic cells in these ovaries. The somatic cells in the *Prdm1-Cre*, *Tcfap2c*$^{-/-}$ and the littermate controls appear the same (Figure 13). *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice were born alive and appeared morphologically normal. Internal reproductive tracts were noticeably smaller in female *Prdm1*-Cre,

Tcfap2c$^{-/-}$ mice compared to control littermates (Figure 14). Both ovaries and testes from *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice were smaller in size compared to littermate controls (Figure 14). Histological analysis revealed that at 30 dpp, *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ ovaries were devoid of oocytes, and germ cells were absent in 30dpp *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ testes (Figure 14).

Discussion

This study sought to elucidate the role of *Tcfap2c* during germ cell development. In order to bypass the embryonic lethality caused by extraembryonic defects that arise in traditional knock-out studies, we used Cre recombinase to produce *Tcfap2c* deletion in specific tissues.

We detected *Tcfap2c* expression from E12.5-15.5 in both ovaries and testes. *Tcfap2c* was initially identified in ovulated mouse oocytes and in human germ cells in developing testes (Hoei-Hansen et al., 2004; Pauls et al., 2005; Winger et al., 2006). Recently, *Tcfap2c* was identified as a potential "specification" gene because it is detected shortly after PGCs begin to be specified from surrounding somatic tissues (Kurimoto et al., 2008). A recent study also identified *Tcfap2c* in developing germ cells from E6.75 until E13.5 (Weber et al., 2010). We show that *Tcfap2c* expression in germ cells continues past E13.5 suggesting that *Tcfap2c* may play more than just a role in specification within germ cells.

In order to study the role of *Tcfap2c* in germ cells, we first used the epiblast specific *Sox2*-Cre mouse model; which allowed us to bypass the

extraembryonic lethality (Auman et al., 2004). *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries showed decreased expression of germ cell and meiotic markers at E12.5. This could be explained by a developmental delay in the germ cells or by a lack of germ cells altogether. Lack of SYCP3 positive cells in *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries indicates that meiotic cells are not present in E18.5 mutants. Normally, XX germ cells enter the first stage of meiosis between E12.5 and 15.5, if the *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries were delayed, we would expect to see a few meiotic cells at E18.5. The lack of SYCP3 in E18.5 *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries indicates that meiosis is not delayed, but rather suggests that germ cells are either unable to enter meiosis or not present. *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries were also devoid of NOBOX positive cells at E18.5. These results along with the lack of germ cell and meiotic markers indicate that *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries do not contain germ cells at E18.5.

The *Sox2*-Cre model produces embryos devoid of *Tcfap2c* starting around E6.5, but *Tcfap2c* is expressed in germ cells well past specification. In order to determine if *Tcfap2c* is necessary for germ cell maturation after birth, we employed the *ERTM*-Cre recombinase mouse model. *ERTM*-Cre does not cause deletion of *Tcfap2c* flox until the mice have been treated with tamoxifen. Mice that had *Tcfap2c* flox deleted in adult stages did not show any histological differences from littermate controls. Testes from *ERTM*-Cre, *Tcfap2c*$^{-/-}$ mice displayed proliferative cells. *ERTM*-Cre, *Tcfap2c*$^{-/-}$ animals were able to produce offspring of all genotypes including *Tcfap2c* flox deleted indicating that *Tcfap2c* is not necessary for oogenesis and spermatogenesis.

Tcfap2c is expressed in a variety of tissues during embryonic development, not just PGCs (Chazaud et al., 1996). Previous *Sox2*-Cre, *Tcfap2c* knock-out studies showed that *Tcfap2c* mutants were smaller than WT littermates and experience an epidermal developmental disruption (Guttormsen et al., 2008; Wang et al., 2008). The role of *Tcfap2c* in other tissues during embryonic development has not been fully explored. In order to ensure that the *Tcfap2c* gonad phenotype we observed in *Sox2*-Cre, *Tcfap2c*$^{-/-}$ mice was due to the loss of *Tcfap2c* in PGCs, we employed a germ cell specific deletion of *Tcfap2c* using *Prdm1*-Cre.

At E7.5 *Prdm1*-Cre,*Tcfap2c*$^{-/-}$ contained *Pou5f1* driven EGFP positive PGCs. *Pou5f1* expression is seen early in PGC specification and has been shown to promote pluripotency in both PGCs and embryonic stem cells (Kehler et al., 2004). The presence of *Pou5f1*-EGFP positive cells at E7.5 indicates that *Tcfap2c*$^{-/-}$ null embryos initially specify PGCs or PGC-like cells that begin to upregulate pluripotency markers. Weber et al., also observed upregulation of *Prdm1* and *Dppa3* in *Tcfap2c*-deficient PGC-resembling cells *in vitro* (2010). At E8.5 alkaline phosphatase positive cells also are observed in *Prdm1*-Cre,*Tcfap2c*$^{-/-}$ embryos; however, the number of positive cells in mutants was reduced and they had not initiated migration away from the allantois. Similar results were observed in *Sox2*-Cre, *Tcfap2c*$^{-/-}$ mice (Weber et al., 2010). The reduced number of PGCs at E8.5 indicates that loss of *Tcfap2c* either inhibits proliferation of the PGCs or prevents PGCs from activating the correct genetic program necessary for PGC survival after specification. At E9.5 *Prdm1*-Cre,

Tcfap2c$^{-/-}$ embryos showed few if any PGCs, and the few PGCs we observed in these embryos had not begun to migrate. These results indicate that *Tcfap2c* is necessary for not only PGC proliferation, but the few surviving cells are incapable of initiating migration towards the gonads. It is likely that *Tcfap2c* plays an important role in regulating the PGC specification and genetic re-patterning of PGCs.

In littermate control embryos, germ cells are specified, proliferate and then migrate to the gonad where they differentiate. Few PGCs are specified in *Tcfap2c* deficient embryos, but none were observed in the E12.5 gonad. *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ ovaries and testes were not positive for *Pou5f1*-EGFP positive cells at E12.5 indicating that germ cells were not present. Lack of PECAM positive germ cells provides further evidence that *Tcfap2c*$^{-/-}$ gonads do not contain germ cells at E12.5. Not surprisingly, adult *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice had noticeably smaller ovaries and testes. This size difference is explained by the lack of germ cells in *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice.

These results indicate that *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ germ cells fail to reach the gonad and are being lost prior to colonization at E10.5. A similar phenotype is observed in mice deficient for *Kit*, a germ cell surface signaling molecule. *Kit* mutants display normal numbers of PGCs at specification, however they fail to proliferate after E8.5 and never reach the gonad resulting in infertile mice (Buehr et al., 1993; Manova et al., 1991). The majority of germ cells that are specified in *Kit* mutants form clumps and fail to leave the base of the allantois. The few cells that do migrate in *Kit* mutants fail to reach the gonad and ultimately undergo

apoptosis (Besmer et al., 1993; Matsui et al., 1991). A similar situation could be occurring in *Tcfap2c* deficient mice. Our study showed PGCs at the base of the allantois, but they did not appear to be migrating at E8.5 or 9.5 and were absent by E12.5. In fact, another study using alkaline phosphatase also showed that *Sox2*-Cre, *Tcfap2c*$^{-/-}$ mice loose PGCs between E8.0 and 9.0 (Weber et al., 2010). The exact mechanism leading to the disappearance of germ cells in *Tcfap2c*$^{-/-}$ is still unknown. However, it does not appear to be due to an increase in cell death (Weber et al., 2010). In fact, in vitro studies indicate that repression of *Tcfap2c* leads to upregulation of somatic markers suggesting that *Tcfap2c* functions to repress somatic fate in early PGCs (Weber et al., 2010). If *Tcfap2c* is necessary to repress somatic cell fate, the loss of *Tcfap2c* in PGCs in the *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ embryos could lead to increased levels of somatic markers causing PGCs not to undergo apoptosis but rather re-initiate the somatic fate and loose the PGC fate.

PGCs are one of the few lineages produced during embryogenesis that, like ES cells, are pluripotent. PGCs are derived from surrounding somatic tissues in the presence of the correct signals. Many recent studies have sought to further elucidate the transcriptional network necessary for PGC specification and establishment of pluripotency, but the exact factors and how they interact is still largely unknown.

Our study identifies *Tcfap2c* as a factor that plays an important role during germ cell development in mouse embryos. PGCs in *Tcfap2c* mutants are specified, but never reach the gonad. It is possible that the *Pou5f1*-EGFP and

alkaline phosphatase positive cells observed in *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ embryos were not fully specified PGCs, but rather PGC-like precursor cells. Loss of *Prdm1* produces a similar phenotype. *Prdm1* mutant mice initially specify fewer PGCs, and the few PGCs that are identified do not proliferate. The PGCs that are present in these mutants fail to suppress Hox genes and do not upregulate PGC genes such as *Dppa3* (Ohinata et al., 2005; Vincent et al., 2005). Loss of *Prdm1* results in aberrant transcriptional signaling in PGCs resulting in a loss of the germ cell population. It appears that loss of *Tcfap2c* also results in disregulation of PGC genetic networks also leading to a loss of PGCs. However, the transcriptional regulator *Pou5f1* does not appear to be effected in *Tcfap2c* mutants. *Pou5f1* expression was observed in *Tcfap2c* mutant embryos indicating that *Pou5f1* is either not a direct target of *Tcfap2c* or loss of *Tcfap2c* is not sufficient to prevent *Pou5f1* expression during PGC specification. *Pou5f1* has been identified in embryonic stem cells as a transcription factor that promotes pluripotency. Conditional germ cell ablation of *Pou5f1* results in normal specification but PGCs undergo apoptosis and are gone by E10.5 (Kehler et al., 2004). The presence of *Pou5f1* in *Tcfap2c* mutant PGCs suggests that these two transcription factors likely function independently.

This study identifies a role for *Tcfap2c* during PGC specification and differentiation. Loss of *Tcfap2c* during embryonic development results in a severe germ cell phenotype where PGCs are lost prior to E12.5. However, the exact regulatory role of *Tcfap2c* has not yet been fully elucidated. Also, *Tcfap2c* is seen well past specification and is potentially playing an important regulatory role

during germ cell differentiation in XX and XY gonads. Determining the molecular regulatory pathways influenced by *TCFAP2C* will provide important insight into understanding the genesis of gametes. Chapter two details the experiments that we conducted to further elucidate direct targets of TCFAP2C and the pathways that might be effected by TCFAP2C in germ cells during fetal gonad development.

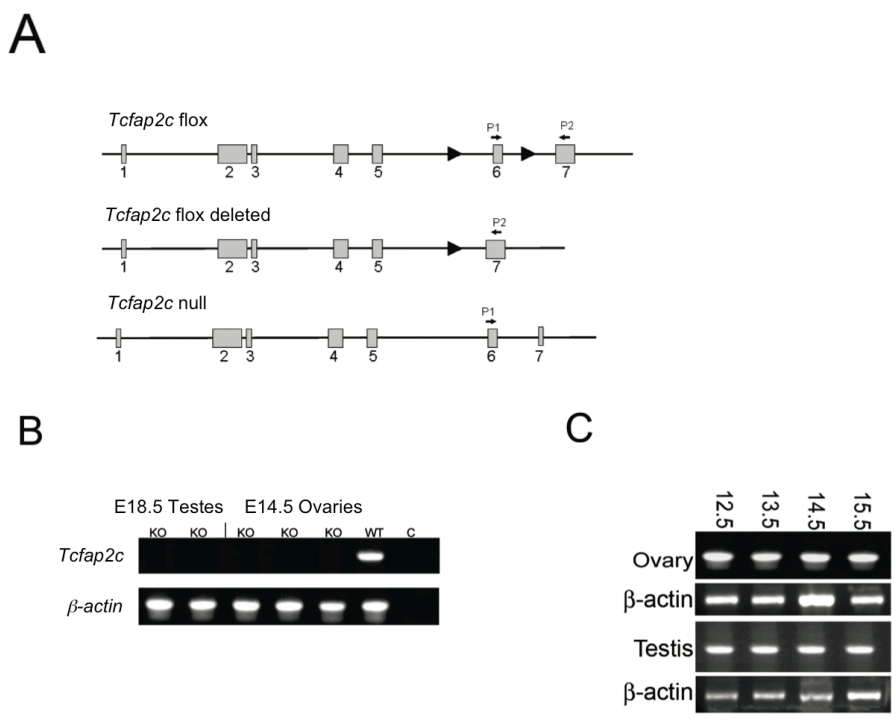


Figure 5 – Deletion of *Tcfap2c*. A) Schematic representation of the *Tcfap2c* flox allele with loxP sites (black arrow heads) surrounding exon 6 (top). After exposure to Cre recombinase, exon 6 and one of the loxP sites have been excised creating the*Tcfap2c* flox deleted allele (middle). The *Tcfap2c* null allele has a truncated exon 7 (bottom). *Tcfap2c* flox deleted and *Tcfap2c* null alleles produce a nonfunctional TCFAP2C protein. *Tcfap2c*$^{-/-}$ animals contain a Cre recombinase transgene, *Tcfap2c* null allele and a *Tcfap2c* flox allele, which is deleted upon exposure to Cre recombinase. P1 and P2 indicate the location of primers that were designed to detect undeleted *Tcfap2c.* B) RT-PCR using the primers indicated in A verified complete deletion of *Tcfap2c* in E18.5 testes and E14.5 Ovaries. C) *Tcfap2c* expression is detected by RT-PCR in wild-type E12.5-15.5 ovaries and testes.

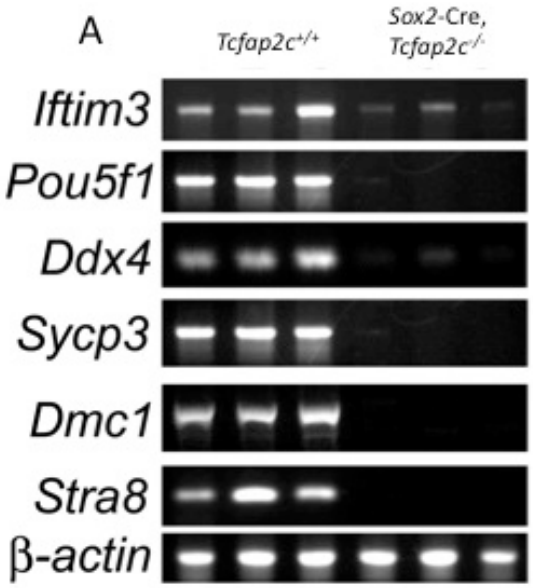
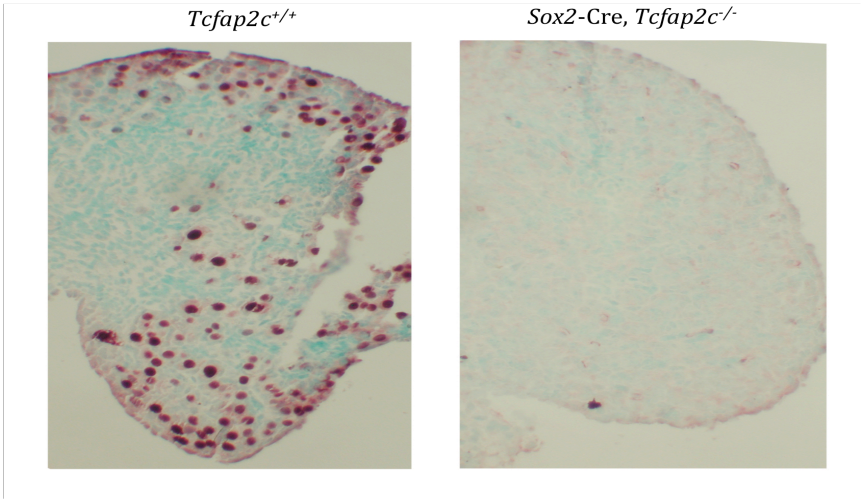


Figure 6 – *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries express lower levels of germ cell and meiotic markers. RT-PCR on E12.5 *Tcfap2c*$^{+/+}$ and *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries show lower levels of the germ cell markers *Ifitm3, Pou5f1* and *Ddx4* and the meiotic markers *Sycp3, Dmc1* and *Stra8*.

A



B

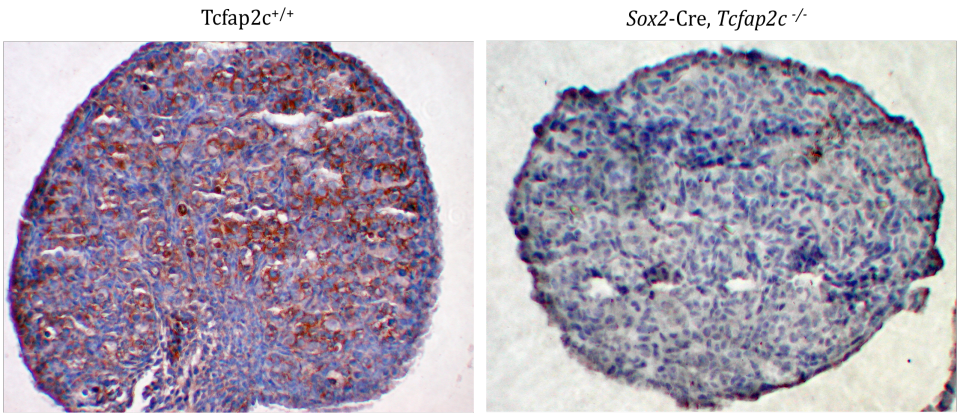
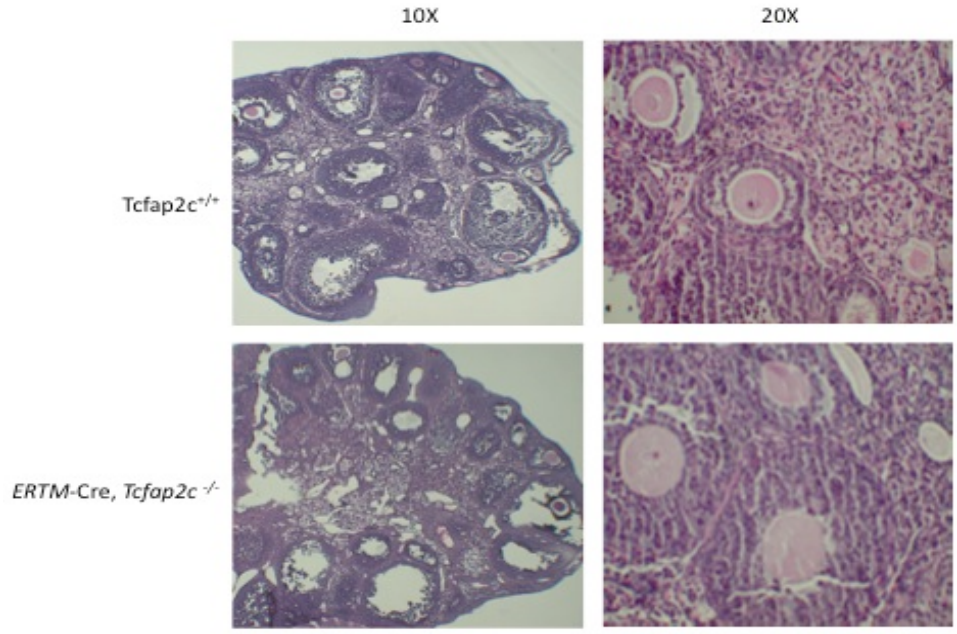


Figure 7 - *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries do not express the germ cell marker NOBOX or the meiotic marker SYCP3. A) E18.5 *Sox2*-Cre, *Tcfap2c*$^{-/-}$ ovaries do not contain any NOBOX positive germ cells (pink). B) E18.5 *Sox2*-Cre, *Tcfap2c*$^{-/-}$ do not have any SYCP3 positive germ cells (brown) when compared to *Tcfap2c*$^{+/+}$. 20X

A



B

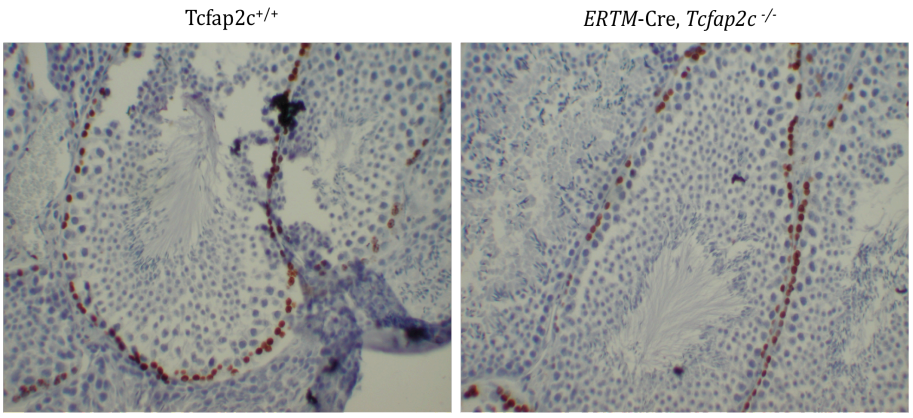


Figure 8 – Loss of *Tcfap2c* after birth does not affect spermatogenesis or oogenesis. A) 30 dpp *ERTM*-Cre, *Tcfap2c*$^{-/-}$ ovaries display folliculogenesis and normal ovarian histology. B) Spermatogenesis is not disrupted in adult *ERTM*-Cre, *Tcfap2c*$^{-/-}$ testes at day 30dpp. Proliferative BrdU positive (brown) cells are seen in both *ERTM*-Cre, *Tcfap2c*$^{-/-}$ and *Tcfap2c*$^{+/+}$ testes.

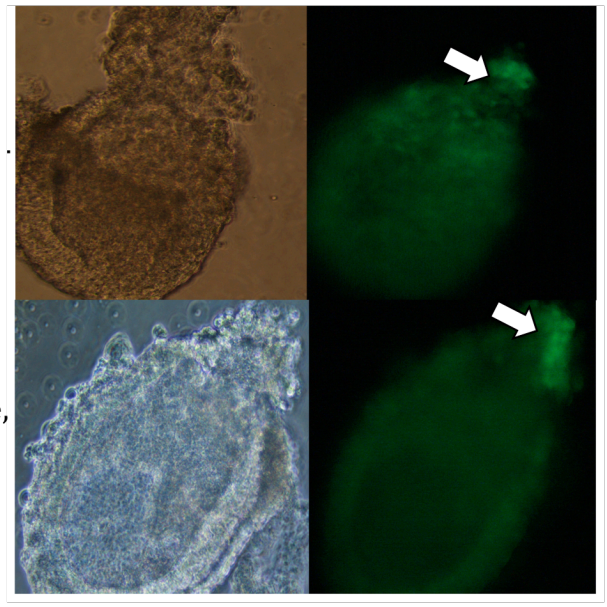


Figure 9 – Germ cells are specified in *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice. E7.5 Oct4-EGFP positive *Tcfap2c*$^{+/+}$ control littermates and *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ embryos both display a cluster of EGFP positive germ cells in the epiblast. EGFP positive primordial germ cells are indicated with arrows.

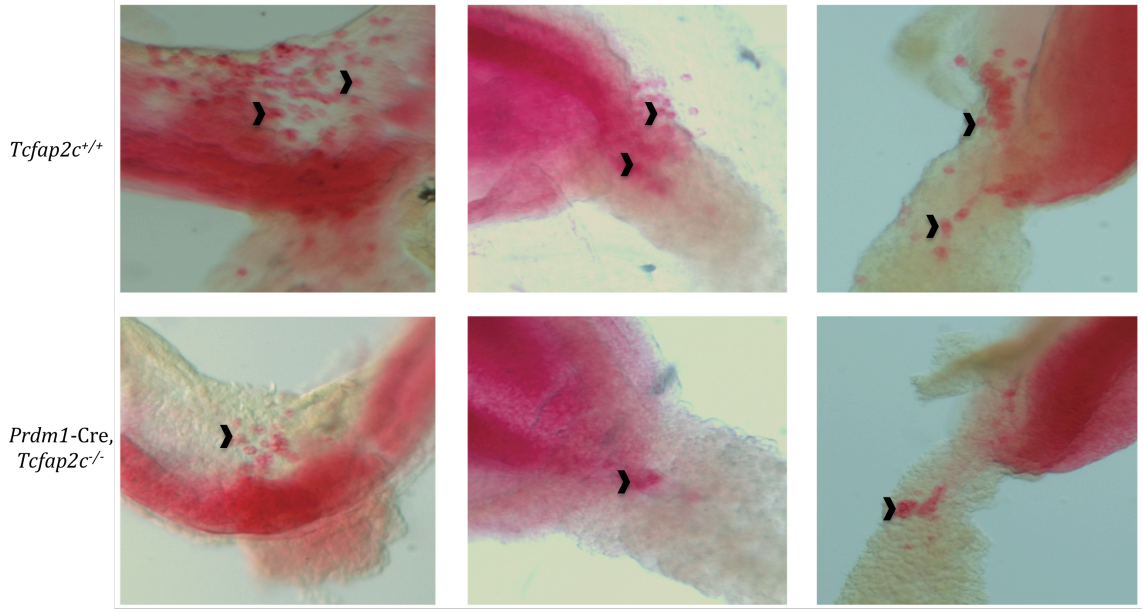


Figure 10 – *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice have fewer germ cells at E8.5 than littermate controls. Whole-mount alkaline phosphatase staining in E8.5 *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice and control littermates. Arrowheads point to alkaline phosphatase positive primordial germ cells.

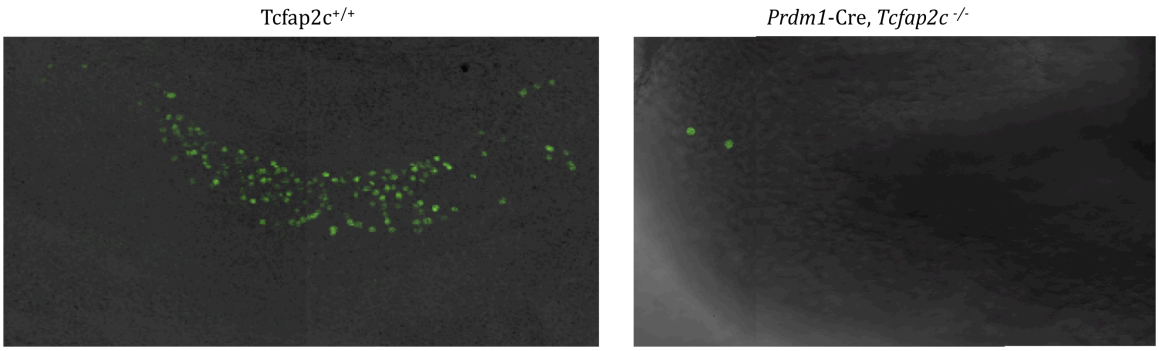


Figure 11 - *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ germ cells do not proliferate or migrate by E9.5. *Pou5f1*-EGFP expression (green) in germ cells of *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ and *Tcfap2c*$^{+/+}$ littermate control at E9.5. Posterior end of embryo to the right and anterior end toward the left. 20X

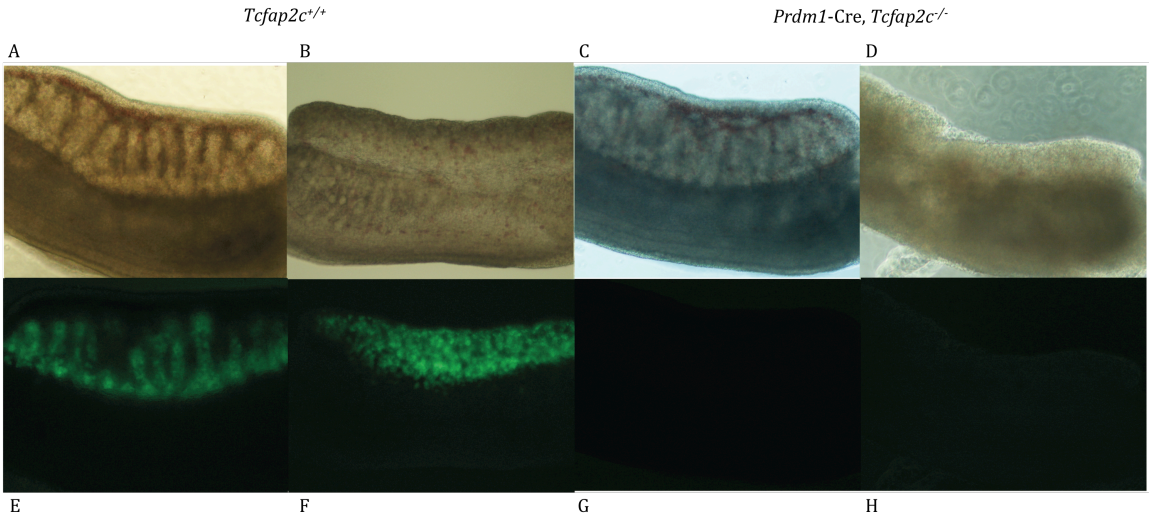


Figure 12 – *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ lack germ cells at E12.5. *Pou5f1*-EGFP expression in *Tcfap2c*$^{+/+}$ littermate control (A,B,E and F) and *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mutant (C,D,G,and H) E12.5 gonads. Brightfield (top) and FITC filter (bottom). 10X

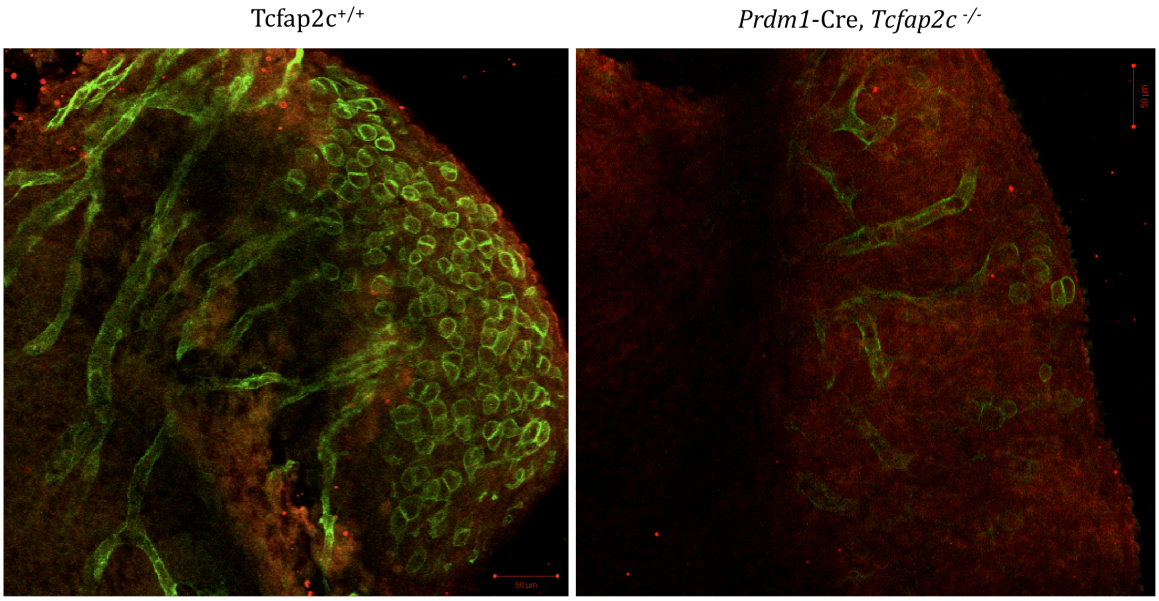


Figure 13 - *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice lack PECAM positive germ cells at E12.5. E12.5 *Tcfap2c*$^{+/+}$ gonads show PECAM positive germ cells and endothelium (green) and GATA4 positive somatic tissue (red). *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ displays some PECAM positive endothelium and very few if any germ cells. 20X

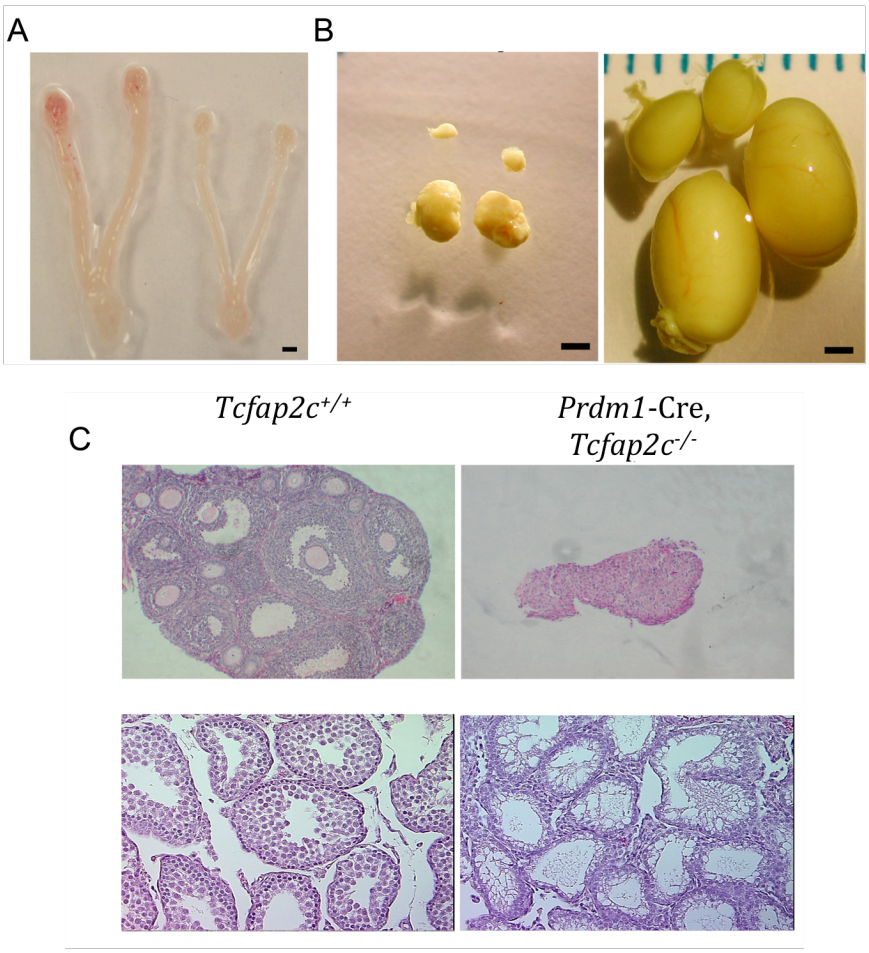


Figure 14 – *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice lack mature gametes. Gonad morphology of adult *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice. A) Female reproductive tracks of *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mutant and *Tcfap2c*$^{+/+}$ littermate control. B) Ovaries and Testes of *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mutants (top) and *Tcfap2c*$^{+/+}$ wild-type controls (bottom) at 30dpp. B) Histology of ovaries (top) and testes (bottom) from *Tcfap2c*$^{+/+}$ wild-type controls and *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mutants. 10X

CHAPTER TWO

Introduction

AP-2 transcription factors play critical roles during embryonic development by regulating proliferation and differentiation in many tissues (Iagawa et al., 1987; Hilger-Eversheim et al., 2000; Guttormsen et al., 2008). Within the murine germ line, *Tcfap2c* is first detected at E6.75 in primordial germ cells and continues to be detected in the germ cell population until E13.5 during embryogenesis (Kurimoto et al., 2008; Weber et al., 2010). In Chapter one we detected *Tcfap2c* in embryonic testes from E11.5-15.5. Expression also is detected in ovulated mouse oocytes and in human germ cells in developing testes from weeks 12-37 of gestation (Hoei-Hansen et al., 2004; Pauls et al., 2005; Winger et al., 2006). During PGC specification, *Tcfap2c* is expressed shortly after PGCs are identified from surrounding somatic tissues and has been classified as a potential "specification" gene (Kurimoto et al., 2008). In Chapter one we show that loss of *Tcfap2c* leads to a loss of germ cells indicating that *Tcfap2c* plays an important role in regulating germ cell development in mice. Another recent study showed that loss of *Tcfap2c* in the epiblast by conditional gene knock-out leads to reduction of PGC numbers at E8.5 and adult *Tcfap2c* mutants lack mature gametes (Weber et al., 2010). These studies show that *Tcfap2c* is indeed an important regulator of germ cell specification, proliferation and migration, but was

not necessary for adult spermatogenesis and oogenesis. However, *Tcfap2c* expression is observed in germ cells throughout development and not only during early PGC specification. In order to further explore the role of *Tcfap2c* in germ cells, the second goal of this study was to determine if *Tcfap2c* plays a role during fetal gonad differentiation.

After PGCs are specified, they proliferate and migrate to the genital ridge by E11.5 (Ginsburg et al., 1990; Ohinata et al., 2005). Between E10.5 and 14.5 germ cells begin to differentiate based on cell-autonomous and somatic cues and ultimately result in meiotic arrest of XX germ cells and mitotic arrest of XY germ cells (Hilscher, 1974; Setchell and Main, 1978; Western et al., 2008). The sex determination events that occur from E11.5-14.5 are of paramount importance in producing functional gametes. The exact machinery and mechanisms controlling the differentiation of germ cells during fetal gonad development is still not completely understood.

During specification and differentiation, germ cells re-establish pluripotency. Several genes that have been identified as pluripotency factors in embryonic stem cells also are upregulated during germ cell development, such as *Lin28*, *Nanog*, and *Hmga2*. *Lin28* is upregulated in PGCs at E7.5 and expression has been observed in germ cells throughout specification, proliferation, migration and gonad colonization (West et al., 2009). *Lin28* also has been observed in mouse type A primitive spermatogonia and in adult human oocytes (Assou et al., 2009; Zheng et al., 2009). LIN28 is responsible for processing let7 microRNAs into their mature and functional form. The let7

microRNA family regulates target gene expression by recognizing and degrading targeted transcripts prior to translation. Let7 microRNAs have been shown to regulate expression levels of *Hmga2* and *Prdm1* (Mayr et al., 2007; Ohinata et al., 2008). Loss of *Lin28* in the germ cells leads to decreased levels of *Prdm1*, a master regulator of PGC specification necessary for transcriptional activation of several germ cell specific genes (Ohinata et al., 2008; West et al., 2009). *Nanog*, another gene implicated in regulating pluripotency, has been extensively studied in embryonic stem cells and is required to maintain pluripotency of these populations. *Nanog* deficient embryos fail to form an epiblast due to loss of pluripotency (Mitsui et al., 2003). In germ cells, *Nanog* expression is detected shortly after specification at E7.5 and continues to be expressed until E14.5 and E16.5 in XX and XY germ cells respectively. *Nanog* expression is downregulated upon initiation into meiosis in XX germ cells, or mitotic arrest in XY germ cells (Yamaguchi et al., 2005). Loss of *Nanog* in germ cells leads to decreased numbers of PGCs at specification and complete loss of germ cells by E11.5 due to apoptosis suggesting that *Nanog* also acts to regulate germ cell proliferation and survival as it does in embryonic stem cells (Yamaguchi et al., 2009). Interestingly, TCFAP2C has been implicated in the regulation of *Nanog*. In trophoblast stem cells, direct binding of TCFAP2C to the *Nanog* promoter results in repression of *Nanog* (Kuckenberg et al., 2010). Another gene involved in regulating cell transcription, *Hmga2* is a DNA binding protein that plays a role in cell proliferation and differentiation in many tissues during embryonic development. Specifically, *Hmga2* has been shown to play a role in chromatin

remodeling and regulation of microRNA levels (Pfannkuche et al., 2009). HMGA2 binds AT-rich sequences within the chromatin functionally changing the DNA architecture altering access to promoter regions of genes. This architectural change can result in either repression or initiation of gene expression, but often these proteins herald a pluripotent transcriptional state. That is why aberrant expression of *Hmga2* in adult tissues often is associated with tumorigenesis (Pfannkuche et al., 2009). Expression of *Hmga2* is seen in most embryonic tissues between E10.5 and 15.5 and mice lacking *Hmga2* are approximately 50% smaller due to a lack of proliferation (Zhou et al., 1996). *Hmga2* has not been extensively studied in germ cell lineages, but expression has been seen in Xenopus laevis oocytes and spermatogonia as well as in mouse spermatogonia (Agostino et al., 2004; Hock et al., 2006). Interestingly, male mice lacking *Hmga2* are sterile due to disrupted spermatogenesis suggesting that *Hmga2* plays an important role during germ cell development (Hock et al., 2006).

Germ cell differentiation requires the coordination of many transcriptional and genetic networks. Along with genes regulating pluripotency, genes such as *Kit* and *Cdh1* play integral roles in PGC differentiation, survival and cell-cell interaction. *Kit* is a cell surface protein that is involved in PGC communication with surrounding somatic tissues. Expression of *Kit* is seen from E7.5 through to E14.0 when XX germ cells enter meiosis and XY cells enter mitotic arrest and expression is re-initiated in both sexes after birth (Manova et al., 1990; Manova and Bacharova 1990). Loss of *Kit* results in a lack of germ cell proliferation and failure to migrate resulting in sterile mice (Buehr et al., 1993, Manova et al.,

1991). Another factor involved in cell-cell interaction within the PGC population is *Cdh1*. A cell surface receptor that mediates cell-cell adhesion between germ cells, *Cdh1* expression is observed in germ cells from E8.5 until 14.5 (Bendel-Stenzel et al., 2000; Okamura et al., 2003). *Cdh1* functions to adhere PGCs to one another, forming clusters during specification, migration and colonization of the gonad, loss of *Cdh1* results in loss of germ cells by E11.5 (Okamura et al., 2003). TCFAP2C has been shown to directly interact with the *Cdh1* promoter in human breast cancer cells, colon cancer cells and MDCK kidney epithelial cells (Baldi et al., 2001; Decary et al., 2002; Dela Croix et al., 2005; Schwartz et al., 2007). Considering the importance of the pluripotency genes, *Lin28*, *Nanog* and *Hmga2*, as well as cell surface molecules, *Kit* and *Cdh1*, during germ cell development it is necessary to determine if TCFAP2C plays a role in regulating expression of these genes during germ cell differentiation.

In Chapter 1 we described the germ cell phenotype observed in *Tcfap2c* null mice. Germ cells were specified but failed to migrate and were lost by E12.5. Previous studies have focused on the role of *Tcfap2c* during PGC specification (Kurimoto et al., 2010; Weber et al., 2010). *Tcfap2c* has been shown to play an important role during specification; however, *Tcfap2c* expression continues in germ cells well past the time of PGC specification. This study sought to elucidate the role of *Tcfap2c* in germ cell differentiation after PGC specification and migration. To better define the functional role of TCFAP2C and identify target genes, we used chromatin immunoprecipitation to identify genomic interactions of TCFAP2C in germ cells at E12.5, a time when both XX and XY gonads are

undergoing drastic changes. Our data indicate that TCFAP2C plays an important role not only during PGC specification, but also during germ cell differentiation.

Materials and Methods

Microarray Data

Microarray data profiling gene expression of E12.5 pre-granulosa cells, Sertoli cells and whole gonads was obtained from the study described in Bouma et al., (2010).

Real-time PCR Expression analysis and Flow Cytometric isolation of PGCs

Matings were set up in the afternoon and females were checked for copulatory vaginal plugs the following morning. Noon of the day a vaginal plug was detected was considered embryonic day (E)0.5 days of gestation. Five XX and XY E12.5 wild-type embryos were dissected and gonads collected, separated from mesonephros and pooled, so each pool contained either 10 XX or XY gonads. Three independent pools were created and the data represents the average of all three pools. Age and sex were determined morphologically as described in 'Manipulating the Mouse Embryo' (2003). Total RNA was isolated using the RNeasy Mini *Kit* according to manufacturer's instructions (Qiagen, CA) and 1μg of total RNA was reverse transcribed into cDNA using the qScript cDNA synthesis *Kit* (Quanta Biosciences, MD). Gene expression levels of *Tcfap2a*, *Tcfap2b*, *Tcfap2c*, *Tcfap2d, Tcfap2e, Kitl, Kit, Hmga2, Lin28, Cdh1, Nanog* and *Dppa3* were evaluated using real-time PCR. Gene specific taqman probes and

reaction buffer were acquired from Applied Biosystems and reactions run according to manufacturer's specifications. Reactions were run on a Biorad iCycler (2 min 50˚C, 10 min 95˚C, cycle 40 times at 15 sec 95˚C and 1 min 60˚C). Relative expression level of transcripts was determined by calculating the average of *Gapdh* expression values and using this as a normalization factor. Statistical differences were assessed at $P < 0.05$ using a Students t-test. Relative expression levels were presented by plotting mean $2^{-\Delta Cp}$ values (Schmittgen and Livak 2008).

Pou5f1-EGFP (B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J) mice were purchased from The Jackson Laboratory (MA). These mice contain the EGFP coding sequence inserted behind the *Pou5f1* promoter, and express EGFP specifically in the germ cell population starting at E7.75. XX and XY gonad-mesonephric complexes were collected from 10 litters of E12.5 F1 (Black Swiss x *Pou5f1*-EGFP; n=7) embryos and placed into phosphobuffered saline (PBS). Mesonephroi were dissected away from the gonad, and XX and XY gonads were pooled separately, disassociated in 500μL pre-warmed trypsin-EDTA (Sigma-Aldrich, MO) for 15 minutes with occasional agitation. 500μL of pre-warmed media was then added to stop trypsin digestion. The cell suspensions were filtered through a 10μm porous film and EGFP positive cells were isolated using a MoFlo Flow Cytometer and High Speed Cell Sorter (Dako Inc., CO). Cells were sorted into 500μL of Lysis Buffer (Qiagen, CA). Total RNA from EGFP positive germ cells was isolated using the RNeasy Mini *Kit* (Qiagen, CA) and cDNA was prepared as described above. Real time PCR analysis was conducted

to compare mRNA levels for the *Tcfap2* family members, the germ cell marker gene *Dppa3*, and the somatic marker gene *KitL* in whole gonads, isolated germ cells and gonadal somatic cells using Taqman probes (Applied Biosystems, CA). Experiments were run on seven pools of either XX either XY isolated germ cell and somatic cell from a litter, at least five gonad pairs went into each pool. Real-time PCR analysis was performed as previously described. Statistical differences were assessed at $P<0.05$ using a Student's t-test. Relative expression levels were presented by plotting $2^{-\Delta Cp}$ values (Schmittgen and Livak 2008).

Taqman Probe IDs (Applied Biosystems, CA)

Tcfap2a – Mm00495574_m1*

Tcfap2b – Mm00493468_m1*

Tcfap2c – Mm00493473_m1*

Tcfap2d – Mm00462523_m1*

Tcfap2e – Mm01179789_m1*

Lin28 – Mm00524077_m1*

Hmga2 – Mm00780304_sH

Nanog – Mm02019550_s1*

Cdh1 – Mm01247357_m1*

Kit – Mm00445212_m1*

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described in Dahl and Collas (2008). Briefly, E12.5 Black Swiss XX and XY gonads were dissected away from the mesonephros and ten pairs of gonads were pooled by

sex in cold PBS and proteins cross-linked to DNA in 37% formaldehyde at room temp for 10 minutes. Fixation was stopped by quenching samples in 125mM Glycine for 5 minutes. Samples were washed in PBS and then lysed in buffer (50mM Tris-HCl pH8.0, 10mM EDTA, 1% SDS, protease inhibitor mix and 1mM PMSF). Samples were sonicated for 10 minutes using a Bioruptor (Diagenode, NJ). RIPA ChIP buffer was added (10mM Tris-HCl pH7.5, 140mM NaCl, 1mM EDTA, 0.5mM EGTA, 1%(v/v) Triton X-100, 0.1%(w/v) SDS, protease inhibitor mix and 1mM PMSF) and samples centrifuged for 10 minutes at 2500rmp. Supernatant was collected and RIPA ChIP buffer added. Input DNA was separated from the sample prior to immunoprecipitation. Immunoprecipitation was performed using TCFAP2C antibodies (Santa Cruz Biotechnologies sc-31935) and the Active Motif ChIP-IT Express Chromatin Immunoprecipitation *Kit* (Active Motif, Carlsbad, CA). Immunoprecipitated and input DNA was isolated and purified using the Gentra Puregene *Kit* (Qiagen, CA). Six pools consisting of ten gonads each were used for ChIP analysis. PCR analysis was performed using primers that targetted potential *Tcfap2c* binding sites ($GCCN_3CGG$) in *Cdh1, Hmga2, Lin28, Nanog* and *Kit* promoter regions (>1000bp upstream of gene start site). Input DNA was analyzed for the presence of the *Cdh1, Hmga2, Lin28, Nanog* and *Kit* promoter sequences as a positive control and a nonspecific mouse IgG antibody was used as a negative control (Vector Laboratories, MI). *Cdh1* 9-10 primers span an area of the *Cdh1* promoter region that does not contain TCFAP2C recognition sites. *Cdh1* Large primers produce a 1000bp product and ensure that sonication sheared DNA to >1000bp products.

ChIP Primer Sequences (5'-3')

Cdh1 F:TGGTCTGGTACCCCACTTG R:AGAGGGTCTTGGGATTGCAT

Nanog F:CAGTCTGGGTCACCTTACAGCT R:GACACCAACCAAATCAGCCTAT

Hmga2 F:GCCCTGTCTTTTTAACCCCACT R:GGCTGCAGGCGAAGCAAGAG

Lin28 F:CCCTTTAAATGGGTTGTAGCTG R:TTCGGGTAGTATGACTGGTGAA

Kit F:GGCTGGCCCGTACCTAAT R:GCTGTGCCCTCTAAGACCAG

Cdh1 9-10 negative control F:TGAGCCTCTGGTAGGTTGCT

R:ACCAGGACTTGCACTGATCC

Cdh1 Large 1000bp negative control F:GAAATGGGCCAAGGGTATT

R:CCCTGCTCTCAGCAACCTAC

Statistical Analysis

Real-time PCR data for E11.5-13.5 XX and XY gonad pools were analyzed as a 2 X 3 (gender, gonad age and gene expression) factorial arrangement of treatments using the GLM procedure (SAS Inst. Inc., Cary, NC). Data were analyzed by least-squares analysis of variance methods (Harvey, 1960). Gonad gender and gonad age were used as fixed effects in the model for gene expression differences. The response variables were gene expression differences and the interactions between different gender pools of the same gonad age (for example, E12.5 XX vs XY gene expression levels for a specific gene) as well as differences between gene expression levels of one gender across all thee time points (for example, XX E11.5 vs 12.5 vs 13.5). Significant differences between means were determined using LSD and were considered significant when $p \leq 0.05$.

Results

Tcfap2 Expression in Fetal Gonads

In order to examine the role of *Tcfap2* family of transcription factors in germ cell development, we first determined expression of *Tcfap2* factors in fetal gonads. A microarray gene profiling experiment of isolated E12.5 pre-granulosa cells and Sertoli cells, and whole E12.5 gonads revealed significantly higher *Tcfap2c* expression in whole gonad compared to isolated pre-granulosa and Sertoli cells (Figure 15; Bouma et al., 2010). Real-time PCR analysis of whole E12.5 fetal gonads revealed that *Tcfap2a*, *Tcfap2b* and *Tcfap2c* were the primary Tcfap2 family members present and *Tcfap2c* has the highest levels in both fetal testis and ovaries (Figure 16). To more acurately determine which cells in the gonad express *Tcfap2c*, *Pou5f1*-EGFP germ cells were isolated from XX and XY fetal gonads at E12.5 using flow cytometry. Purity of the isolated germ cell population was confirmed by expression of the germ cell specific gene *Dppa3* and absence of the somatic cell marker *Kitl* (Figure 17). Real-time PCR on both isolated fractions (germ cells and somatic cells) confirmed that *Tcfap2c* was expressed exclusively in germ cells (Figure 17). *Tcfap2a* and *Tcfap2b* was below detectable levels.

TCFAP2C Interacts With the Promoter Regions of Differentiation and Pluripotency Factors

Putative TCFAP2C binding sites (5'-GCCN_3GGC-3') were identified in the proximal promoter regions of key genes involved in germ cell-cell interaction,

Cdh1 and *Kit*, as well as genes that regulate germ cell pluripotency*, Lin28, Nanog* and *Hmga2*. The 1000bp upstream of the target gene's start site were examined for TCFAP2 consensus sequences. In some cases we found many putative TCFAP2 binding sites; however, we focused on the site most proximal to the start site. Chromatin immunoprecipitation (ChIP) was performed on E12.5 gonads to determine whether TCFAP2C binds the putative target sites found in *Cdh1, Kit, Hmga2, Nanog* and *Lin28* promoters *in vivo*. Antibodies against TCFAP2C were able to precipitate target fragments containing putative TCFAP2C binding sites from the target promoter sequence (Figure 18). Specificity of the immunoprecipitation was verified by a non-specific IgG. TCFAP2C antibody immunoprecipitated segments of the *Cdh1, Kit, Hmga2, Nanog* and *Lin28* promoters that contain TCFAP2C recognition sites in male and female pools of sonicated gonad DNA. These results indicate that TCFAP2C binds to promoter regions of the *Cdh1, Kit, Hmga2, Nanog* and *Lin28* genes in E12.5 gonads.

Expression Profile of Selected Pluripotency and PGC Markers During Gonad Differentiation

Microarray analysis verified that *Hmga2, Lin28, Cdh1* and *Kit* were all expressed in E12.5 gonads (Figure 15; Bouma et al., 2010). *Hmga2, Lin28, Nanog* and *Cdh1* were higher expressed in whole gonads than somatic cells suggesting these genes are specifically expressed in germ cells (Bouma et al., 2010).

In order to determine if TCFAP2C might be regulating gene expression of the transcriptional targets identified using chromatin immunoprecipitation, expression levels of *Cdh1, Kit, Hmga2, Nanog* and *Lin28* were analyzed. Whole gonads from E11.5, 12.5 and 13.5 were analyzed for gene expression differences between XX and XY gonads as well as between time points within either XX or XY gonads (Figure 19).

Cdh1, Kit, KitL, *Tcfap2c* and *Lin28* significantly increased XX gonads from E11.5 to E13.5 indicating that these genes were upregulated during these time points ($p \leq 0.05$; Figure 19). Expression of *Hmga2* and *Nanog* trended to increase from E11.5 to 13.5 in XX gonads; however, this trend was not significant. *Cdh1, Tcfap2c, Nanog* and *Lin28* also trended to increase in XY gonads, but again these interactions were not significant. Significant differences were seen between XX and XY gene expression of *Cdh1, Kit, KitL, Tcfap2c* and *Lin28*, with the most significant differences often seen at E13.5. XX gonads generally showed higher gene expression than XY gonads (Figure 19). *Hmga2* decreases in XY gonads from E11.5 to E13.5 indicating potential down regulation of this gene.

Discussion

While *Tcfap2c* is hypothesized to repress somatic fate and induce pluripotency in PGCs shortly after specification, the continued role of *Tcfap2c* has not previously been explored (Chapter 1; Weber et al., 2010). We have identified *Tcfap2c* in germ cells from E11.5-13.5. Previous studies have indentified *Tcfap2c* in germ cells begins around E8.5 and continues through to

adult oocytes (Hoei-Hansen et al., 2004, Pauls et al., 2005, Winger et al. 2006; Weber et al., 2010). This study identifies *Tcfap2c* as the main member of the *Tcfap2* family in the XX and XY germ cell population during embryonic development. This is important due to the fact that *Tcfap2* binding sites are highly conserved and often more than one *Tcfap2* family member may be able to bind to a consensus sequence in a target promoter (Kohlbecker et al., 2002; Woodfield et al., 2010). It has been shown that *Tcfap2* family members are capable of acting as heterodimers or have compensatory roles (Winger et al., 2006 and Kohlbecker et al., 2002). Isolated germ cells solely expressed *Tcfap2c* and somatic cells did not show expression of any Tcfap2 family members. This indicates that no other *Tcfap2* family memebers are present to play a compensatory role in the absence of *Tcfap2c*.

TCFAP2 transcription factors bind the DNA consensus sequence 5'-$GCCN_3GGC$-3' in genomic promoter regions, and either promote or inhibit gene transcription (Eckert et al., 2005). In this study, we identified TCFAP2 binding sites within the promoter sequences of *Hmga2*, *Lin28*, *Nanog*, *Kit* and *Cdh1*. As *Tcfap2c* is the most highly expressed member of the AP-2 family, we performed chromatin immunoprecipitation to determine if TCFAP2C binds these sequences in E12.5 mouse gonads. This study showed that TCFAP2C binds the promoter regions of *Hmga2*, *Lin28*, *Nanog*, *Kit* and *Cdh1* in XX and XY germ cells. While there is currently no definitive *in vivo* method to determine how this binding may relate to the regulation of gene transcription, TCFAP2C binding to the promoter region of these targets may be either inhibiting, upregulating or maintaining

transcription. Further studies, such as targeted-promoter mutation studies or reporter construct assays, could determine the exact regulatory role of *Tcfap2c* for these genes.

This study identified interaction of TCFAP2C with genes involved in pluripotency and cell differentiation. *Hmga2* has not been explored previously in germ cells. This study found *Hmga2* in E11.5-13.5 XX and XY gonads. Identified as a HMG architectural protein that binds DNA, *Hmga2* expression is seen widely throughout the embryo from E10.5 to 15.5 (Zhou et al., 1996). *Hmga2* is believed to regulate cell proliferation and differentiation and has not been identified in adult mouse tissues other than spermatocytes (Hock et al., 2006). TCFAP2C binds the promoter region of *Hmga2* and therefore may play a role in regulating this differentiation factor. Indeed, as *Tcfap2c* increases in both XX and XY gonads, *Hmga2* increases in XX gonads and decreases in XY gonads. The difference between *Hmga2* expression in XX and XY gonads is likely due to the differing differentiation states of the two germ cells at this stage. XY germ cells enter mitotic arrest around E12.5 and cease to proliferate, while XX germ cells continue to proliferate until they enter meiotic arrest. The increased expression of both *Tcfap2c* and *Hmga2* in XX germ cells could be indicative of the continued proliferative cells and lower levels of *Tcfap2c* and decreasing levels of *Hmga2* reflect XY germ cell arrest. Interestingly, *Hmga2* deficient mice are sterile indicating that *Hmga2* plays an important role in germ cell development that has not been explored previously. The evidence that *Tcfap2c* binds *Hmga2* indicates that *Tcfap2c* plays a role in regulating *Hmga2* in the germ cell population.

Lin28 also has not been studied in late germ cell development. *Lin28* has been identified in germ cells shortly after specification at E7.5 where it has been shown to regulate the expression of a master germ cell differentiation regulator *Prdm1* (West et al., 2009). This study detected *Lin28* in both XX and XY gonads from E11.5 to 13.5. *Lin28* inhibits the processing and maturation of the let7 microRNA family. Let7s effectively suppresses PRDM1 in vitro, and it is believed that in PGCs *Lin28* represses *let7* allowing upregulation of *Prdm1* (Nie et al., 2008; West et al., 2009). Knock-down of *Lin28* in PGCs led to a reduction in *TNAP* positive cells and a reduction of *Prdm1* expression, while overexpression of *Lin28* produced increased numbers of PGCs (West et al., 2009). The role of *Lin28* during later germ cell differentiation has not been studied. *Tcfap2c* bound the promoter of *Lin28*, indicating a regulatory role for *Tcfap2c* in the regulation of *Lin28*. As *Tcfap2c* increases from E11.5 to 13.5 in both XX and XY gonads, *Lin28* increases as well. This implies that *Tcfap2c* plays a role in upregulating *Lin28* expression in both XX and XY gonads during germ cell differentiation.

The pluripotency factor *Nanog* has been well studied during germ cell specification and development. *Nanog* is first detected at E7.5 and is downregulated at E14.5 in XX germ cells as they enter meiotic arrest and 16.5 in XY germ cells after mitotic arrest (Yamaguchi et al., 2005; Yabuta et al., 2006). We observed *Nanog* in both XX and XY gonads, but only observed increased *Nanog* in XY gonads. Due to TCFAP2C binding the *Nanog* promoter it is likely to play a role in the regulation of *Nanog*. *Nanog* is required to maintain pluripotency and since *Tcfap2c* acts as a regulator of *Nanog* expression it implicates *Tcfap2c*

as a regulator of pluripotency in germ cells as well (Mitsui et al., 2003). Loss of *Nanog* in germ cells leads to germ cell depletion due to apoptosis (Yamaguchi et al., 2009). Loss of pluripotency and apoptosis in germ cells may provide an explanation for the lack of germ cells in *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ embryos.

TCFAP2C was also shown to bind to targets in the promoters of genes involved in germ cell-cell interaction. *Kit* and *Cdh1* are both cell surface markers that have been shown to play key roles during specification, migration and colonization of the gonad. *Kit* interacts with *Kit*l, which is secreted by surrounding somatic tissues prompting germ cell motility and clustering after gonad colonization (Rynyan et al., 2006; Gu et al., 2009). *Cdh1* facilitates cell-cell interaction between germ cells allowing them to remain clustered during specification, migration and finally during gonad colonization. *Cdh1* and *Kit* were both identified in this study from E11.5 to 13.5. The promoters of *Cdh1* and *Kit* were bound by TCFAP2C. Interaction of TCFAP2C with the *Cdh1* promoter has been previously reported in human breast cancer, colon cancer and MDCK kidney epithelial cells (Baldi et al., 2001; Decary et al., 2002; Dela Croix et al., 2005; Schwartz et al., 2007). TCFAP2C has been shown to act to either upregulate or repress *Cdh1* expression depending on the tissue. In germ cells, it remains unclear exactly how TCFAP2C regulates *Cdh1* and *Kit*. Expression of *Cdh1* does not show any significant differences between E11.5 to 13.5 in XX and XY gonads. However, *Kit* expression increases in XX gonads perhaps in accordance with the increase in germ cell numbers as some XX germ cells are proliferative. Loss of either *Kit* or *Cdh1* leads to loss of germ cells prior to E11.5.

The role of these cell surface markers at later stages are not well studied. *Cdh1* and *Kit* are both downregulated shortly after onset of meiosis in XX germ cells and mitosis in XY germ cells. The binding of TCFAP2C to the *Cdh1* and *Kit* promoters likely maintains expression of these genes during specification, migration and colonization.

These genes all play important roles in regulating germ cell differentiation and production of mature gametes. Given the role of TCFAP2C as a transcription regulator in germ cell differentiation, this suggests that *Tcfap2c* plays a longer lasting role in germ cell development than previously thought. Determining the molecular regulatory pathways influenced by TCFAP2C will provide important insight into understanding the genesis of gametes.

PGCs are one of the only lineages produced during embryogenesis that, like ES cells, are pluripotent. Our studies elucidate *Tcfap2c* as a factor that plays an important role in regulating gene expression pathways within the transcriptional network necessary in germ cells during fetal gonad differentiation.

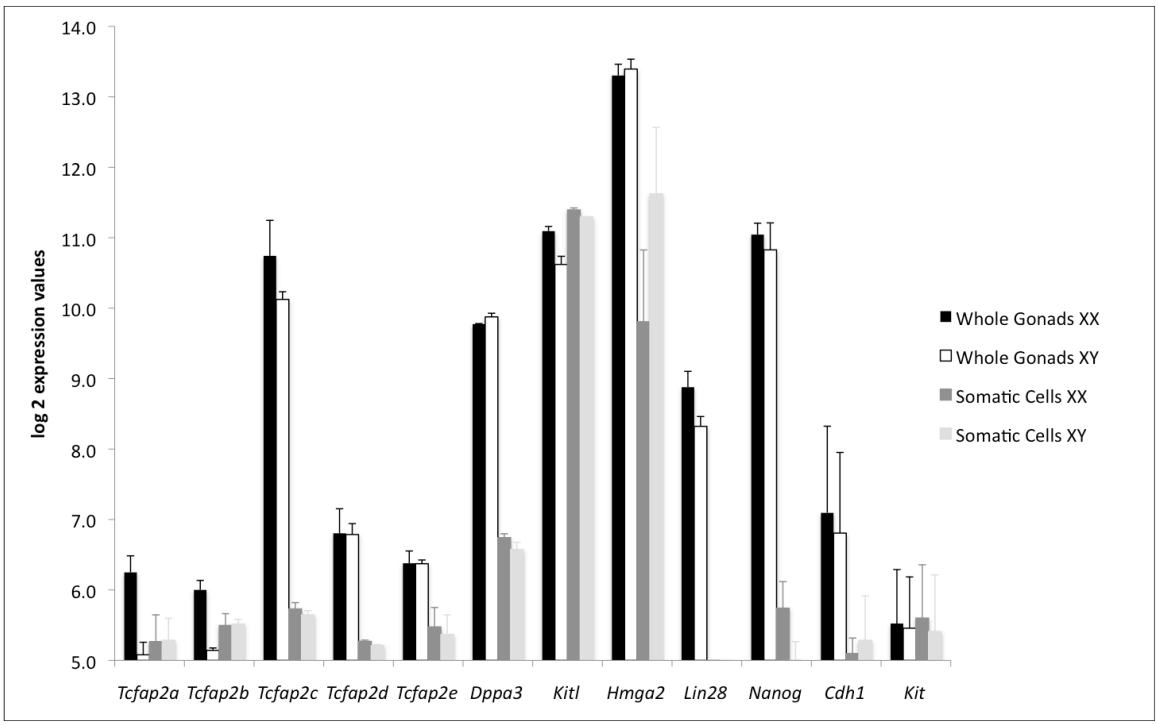


Figure 15 – Expression of AP-2 family, germ cell and pluripotency markers in E12.5 gonads. Microarray expression data from E12.5 whole gonads and isolated E12.5 Sertoli cells and precursor granulosa cells (Bouma et. al., 2010). *Dppa3* and *Kitl* are positive markers for germ cells and somatic cells, respectively. Error bars represent SEM.

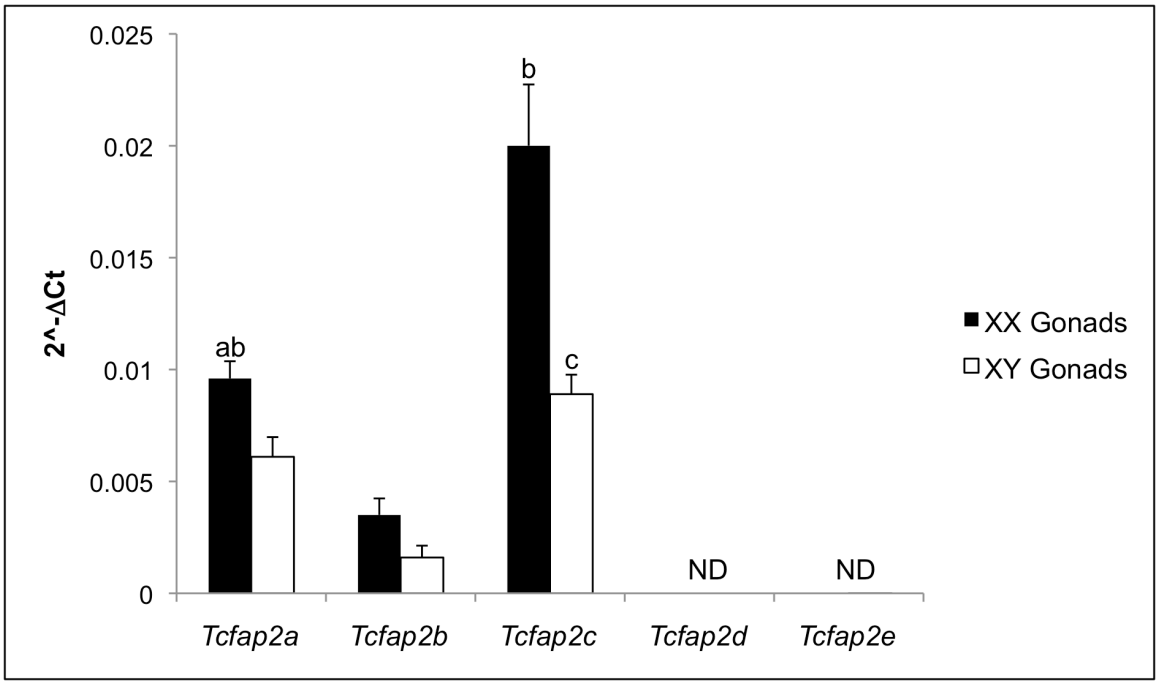
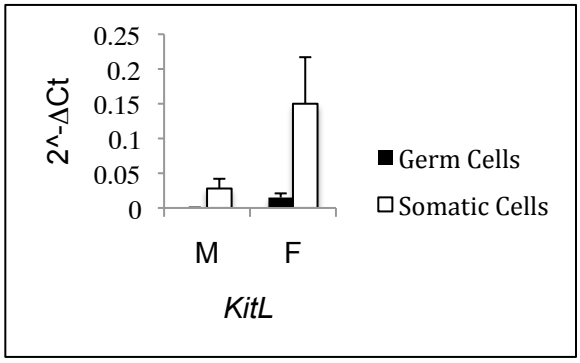
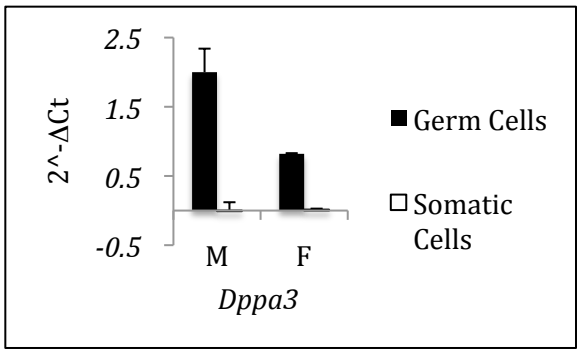


Figure 16 – *Tcfap2* expression in E12.5 gonads. Real-time PCR was performed on E12.5 gonads to determine relative expression levels of the *Tcfap2* family members. Different superscripts denote significant differences between family members by genetic sex. ND – not detected. p≤0.05. Error bars represent SEM.

A


0.25
0.2
0.15
0.1
0.05
0
2^-ΔCt
Germ Cells
Somatic Cells
M
F
KitL

B


2.5
1.5
0.5
-0.5
2^-ΔCt
Germ Cells
Somatic Cells
M
F
Dppa3

C

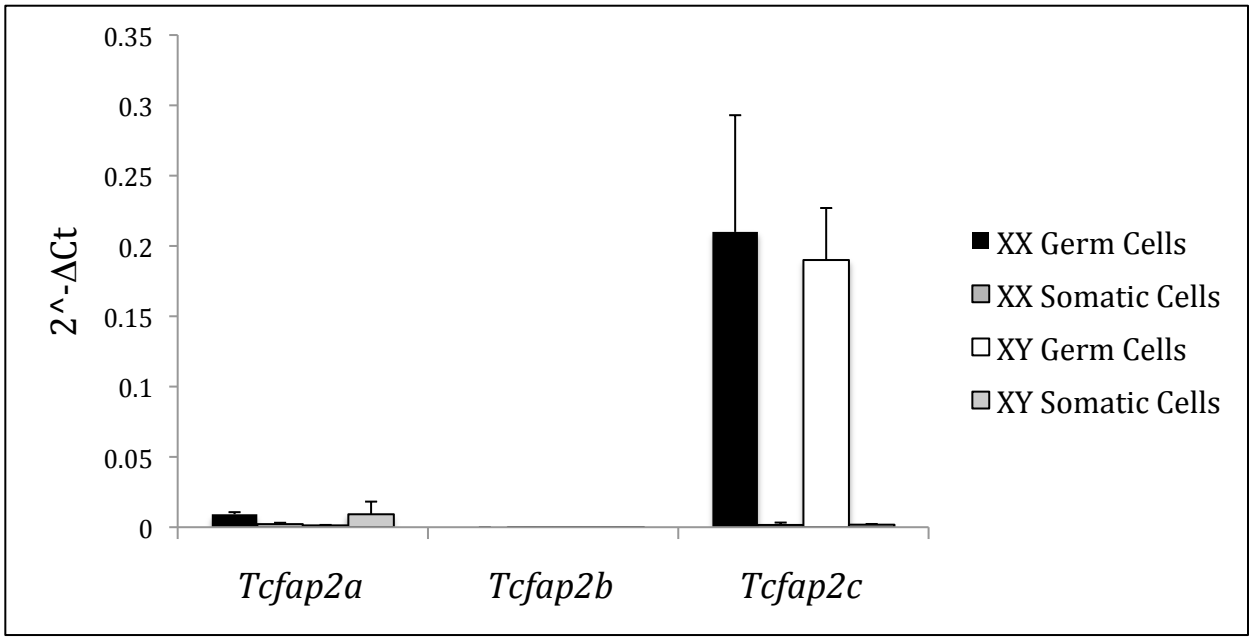
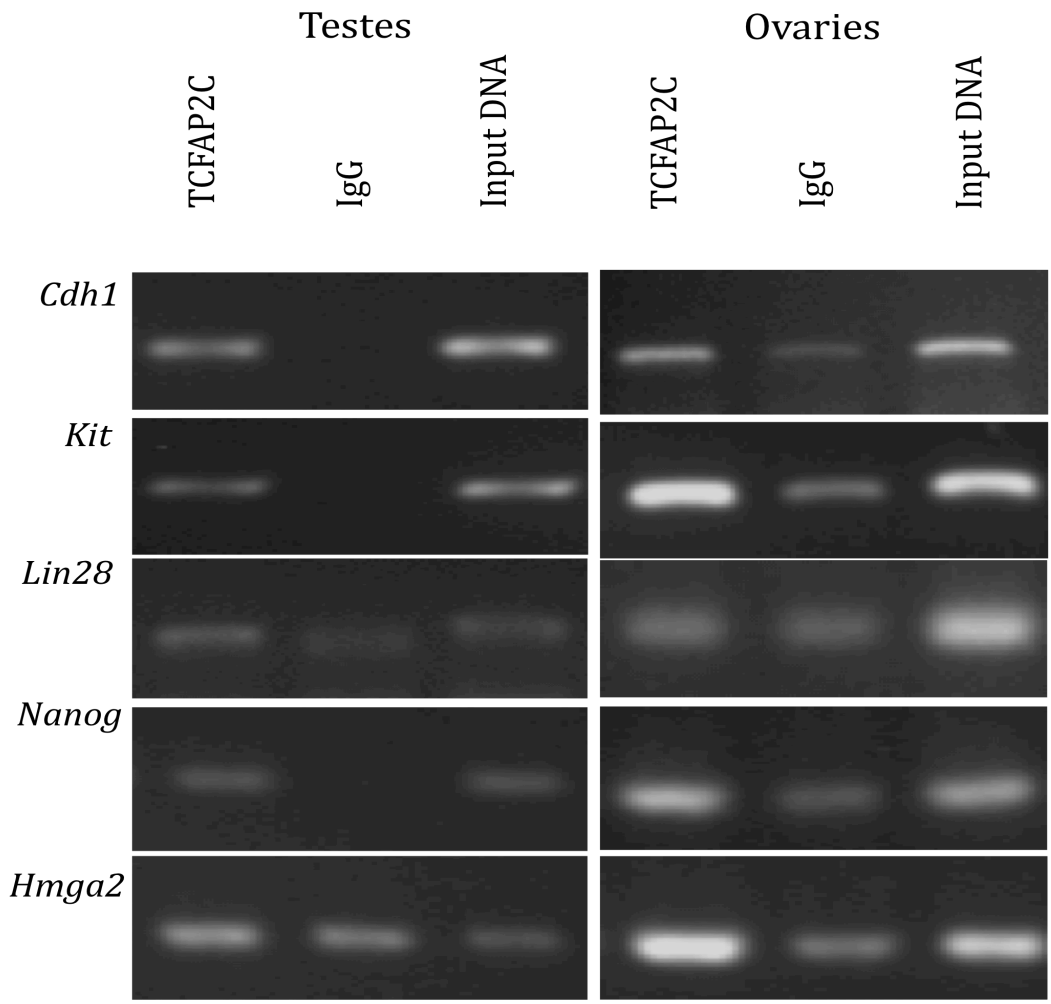

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
2^-ΔCt
XX Germ Cells
XX Somatic Cells
XY Germ Cells
XY Somatic Cells
Tcfap2a
Tcfap2b
Tcfap2c

Figure 17 – Expression of *Tcfap2* family in isolated cells of E12.5 gonads. Germ cells were isolated from somatic cells using *Pou5f1*-EGFP expression and flow cytometry. *Kitl* (A) and *Dppa3* (B) are positive markers for somatic cells and germ cells, respectively. C) Expression of *Tcfap2a*, *Tcfap2b* and *Tcfap2c* in germ cells and somatic cells were determined using real-time PCR. Error bars represent SEM.


Testes
Ovaries
TCFAP2C
IgG
Input DNA
TCFAP2C
IgG
Input DNA
Cdh1
Kit
Lin28
Nanog
Hmga2

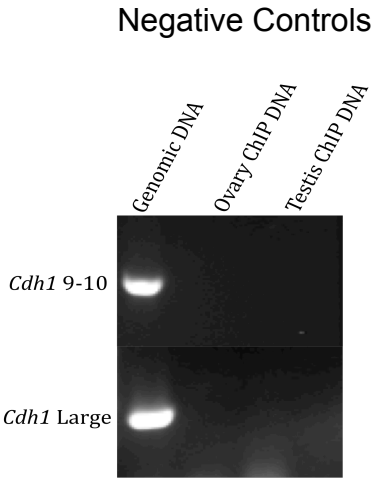
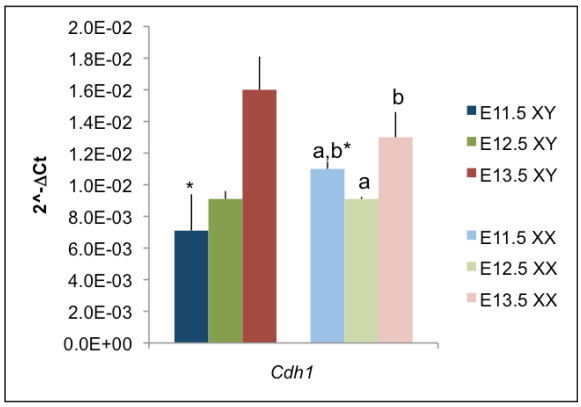

Negative Controls
Genomic DNA
Ovary ChIP DNA
Testis ChIP DNA
Cdh1 9-10
Cdh1 Large

Figure 18 –TCFAP2C binds putative TCFAP2 recognition sites in the promoter regions of *Cdh1, Kit* and *Lin28* at E12.5 in *Tcfap2c*$^{+/+}$ ovaries and testes. Lane 1, PCR product derived from template immunoprecipitated by anti-TCFAP2A antibody; Lane 2, PCR product derived from template immunoprecipitated by IgG; Lane 3, PCR product derived from direct input DNA template without immunoprecipitation. Negative controls – *Cdh1* 9-10 primers span an area of the *Cdh1* promoter region that does not contain TCFAP2C recognition sites. *Cdh1* Large primers produce a 1000bp product and ensure that sonication sheared DNA to >1000bp products.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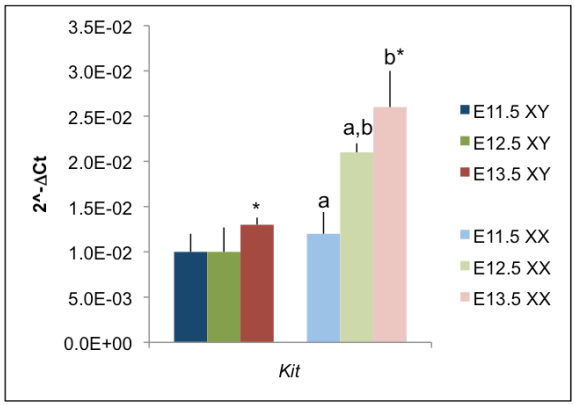
1.8E-02
1.6E-02
1.4E-02
1.2E-02
1.0E-02
8.0E-03
6.0E-03
4.0E-03
2.0E-03
0.0E+00
2^-ΔCt
*
a,b*
a
b
E11.5 XY
E12.5 XY
E13.5 XY
E11.5 XX
E12.5 XX
E13.5 XX
Cdh1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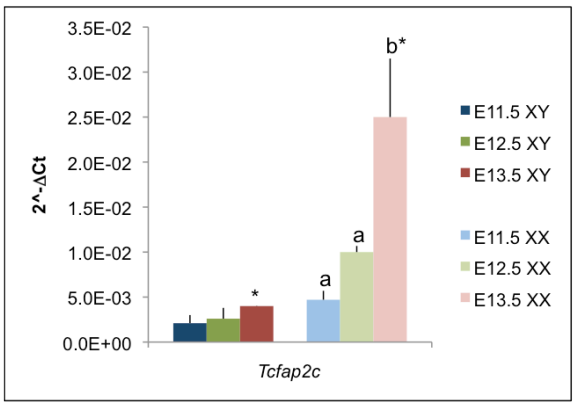
3.0E-02
2.5E-02
2.0E-02
1.5E-02
1.0E-02
5.0E-03
0.0E+00
2^-ΔCt
*
a
a,b
b*
E11.5 XY
E12.5 XY
E13.5 XY
E11.5 XX
E12.5 XX
E13.5 XX
Kit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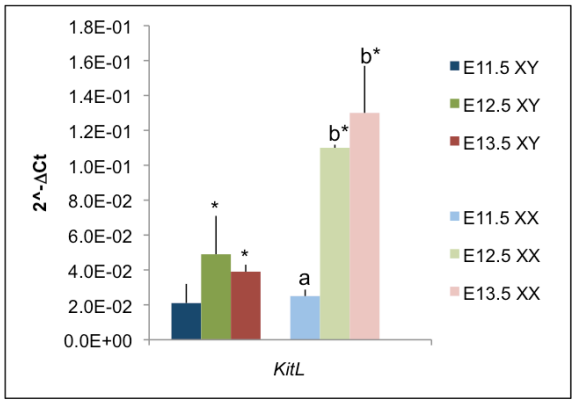
3.0E-02
2.5E-02
2.0E-02
1.5E-02
1.0E-02
5.0E-03
0.0E+00
2^-ΔCt
*
a
a
b*
E11.5 XY
E12.5 XY
E13.5 XY
E11.5 XX
E12.5 XX
E13.5 XX
Tcfap2c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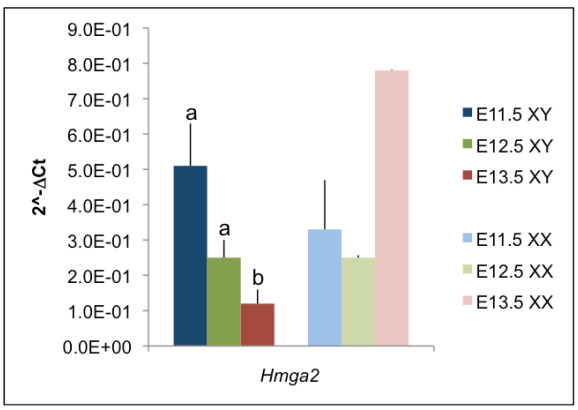
1.6E-01
1.4E-01
1.2E-01
1.0E-01
8.0E-02
6.0E-02
4.0E-02
2.0E-02
0.0E+00
2^-ΔCt
*
*
a
b*
b*
E11.5 XY
E12.5 XY
E13.5 XY
E11.5 XX
E12.5 XX
E13.5 XX
KitL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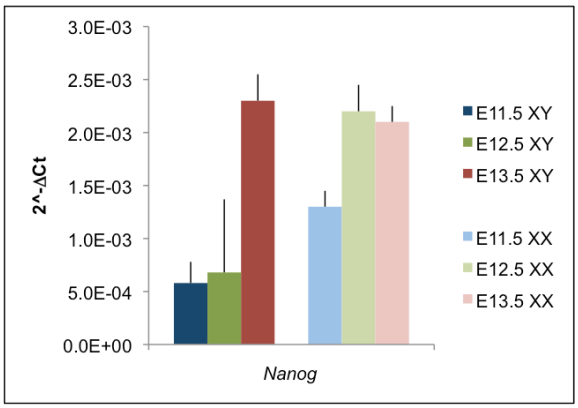
8.0E-01
7.0E-01
6.0E-01
5.0E-01
4.0E-01
3.0E-01
2.0E-01
1.0E-01
0.0E+00
2^-ΔCt
a
a
b
E11.5 XY
E12.5 XY
E13.5 XY
E11.5 XX
E12.5 XX
E13.5 XX
Hmga2


3.0E-03
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1.5E-03
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5.0E-04
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2^-ΔCt
E11.5 XY
E12.5 XY
E13.5 XY
E11.5 XX
E12.5 XX
E13.5 XX
Nanog

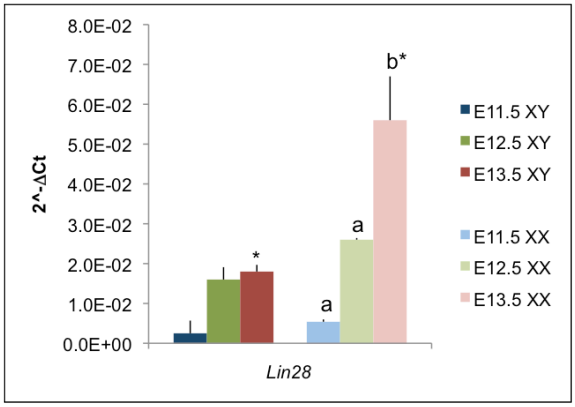
8.0E-02
7.0E-02
6.0E-02
5.0E-02
4.0E-02
3.0E-02
2.0E-02
1.0E-02
0.0E+00
2^-ΔCt
*
a
b*
E11.5 XY
E12.5 XY
E13.5 XY
E11.5 XX
E12.5 XX
E13.5 XX
Lin28

Figure 19 – Expression of PGC and pluripotency markers in developmental gonads. Real-time PCR was performed on pools of either XX or XY E11.5, 12.5 and 13.5 whole gonads and expression levels of the PGC markers *Cdh1* and *Kit*, somatic cell marker *Kitl*, and cell differentiation regulators *Nanog, Hmga2, Lin28* and *Tcfap2c*. Super script '*' denotes significant differences between XX and XY gonads at the same time point. Letter superscripts differences between time points within XX or XY gonads, different letters indicate significant differences between groups. $p \leq 0.05$, error bars represent SEM.

CHAPTER THREE

Conclusions

The overall objective of this study was to determine the role of *Tcfap2c* during different stages of germ cell development. In order to achieve this objective, this study looked at the major stages of murine germ cell development, namely specification, proliferation, migration, differentiation and adult maturation. The Cre/loxP conditional mutation system was used to produce *Tcfap2c* knock-out models that helped to elucidate the role of *Tcfap2c* during these different stages. We also sought to identify regulatory targets of *Tcfap2c* during fetal gonad differentiation.

In chapter one we characterized the loss of *Tcfap2c* using three different Cre/loxP models. The epiblast-specific *Sox2*-Cre deletion of *Tcfap2c* produced mutant mice that lacked meiotic and germ cell markers by E14.5, indicating that these mice did not possess germ cells. This phenotype was observed in the study that also used *Sox2*-Cre performed by Weber et al., (2010). However, the *Sox2*-Cre model had a few significant drawbacks. This model deleted *Tcfap2c* in the entire epiblast, *Tcfap2c* expression is seen in many tissues of developing embryos. Loss of *Tcfap2c* in the entire embryo could result in detrimental phenotypes in other tissues that could effect germ cell development. Also, *Sox2*-

Cre, *Tcfap2c*$^{-/-}$ mice died at or before birth preventing the study of adult stages of spermatogenesis and oogenesis. To that end, I used ERTM-Cre to cause deletion of *Tcfap2c* in mice after puberty. These mice did not show any differences from control littermates spermatogenesis, oogenesis and fertility. These results show that while *Tcfap2c* is still expressed in oocytes and spermatogonia, *Tcfap2c* is not necessary in adult testis and ovaries. Finally, *Prdm1*-Cre was used to produce *Tcfap2c* deletion specifically in the germ cells during specification. *Prdm1*-Cre was a perfect model to study *Tcfap2c* in PGCs, as this model allowed all other tissues of the embryo to develop without disruption of *Tcfap2c* except for the germ cells. *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice initially specified PGCs at E7.5, but these cells failed to proliferate and migrate. Germ cells were lost in *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ by E12.5. Adult ovaries and testes from *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ were drastically smaller than control littermates and did not display spermatogenesis or oogenesis. *Prdm1*-Cre, *Tcfap2c*$^{-/-}$ mice verified the loss of germ cells seen in *Sox2*-Cre. This study showed *Tcfap2c* is necessary for complete specification, proliferation and migration of germ cells during fetal development. This study identified the role of *Tcfap2c* in germ cell development, but the exact mechanism and pathway that *Tcfap2c* functions through during these stages are still unclear.

Chapter two sought to elucidate some of the molecular targets of *Tcfap2c* in germ cells during fetal gonad differentiation. *Tcfap2c* expression is seen throughout germ cell development past the initial specification, proliferation and migration stages during which we have shown *Tcfap2c* to be necessary. The

persistent expression of *Tcfap2c* suggests that it continues to play a regulatory role during the events that characterize fetal gonad differentiation. This study identified *Tcfap2c* as the primary member of the *Tcfap2* family expressed in germ cells during fetal gonad development. This means that any *Tcfap2* binding sites identified in genes involved in germ cell differentiation during this stage likely are regulated by TCFAP2C. TCFAP2C binding sites were identified in the germ cell-cell interaction genes *Cdh1* and *Kit* as well as in the pluripotency genes *Nanog*, *Lin28* and *Hmga2*. TCFAP2C binds the promoter regions of *Cdh1, Kit, Nanog, Lin28* and *Hmga2* in E12.5 ovaries and testes. This shows active interaction between TCFAP2C and these genes indicating that TCFAP2C plays a role in regulating these genes and their respective pathways. Notably, *Hmga2* and *Lin28* expression has never been identified or explored in germ cells during fetal gonad differentiation. *Hmga2* and *Lin28* have been shown to be important regulators of pluripotency. LIN28 function to process let7 microRNAs into a mature functional state, these are responsible for regulating the expression levels of genes necessary for pluripotency and differentiation. *Lin28* has been shown to play an important role during germ cell specification; this study provides initial findings that this pathway also is active in regulating the differentiation of germ cells in the fetal gonad. The next step in understanding the role of *Tcfap2c* in germ cells will be to look at the expression levels of *Tcfap2c* targets in *Tcfap2c* mutant mice. Also, future studies to elucidate the role of this pathway and the part that *Tcfap2c* plays within it would greatly expand the understanding of fetal gonad differentiation. Conditional mutation of *Tcfap2c* at E10.5, after the germ

cells have been specified and migrated to the gonad, would provide significant information that would solidify the regulatory role of *Tcfap2c* during fetal gonad differentiation. Overall, this study identifies *Tcfap2c* as an important player in the regulation of germ cell differentiation and differentiation.

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