DISSERTATION

ROLE OF ENDOGENOUS RETROVIRUS IN CONTROL OF FELINE LEUKEMIA VIRUS INFECTION AND IMPLICATIONS FOR CROSS SPECIES TRANSMISSION

Submitted by

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ABSTRACT

ROLE OF ENDOGENOUS RETROVIRUS IN CONTROL OF FELINE LEUKEMIA VIRUS INFECTION AND IMPLICATIONS FOR CROSS SPECIES TRANSMISSION

Endogenous retroviruses (ERV) are markers of ancient retroviral infections, though evolutionary forces have limited the capacity for ERV replication and virulence. While they are seldom considered infectious alone, they maintain the ability to interact with their exogenous retroviral (XRV) progenitors. In Chapter One, we review the interactions that exist between ERV and XRV dyads. One such couplet includes feline leukemia virus (FeLV), a common domestic cat pathogen. In Chapter Two, we review FeLV subgroup taxonomy and the methods used from which they were originally characterized. Though the domestic cat is regarded as the natural host for the virus, recent reports have documented FeLV infections in wild felids with pathogenic consequences. Chapter Three examines the root of a contemporary FeLV outbreak in Florida panthers (*Puma concolor coryi*), a species that lacks endogenous FeLV. Our phylogenetic analysis of the contemporary FeLV outbreak has further implicated domestic cats (*Felis catus*) as the origin of FeLV infections in wild felids. Furthermore, we detected a recombinant oncogenic variant in Florida panthers that is believed to be non-horizontally transmissible. These field studies have prompted us to examine the cellular basis of infection and intrinsic resistance to the virus. In Chapter Four, we interrogate the cellular basis of FeLV infections between puma (*P. concolor*) and domestic cat cells using *in vitro* approaches. We demonstrated that puma cells support greater infection and replication. Additionally, we documented enFeLV long terminal repeats (LTR) in domestic cats are negatively correlated to FeLV infection outcomes in vitro. Natural FeLV infections in both Florida panther and domestic cat tissues offered us the opportunity to examine end stage disease dynamics, which demonstrate that Florida panthers have the ability to produce more virus despite having lower proviral loads than domestic cats. The results of both *in vivo* and *in vitro* experiments prompted us to further

investigate enFeLV-LTRs and their role in FeLV infection. Chapter Five took advantage of the publicly available data in the NCBI Sequence Read Archive (SRA) to evaluate enFeLV-LTR basal transcription levels. Data-mining the domestic cat transcriptome showed that lymphoid cells, which are relatively resistant to *in vitro* FeLV infection, transcribe more enFeLV elements than relatively susceptible cells (i.e., fibroblasts). We also identified microRNA transcripts are produced that have the potential ability to down-regulate FeLV RNA transcripts. In Chapter Six, we innovated a new methodology to characterize the enFeLV-LTR integration sites across the entire genome of 20 related and unrelated domestic cats in an attempt to uncover genes that may be influenced by LTR enhancement of gene expression. We found one LTR integration site in a limited number of cats that is within 1MB of APOBEC1, an antiviral gene, and that the most common gene found in close proximity to LTR integration sites are zinc fingers, a broad-acting class of regulatory proteins. Collectively, this groundwork provides future directions to uncover direct and indirect mechanisms of enFeLV-mediated restriction of FeLV infection. We conclude that because wild felids lack enFeLV, they may be more vulnerable to FeLV infection. As urbanization forces niche overlap and contact between wild and domestic felids, the risk of infection of these species is likely to increase, and thus it will be important to consider contacts between FeLV-infected domestic cats and wild felid populations during development of conservation action plans.

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CHAPTER ONE

Interactions Between Endogenous Retroviruses and Their Exogenous Counterparts

INTRODUCTION

Animal genomes are riddled with evolutionary fossils left over from ancient viral infections; notable among these are remnants of retroviruses. Retroviruses require conversion of RNA genomes into DNA that stably integrate into host DNA (Coffin, 1992). Following integration, retroviruses hijack host cellular machinery for replication. In normal somatic cell infections, this process allows for the establishment of life long infections, as integrated viral genomes are not easily excised. In the special case of retroviral germline infections, integrated viral genomes may be passed along to progeny by vertical transmission (Figure 1). This results in permanent viral penetrance into the animal's cellular genome, resulting in an endogenous retrovirus (ERV), in contrast to horizontally transmitted counterparts, referred to as exogenous retroviruses (XRV) (Coffin, 2004). Early on in infection, ERVs are identical to their XRV progenitors. At this stage, they may be transcribed and can be infectious. After generations, ERV mutations accumulate, mitigating deleterious impacts of fully functional viruses. Defective ERVs are selected through generations of inheritance and become fixed in host populations (Lober et al., 2018). This phenomenon is correlated with diminished virulence, marked by mutations and deletions of the exogenous virus. Through the process of retrotransposition, ERV genomes may also be translocated, or 'retrotranspose' to other loci, leaving behind solo-long terminal repeats (LTRs), which harbor enhancer and promoter functions (Britten, 1997) and other remnant ERVs (Boeke and Stoye, 1997).

Once ERVs integrate and become fixed within a population, they can be regarded as markers of ancient retroviral infections (Coffin, 2004) and become integrated as a component of normal host genomic function. Analysis of retroviral-like sequences within host genomes has provided evidence that retroviruses were present during the Paleozoic Era (Aiewsakun and Katzourakis, 2017). Exogenous retroviruses that are responsible for seeding ERVs often become extinct, but in some cases, related

viruses still exist and interact with their endogenous counterparts in a variety of ways. Evaluation of exogenous-endogenous retroviral interactions provides a unique opportunity to witness host 'evolution in action', and document ways that viral infections can perpetuate through means other than horizontal transmission.

In consideration of the interesting relationship between ERVs and their exogenous counterparts, this review will examine and catalogue reported interactions between endogenous and exogenous retroviruses in avian and mammalian hosts, predominantly domestic animals which account for most of the scientific literature in this area (Garcia-Etxebarria et al., 2014). This work will update a thorough review conducted in 1998 (Rasmussen, 1997) and build upon a recent review detailing the origin and evolutionary consequences of endogenous retroviruses (Johnson, 2019). Endogenous retroviruses in non-avian and non-mammalian hosts have only recently been documented (Xu et al., 2018), and there are likely many other species and systems where similar mechanisms have not yet been described.

We will review the interactions between ERVs and their extant XRV counterparts. This includes ERV-XRV interactions in Jaagsiekte sheep retrovirus (JSRV), Rous sarcoma virus and avian sarcoma leukosis virus (RSV/ASLV), murine leukemia Virus (MMLV), mouse mammary tumor virus (MMTV), feline leukemia virus (FeLV), and koala retrovirus (KoRV) infections (Table 1). Understanding mechanisms by which ERV-XRV pairs interact provide a basis for study of ERVs that constitute approximately 8% of human genomes, relating potential interactions with past, present or future XRV epidemics. This is of particular importance as newly introduced retroviruses, such as HIV, have the significant potential of becoming endogenized. Beyond that, ERV-XRV interactions have and will continue to shape animal disease and biology. This review will not specifically discuss the role of endogenous retroviruses in placentation and mammalian evolution, normal biological processes, immune modulation, oncogenesis, and disease progression outside the scope of ERV interactions, which have all been thoroughly reviewed recently (Bannert et al., 2018; Denner, 2016; Frank and Feschotte, 2017; Grandi and Tramontano, 2018; Johnson, 2019; Morris et al., 2018). Readers are also referred to a recent review on the biology and early actions of endogenization (Greenwood et al., 2018).

NATURE OF ENDOGENOUS/EXOGENOUS RETROVIRAL INTERACTIONS

Endogenous and exogenous retroviruses exist in a unique system in which a virally derived host genetic component has the potential to come in contact with a related transmissible virus. Due to their high nucleotide similarity, these two entities can often interact in predictable ways that transcend the normal paradigms of host immunity. As a primer to the specific interactions detailed below, ERV-XRV interactions can be classified in a number of different groups (Table 2). Briefly, recombination is commonly encountered as the retroviral life cycle establishes the conditions for two genetically related viral RNAs to be co-packaged in virions. ERVs have the capacity to modulate viral recognition by immune or host cells through receptor interference and immunological self-tolerance. Finally, ERVs have the capacity to restrict and/or promote viral restriction (occasionally simultaneously) using different cellular and molecular mechanisms.

ROUS SARCOMA VIRUS/AVIAN SARCOMA LEUKOSIS VIRUS (RSV/ASLV; *Alpharetrovirus)*

In 1910, Rous sarcoma virus became the first oncogenic virus that was described in any species after Dr. Peyton Rous demonstrated that cell-free filtrates from avian sarcomas were capable of transmitting sarcomas in chickens (Rous, 1911). RSV and ASLV nomenclature has evolved as different genetic elements and functions have been identified (Rubin, 2011). RSV is a replication-competent rapidly transforming virus closely related to the ASLV complex. It was not until 1953 that additional ASLVs were described by using focus formation assays. Initially these agents were called resistance-inducing factors (RIFs) due to their ability to restrict RSV infection (Rubin, 1955). This ultimately led to the identification of additional ASLV subgroups including subgroup E and J that comprise endogenous ASLV. Subgroups F through I are also endogenous ASLVs found in species other than the domesticated chicken (*Gallus gallus*) (Payne and Nair, 2012; Venugopal, 1999). The major difference between RSV and other members of the ASLV complex is that ASLVs lack the *v-src* gene that conveys the rapidly transforming properties of RSV (Swanstrom and Wills, 1997). LTR sequences from the endogenous ASLVs are also defective. While they contain motifs that resemble enhancer regions, these regions are

not complete and as a result, are insufficient for enhancer function (Habel et al., 1993). Barring these differences, RSV is indistinguishable from the ASLV complex viruses.

Chickens have on average, 5 endogenous ASLV integration sites per individual with up to 2 dozen identified loci, though only one locus exists in the red jungle fowl (*G. gallus* wild ancestor) reference genome (Benkel and Rutherford, 2014). Certain chicken cell lines (i.e., line 0) important in the agricultural production industry were specifically developed to delete ERVs from the genome. As noted above, ASLV subgroup E loci harbor mutations that render the ERV incapable of generating infectious virus. One notable exception is loci *ev*-2, which is capable of producing Rous-associated virus-0 (RAV-0), a non-oncogenic ASLV (Astrin et al., 1980). While not completely replication-defective, RAV-0 U3 LTR and surrounding segments vary from RSV, which is believed to account for a reduction in virulence (Hughes, 1982). Endogenous ASLV has been documented to both promote and protect against disease following exogenous virus RSV/ASLV infection. Interactions between RAV-0 and RSV were described following quail cell infections with non-producing foci RSV (RSV(O)). Japanese quail cells lack RAV-0, which limited replication of RSV(O), a phenomenon that was not observed in chicken cells that harbor RAV-0. Co-inoculation of RAV-0 with RSV(O) in Japanese quail cells rescued the ability for RSV replication, demonstrating its helper virus activity (Figure 2) (Vogt and Friis, 1971).

Unsurprisingly, recombination occurs frequently between exogenous retroviruses and their related endogenous counterparts as nucleotide similarities predispose polymerases resuming stalled transcription on a different strand that resembles the original template (Luo and Taylor, 1990). These recombinants may increase virulence in many cases as mutations that have been accumulated in ERVs may be rescued by replacing the defunct sequence with functional protein-encoding sequences. ASLV subgroup E-RAV-60 recombinants were shown to induce lymphoid leukosis in response to exogenous virus-origin *c* regions in recombinant viruses (Crittenden et al., 1980). More recently, a rapidly evolving oncogenic subgroup (subgroup J) has been identified, and is believed to have emerged through recombination of a previously uncharacterized exogenous ASLV and an endogenous ERV distantly related to ASLVs (Sacco et al., 2004; Smith et al., 1999; Venugopal, 1999).

Interference between endogenous and exogenous ASLV/RSV is dependent ERV structure and age at time of infection. Chicken embryos inoculated with RAV-0 challenged with exogenous RAV-1 and RAV-2 (RSV pseudotype viruses) supported greater infection with RAV-1 or RAV-2, as measured length of viremia, or delayed or absent development of an adaptive immune response to the challenge virus. The mechanism proposed was that chicks were immunologically tolerant to envelope group-specific glycoproteins similar between both endogenous and exogenous viruses (Crittenden et al., 1987). Particular *ev* loci have since been identified to support this hypothesis. While some loci may exacerbate infection (*e.g. ev*12, *ev*21), others may work to help restrict immune tolerance (*e.g. ev*6, *ev*9); others still have no influence on ASLV infection (*e.g., ev*1, *ev*3) (Gavora et al., 1995; Kuhnlein et al., 1992; Smith et al., 1990). *ev*21 tolerance is most restrictive if the endogenous virus is genetically transmitted (versus experimentally horizontally transmitted) and if ASLV infection occurs prior to 4 weeks of age (Fadly and Smith, 1997).

Specific endogenous integration sites have been characterized to protect against disease. Crittenden *et al*. demonstrated that variation in *ev* gene expression and the RAV strain inoculated influenced ALV shedding rates and horizontal transmission characteristics of the exogenous infection (Crittenden et al., 1984). They measured relationship between genomic variation at *ev*2 and *ev*3 loci versus infection with four strains of ALV (RAV-1, RAV-2, RPL-40, and RPL-42). Animals lacking both *ev* loci exhibited higher mortality from non-neoplastic syndromes following RAV-1 infection. Line-0 chickens lacking both *ev* loci (which were developed separately from *ev*2-/*ev*3- negative chickens described above) had lower viremia and higher antibody production following RAV-1 infection. Inexplicably, line-0 chickens did not develop non-neoplastic syndromes. The other three ALV strains tested were more variable, generating fewer non-neoplastic syndromes. *ev*-positive chickens developed less robust neutralizing antibody responses. In a separate experiment, Denesvre et al. indicated that Subgroup J ASLVs expression of endogenous ev/J 4.1Rb envelope protein completely interfered with exogenous infection (Denesvre et al., 2003). These studies provide strong evidence for immunological tolerance as a mechanism for ERV-XRV interference.

New developments in transcriptomic analysis has allowed for the investigation of endogenous ASLVs in light of disease progression and infection. Interestingly, ALV-E *env* expression is increased in embryonic fibroblasts infected with Mareck's disease virus (herpesvirus), but is decreased in cells infected with ALV subgroup J viruses and reticuloendotheliosis virus (Hu et al., 2017; Hu et al., 2016). The significance of these results has yet to be determined, but indicate a possible role for ALV expression during development in generation of immunological tolerance to a variety of exogenous infections.

JAAGSIEKTE SHEEP RETROVIRUS (JSRV; *Betaretrovirus*)

Ovine pulmonary adenocarcinoma (OPA) was first recorded in domesticated sheep herds in the 1800's; however, its etiological agent, JSRV, was not identified until 1995 (Palmarini et al., 1996; Sharp and De Martini, 2003). OPA, originally known as jaagsietke for the respiratory clinical symptoms in affected animals, is the cause of transmissible neoplastic disease that occurs in approximately 30% of JSRV-infected individuals. Prevalence of JSRV varies from 40-80% of sheep and is found rarely in goats. Much like other retroviruses that exist both exogenously and endogenously, early molecular detection was hampered by the presence of related endogenous entities due to their high similarity (Palmarini et al., 1996). Historical perspective of the natural history of JSRV in sheep and in depth examination of JSRV induced oncogenesis is reviewed in (Sharp and De Martini, 2003) and (Leroux et al., 2007).

Fluorescent *in situ* hybridization and qPCR have identified approximately 30 copies of the endogenous virus in both sheep and goats (both domestic and wild breeds). Chromosomes 6 and 9 appear to harbor multiple copies of endogenous JSRV (enJSRV) (Carlson et al., 2003). Two chromosomal integration sites (1q45 and 2q41) are common between the two species (Carlson et al., 2003; Hecht et al., 1996; Palmarini et al., 2000). enJSRV appear to be necessary in placenta formation and are required for normal reproductive function in sheep (Dunlap et al., 2006), as reviewed in (Spencer and Palmarini, 2012a; Spencer and Palmarini, 2012b).

While enJSRV and JSRV share 90-98% amino acid similarity (Palmarini et al., 2000) across the majority of its genome, there is extensive difference within U3 region that shares 56% sequence identity,

and is markedly longer in enJSRV (Bai et al., 1996). Aside from being useful as makers of differentiation by molecular methods, the differences in the LTR have been discovered to drive differential expression of JSRV and enJSRV and underlie cellular tropism. JSRV LTR is specifically active in lung epithelia through the recruitment of lung-specific transcription factors, including hepatocyte nuclear factor-3β and NF-κB. enJSRV LTR, on the other hand, contain regions that favor transcription in the reproductive tract relating to reproductive competence (McGee-Estrada and Fan, 2007; Palmarini et al., 2000).

Unlike ASLV, interactions between enJSRV and JSRV described to date are predominately antagonistic, and recombination between the enJSRV and JSRV has not been documented. Expression of enJSRV during ontology of the embryo may induce immunologic tolerance to JSRV (DeMartini et al., 2003; Varela et al., 2009). Spencer *et al.*, demonstrated transcription of enJSRV in developing fetal sheep thymuses, and showed that animals that developed OSA lacked virus-specific antibody responses (Spencer et al., 2003). There is evidence that enJSRV may mediate protection against the exogenous form of the virus both in early and late stages on infection. enJSRV restricts JSRV's ability to enter the cell through receptor interference by saturating hyaluronidase-2, the entry receptor for JSRV, limiting the number of receptors that are expressed on the surface of the cell (Spencer et al., 2003). The action of one enJSRV ERV (enJS56A1) represents a unique restriction factor in the realm of ERV/XRV interactions. In the course of a normal JSRV (betaretrovirus) infection, JSRV Gag organizes around the microtubule organization center/pericentriolar region for viral assembly and budding. Whole provirus enJS56A1-expressed Gag co-assemble with JSRV Gag and these aggregates block normal intracellular trafficking of JSRV Gag, leading to reduced formation of mature virions (Arnaud et al., 2007b; Murcia et al., 2007). Aggregates that form in the cytoplasm are then degraded through proteasomal machinery(Arnaud et al., 2008). The amino acid residue responsible for centrosomal trafficking is residue 21, determined as mutated JSRV Gag at this site modifies this activity (Arnaud et al., 2007a). Wild-type (R21) and mutant (W21) variants of the Gag are found in normal individual sheep in a breed-specific manner (Viginier et al., 2012).

MOUSE MAMMARY TUMOR VIRUS (MMTV; *Betaretrovirus*)

Mouse mammary tumor virus is an exogenous virus that is transmitted in the milk of lactating animals and as the name suggests, can result in mammary tissue tumorigenesis. The endogenous form of the virus (*Mtv*) is found at varying copy numbers between 2 and 8 copies in laboratory mouse strains. There are more than 30 different characterized *Mtv*. MMTV-*Mtv* interactions have been extensively reviewed in (Holt et al., 2013). To date, recombination between MMTV and *Mtv* has not been recorded. This section will briefly address what has been established as a comparison to other systems described in this review, and focus on updates since the most recent review (Holt et al., 2013). Indirect impacts of *Mtv* on MMTV infection relate to immunological alterations that impact MMTV infection parameters and disease progression non-specifically. With respect to direct MMTV-*Mtv* interactions, two main areas have been investigated in detail: tumorigenesis and *Mtv* superantigen (Sag) production as well as its impacts on subsequent infection. Immunological tolerance driven by immune response development in the presence of *Mtv* is perhaps the clearest evidence of this mechanism underlying ERV interference diminishing the clinical and virologic outcome of ERV infection.

Both MMTV and *Mtv* encode for Sag, a type-2 transmembrane protein (Choi et al., 1991; Marrack et al., 1991). With respect to MMTV, Sag is a viral accessory protein encoded for by the 3' LTR and is necessary for dissemination from infected gut-associated lymphoid tissue to mammary glands (Golovkina et al., 1992). Mouse lineages that are defective in processing MMTV Sag are thus resistant to MMTV infection and dissemination (Holt et al., 2013). Sag also mediates B-cell-T-cell interactions and immune cell activation (Held et al., 1993). Some endogenous *Mtv* delete specific T-cell Vβ through negative T-cell selection during thymus organogenesis, offering a protective advantage against MMTV infection (Holt et al., 2013). A library of transposable repetitive elements (TREome) of 56 individual laboratory mouse strains has been characterized, revealing high amounts of diversity among strain specific Sag coding sequences that may drive strain immune phenotypes (Lee et al., 2016). This has led to the characterization of strain-specific responses to stress and infection based on *Mtv* loci and genotypes (Hsu et al., 2017). In 2017, additional *Mtv* Sags have been implicated in the clonal deletion of specific T-

cell populations with variable regions Vβ5.1, Vβ6, Vβ8.1, Vβ8.2, Vβ9, and Vβ11 in NC/Nga mice, a model for atopic dermatitis (Ohkusu-Tsukada et al., 2017). While the direct restriction of MMTV through immune modulation following *Mtv* SAG production is possible since MMTV infects T-cells, it is possible that other systems undergo the same clonal deletion, but leads instead towards immune tolerance and increased virulence to the exogenous virus that are not lymphotropic such as bovine viral diarrhea virus (Bolin, 1995).

Mtv has been implicated in tumorigenesis via Sag-dependent and Sag-independent mechanisms. For example, mammary tumor development has been shown to be dependent upon *Mtv-1, 2,* and *4* expression, where presence is associated with an increased incidence of mammary tumors, potentially through the up-regulation of host proto-oncogenes (Bruno et al., 2013; Imai et al., 1983; Matsuzawa et al., 1990; Van Nie and Vaerstraeten, 1975). Even though Sag-reactive T-cells may be deleted, in some cases *Mtv*-Sag engenders the conditions for tumorigenesis, by activating specific T-cell populations that support the development of certain neoplasms. Specifically, *Mtv*-29 Sag stimulates Vβ16 CD+ T cells preempting B cell lymphomagenesis (Sen et al., 2001). *Mtv*-knock out mouse strains infected with MMTV had decreased tumor development by 90% (Bhadra et al., 2006), and deletion of *Mtv* is associated with increased resistance to tumorigenesis by MMTV strains that encode 'strong' Sag. Thus, Mtv appears to increase pathogenicity of MMTV by increasing the likelihood of the development of tumors, however exact mechanisms are not yet known.

MURINE LEUKEMIA VIRUS (MuLV; *Gammaretrovirus*)

Much like MMTV, MuLVs has been thoroughly characterized in mouse strains used in biomedical research. The origin of endogenous MuLVs and the interactions that have been documented with exogenous MuLV have been extensively reviewed (Kozak, 2014). Ecotropic MuLVs (E-MuLVs) are found in both lab strains and wild mice, and are unable to infect other species. Polytropic MuLVs (P-MuLVs/MCF-MuLV) are found in lab strains and wild mice that also have the ability to infect a limited host range group in vitro, including human and mink cells. Xenotropic MuLVs (X-MuLVs) have not been

identified in lab strains, but are found in wild mice and have a very broad host range that include human, rabbits, cat, bat, and dog cells (Kozak, 2014). Endogenous MuLVs from all three subgroups have been documented in C57BL mouse genomes (>50 loci identified) and other mouse strains have diverse ERV MuLV genotypes at a wide variety of integration sites (Kozak, 2014).

Endogenous MuLV is capable of producing infectious MuLV, which is a relatively unique property among ERVs. The vast majority of endogenous E-MuLVs (*Emv*) are produced from fully functional MuLVs with few mutations resulting in defective replication (Jenkins et al., 1982). Some laboratory mouse strains such as NZB and F/St also have the ability to produce infectious X-MuLVs from their endogenous X-MuLV (*Xrv*) (Kozak, 2014). As such, MuLV viral particles may exist simultaneously as endogenous and exogenous retroviruses. In contrast, endogenous P-MuLVs (*Pmvs*) are incapable of producing infectious virus, despite having coding regions with open reading frames (Jern et al., 2005; Kozak, 2014). The transcriptional activity of many endogenous MuLVs has resulted in high levels of recombination between viral strains, and this has become a very important part of their biology and relates to disease outcoms. While *Pmvs* do not generate viruses alone, they may still generate transmissible P-MuLV when opportunistically packaged along with E-MuLVs (Evans et al., 2009). Transmission of P-MuLV may induce leukemia through insertional mutagenesis following activation of *Myc* or deactivation of *Trp53* (Kozak, 2014). Infectious P-MuLVs appear to be generated *de novo* in infected mice and do not appear to be horizontally transmitted. Mice that do not generate P-MuLVs do not develop viral-induced lymphomas or leukemia (Kozak, 2014). Other examples of MuLV recombination result in other neoplastic syndromes. A recombination event has been documented in xenografts of human tumors passaged in immunosuppressed mice; these recombinant viruses may result in neoplasms on their own (Kozak, 2014).

Historically, recombination of P-MuLV was believed to occur primarily between *Pmv* and E-MuLV within *env* and LTR sequences. Recently, additional sequencing targeting the origins of pathogenic and nonpathogenic recombinant MuLVs has shown that recombination occurs across the entire E-MuLV genome, although recombination in *gag* is limited due to host antiviral restriction factors.

Pathogenicity was not linked with specific changes to LTR, yet, all pathogenic P-MuLV LTRs had undergone recombination, duplication, or mutation (Bamunusinghe et al., 2017).

Beyond recombination, MuLV ERVs have been implicated in restriction against exogenous MuLV infection. This has been documented through a number of different mechanisms. *Fv1,* the first antiviral host restriction factor described, is related to the *gag* gene of a murine endogenous retrovirus (Young et al., 2018). While it only shares 43% amino acid identity to its closest ERV relative, it has been shown to target MuLV capsid proteins, inhibiting viral replication (Bénit et al., 1997). Specific alleles for *Fv1* display differential abilities to restrict different MuLV subgroups. When exposed at physiologic expression levels in cell culture, $Fv1^n$ and $Fv1^b$ restrict B-tropic MuLV and N-tropic MuLV, respectively. When concentrations of these gene products are increased above physiologic expression levels, $Fv1^b$ gains the ability to partially restrict B-tropic MuLV, whereas $Fv1^n$ restriction profiles remain the same regardless of expression level (Li et al., 2016). This may not be biologically significant, but points at the need to consider individual loci and alleles of endogenous retroviral elements and the extent that these genes are transcribed with respect to their antiviral restriction capabilities.

Specific MuLV ERV *env* genes have been co-opted by the host to function as restriction factors against retroviruses, including MuLV. A number of restriction genes (*e.g., Fv-4, Rmcf, Rmcf2*) have MuLV origins and restrict both E-MuLV and P-MuLV infection (Kozak, 2014). Other endogenous MuLV derived structures (*i.e.,* X-MuLV solo LTR) modulate host gene expression. For example, X-MuLV LTR insertions in the mouse APOBEC3 (mA3) intron increase mA3 gene expression, leading to restricted viral replication (Sanville et al., 2010). APOBEC3 is a potent antiviral restriction factor that acts as a cytidine deaminase that interferes with viral replication and this interaction represents one example in which a specific LTR integration site indirectly restricts viral infection through host gene exaptation (Harris and Dudley, 2015).

Recent investigations have characterized the diversity and interrelationship of endogenous MuLVs in laboratory and wild mice, (Bamunusinghe et al., 2016; Lee et al., 2017). Next generation sequencing techniques have revealed the penetrance of endogenous MuLVs in wild and domesticated

Mus musculus (Hartmann et al., 2015). These analyses have revealed a high degree of variation in the location and copy number of endogenous MuLVs across and even within individuals (Lee et al., 2017). Variation in endogenous MuLVs across tissues in single individuals indicated that some MuLV ERVs are transcriptionally active and maintain the ability to replicate infectious virions in a pattern that is strain-specific.

Friend MuLV infection has been documented to mobilize endogenous MuLVs (Boi et al., 2016). Endogenous MuLV transcripts increase one-day post infection with Friend-MuLV, and transcription remains higher throughout the course of infection. It is hypothesized that this process may represent an increased risk of recombination which contributes to pathogenesis and oncogenesis (Boi et al., 2016). This discovery is similar to previous investigations of *Mtv* viral infections; following certain viral infections, *Mtv* expression is increased, while in other cases, *Mtv* expression is decreased.

FELINE LEUKEMIA VIRUS (FeLV; *Gammaretrovirus*)

Feline leukemia virus (FeLV) is a common pathogenic retrovirus that is capable of causing fulminant disease in domestic cats (Hartmann, 2012) originally discovered in a cluster of group housed cats that developed feline malignant lymphoma (Schneinder et al., 1967). Endogenous FeLV (enFeLV) is in genomes of domestic cats (*F. catus*) and related small felids (Felis spp.) at variable copy numbers (8–12 copies per haploid genome and up to 19 per diploid genome), but does not exist in other Felidae (Chiu et al., 2018; Polani et al., 2010). While the genetics and mechanisms of interactions between enFeLV and its exogenous counterpart are not well understood, we perhaps know the most about the clinical outcomes of exogenous FeLV infection on its host. The availability of several effective vaccines, and the ability of cats to 'self-cure' from infection are unique aspects of FeLV infection that could be exploited to elucidate better understanding of exogenous-endogenous retroviral interactions and the role of host immunity in controlling retroviral disease.

The group of viruses known as FeLV is actually made up of 6 recognized subgroups in addition to an endogenous form of the virus (enFeLV) (Chiu et al., 2018) . FeLV is found worldwide in feral and

outdoor cats at prevalence of 3-18% (Bandecchi et al., 2006; Gleich et al., 2009; Muirden, 2002; Yilmaz and Ilgaz, 2000). The most common exogenous subgroup of the virus (FeLV-A) is believed to be the only transmissible subgroup and is found in nearly 100% of FeLV clinical cases (Jarrett et al., 1978; Jarrett and Russel, 1978). enFeLV has long been thought to interact with its exogenous counterparts resulting in recombination, oncogenesis, or interference (Polani et al., 2010). Approximately 50% of cats that are infected with FeLV-A develop a recombinant form (FeLV-B) that arises from recombination between FeLV-A and enFeLV *env* and 3'-LTR regions (Powers et al., 2018). This recombination event leads to a change in cellular tropism as well as accelerated disease progression and formation of lymphoid and other tumors (Anderson et al., 2001; Bechtel et al., 1999).

FeLV-B is known as an oncogenic variant, leading to lymphosarcoma in cats that are infected with the recombinant due to insertional mutagenesis (Fujino et al., 2008). FeLV-B is believed to primarily arise in individual hosts versus being spread by horizontal transmissible in domestic cats, though there have been two FeLV-B cases in domestic cats documented in the absence of FeLV-A infection (Stewart et al., 2013). FeLV-B has also been detected in the endangered Florida panthers that lack enFeLV sequences, the subject of Chapter 3 of my PhD studies, (Chiu et al., 2019), and was also potentially detected in a jaguar (Silva et al., 2016). While enFeLV is responsible for generating virulent FeLV-B, it also has been hypothesized to provide protection against horizontal transmission. One hypothesis for restricted FeLV-B horizontal transmission is the receptor interference. As enFeLV imparts a full or partial *env* gene to FeLV-B, the envelope proteins produced are very similar, and expression of defective enFeLV-env may neutralize FeLV-B receptors (McDougall et al., 1994), as has been described in JSRV.

While recombination and receptor neutralization have been documented prior to the early 2000's, mystery still surrounds how enFeLV may interact with FeLV-A infection. Given the fact that experimental FeLV infection can result in a wide range of disease outcomes, the possibility of immune self-tolerance has been hypothesized, but has not yet been proven (Charreyre and Pedersen, 1991). Thorough examination of infection outcomes has defined at least 4 types of infection – persistent, regressive, latent, and abortive (Torres et al., 2005). Experimental infection of SPF cats has previously

shown that higher enFeLV proviral copy numbers bolster FeLV viral replication (Tandon et al., 2008); however, a natural infection of a colony of domestic cat-Asian leopard cat (*Prionailurus bengalensis*) backcrossed hybrids demonstrated the opposite, where higher enFeLV proviral led to lower infection and viral replication (Powers et al., 2018).

Do puma cells lacking enFeLV support greater FeLV infection when compared to domestic cat cells? This is the question posed in Chapter 4 of this dissertation. We discovered that FeLV infection displayed a negative correlation with increasing enFeLV copy number, suggesting a protective capacity of enFeLV in light of FeLV-A infection. When primary PBMCs (high levels of enFeLV expression) were infected with exogenous FeLV-A, they supported less infection that fibroblasts, which transcribe 10-fold less enFeLV. We confirmed that enFeLV transcription is tissue-specific and enFeLV transcription is highest in lymphoid tissues. We are in the process of testing two hypotheses in Chapters 5 and 6 to explain these observations: 1) enFeLV transcription interacts in a virus specific manner due to direct interference not yet described in other ERV-XRV systems, and/or, 2) solo-LTRs may be positioned near anti-viral host genes that restrict FeLV infection indirectly, as has been documented in MuLV.

KOALA RETROVIRUS (KoRV; *Gammaretrovirus*)

Koala retrovirus represents the most recent ERV described in the literature and is currently undergoing the process of endogenization, with earliest estimation of invasion set at 22,200-49,900 years (Ishida et al., 2015). As such, it has been studied as a model to understand how early retroviral endogenization impacts host biology (Greenwood et al., 2018; Lober et al., 2018). Koala retrovirus was first associated in a case of spontaneous leukemia in a captive koala (Canfield et al., 1988) and identified as a gammaretrovirus closely related to gibbon ape leukemia virus present in koala populations dating back to the 19th century (Hanger et al., 2000). While the virus has endogenized in koala populations in Northern Australia, the virus has not entirely penetrated the koala populations in South Australia. Prevalence is reported at 14.8% in Kangaroo Island koalas and is apparently absent in Phillip Island koalas (Simmons et al., 2012). To date, 10 subgroups of KoRV (A-J) have been identified, although only

one exists as an ERV (KoRV-A) with 39-133 distinct loci described (Chappell et al., 2017; Hobbs et al., 2017; Ishida et al., 2015; Shojima et al., 2013; Xu et al., 2015; Xu et al., 2013). Much like MuLV, KoRV-A as a newly endogenizing retrovirus behaves like an exogenous virus in that it maintains the ability to generate infectious virions and can be transmitted horizontally; however, it has been confirmed to be endogenous as germ cells giving rise to inherited transmission, and all cells contain proviral DNA (Tarlinton et al., 2006). Despite this, there is high levels of variation displayed in copy number which may be due to the maintained ability for the ERV to replicate (Tarlinton et al., 2006). KoRV biology, evolution, and disease association is reviewed further in (Denner and Young, 2013) and (Xu and Eiden, 2015).

As a newly endogenizing virus, not enough time has elapsed on an evolutionary scale for endogenous KoRV-A genomes to become fixed in all koala genomes. KoRV-A is not pathogenic, nor is it associated with an altered immune profile (Maher and Higgins, 2016). However, KoRV-B is associated with chylamydiosis (Waugh et al., 2017). Much like other ERVs, KoRV-A induces immune tolerance, deleting lymphocytes that recognize KoRV-A (Fiebig et al., 2015). This has implications for vaccination and for recombination, as KoRV-A replication is not restricted by host adaptive immune mechanisms. Recombination of endogenous KoRV-A and exogenous KoRV has not been detected, but detection is difficult due to the high number of endogenous proviruses and the degree of variation that exists amongst these endogenous KoRV-As. Recombination has, however, been described between KoRV-A and a unique ERV, Phascolarctos endogenous retroelements (PhER) (Hobbs et al., 2017; Lober et al., 2018). While there are few consistent established ERV-XRV interactions with respect to KoRV, this new virus remains an important model of endogenization, which may give us more insight as to how ERV-XRV interactions evolve during the early phase of viral endogenization.

SIGNIFICANCE

While we no longer regard non-coding DNA (ncDNA) as genomic junk, there is an enormous amount to learn about the variety of ways that ncDNA impacts an animal's biology. Here, we have provided an

overview of documented interactions between endogenous and exogenous retroviruses as one step in considering ERV impacts on host biology. While the endogenous retroviral genes are believed to emerge and proliferate following similar mechanisms, the interactions that exist are dependent on the biology of specific viruses highlighted in Table 1. Following initial description in chickens, ERV-XRV interactions have been most thoroughly characterized in mouse models. The primary interactions between endogenous and exogenous viral pairs can be characterized as recombination, receptor interference, immunological self-tolerance, superantigen interference, and action as an antiviral restriction factor (Figure 3; Table 2). On the surface, endogenous retroviruses appear to have common ways in which they interact with their exogenous counterparts. Almost all display the ability to both restrict and promote exogenous infection, though the mechanisms by which this action occurs is virus-specific and act independently from one another. Most virus dyads do experience receptor interference, recombination, and immune self-tolerance. Mouse mammary tumor virus is the only retrovirus shown to interfere via superantigens, and murine leukemia virus is the only retrovirus reviewed where its corresponding ERV hijacks host gene expression to restrict broadly against XRV infection, including its exogenous counterpart. However, more in depth investigations of other retroviruses may reveal these as more general mechanisms.

Despite the fact that approximately 8% of human genomes are made of ERV elements, humans ERVs with related extant exogenous retroviruses have not been defined (Seifarth et al., 2005; van der Kuyl, 2012). One possible exception is HTLV and HTLV type I-related endogenous sequences (HRES) identified in 1989, sharing low sequence homology (>30% in the *gag* region) encoding a 28-kDa expressed in H9 human T cells that is cross-reactive with HTLV-I gag antigens (Banki et al., 1992; Perl et al., 1989). While it remains entirely possible that exogenous retroviruses will be discovered that have human ERVs correlates, the fact that we have not identified such circumstances raises the tantalizing question of whether ERV establishment ultimately allows a host to overcome the exogenous viral infection that preempted its existence. This is incredibly important, as approximately 70% of infectious diseases are zoonotic in nature that may have the potential to interact with human ERVs (Blancou et al., 2005). Furthermore, unfixed human ERVs are continually undergoing transposition, which may lead to

new host interactions (Wildschutte et al., 2016). It is for this reason that some groups are looking to remove ERVs in domesticated pigs with sights of reducing risks of ERV interactions in xenografts and organ transplants (Niu et al., 2017). Ultimately, understanding how our genome elements relate directly to susceptibility or resistance to infection through ERV interactions may be key in preparation for defending against new retroviral threats, assessing individual risks to infections, and overcoming many infectious and non-infectious diseases

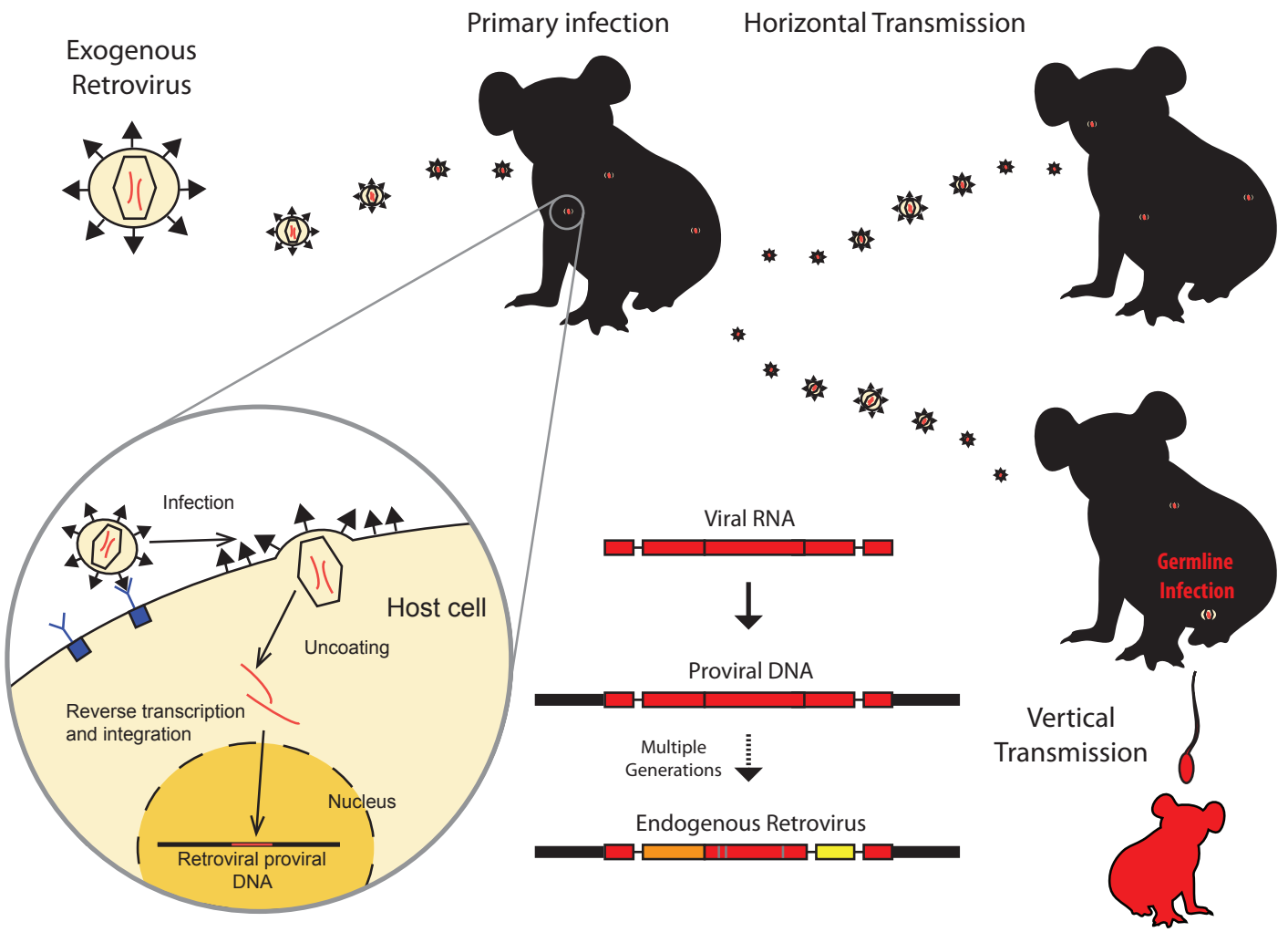

Figure 1.1: Retrovirus infection results in the integration of reverse transcribed viral DNA into the host genome that results in lifelong infection. In a small proportion of infections, infection can occur in the germline. When infected germ cells result in a developing embryo, all fetal cells are infected with the new endogenous retrovirus. Over many generations, the virus undergoes multiple mutations including single nucleotide polymorphisms, insertions, and deletions, often times leading to the inability for endogenous retrovirus to produce infections virus.

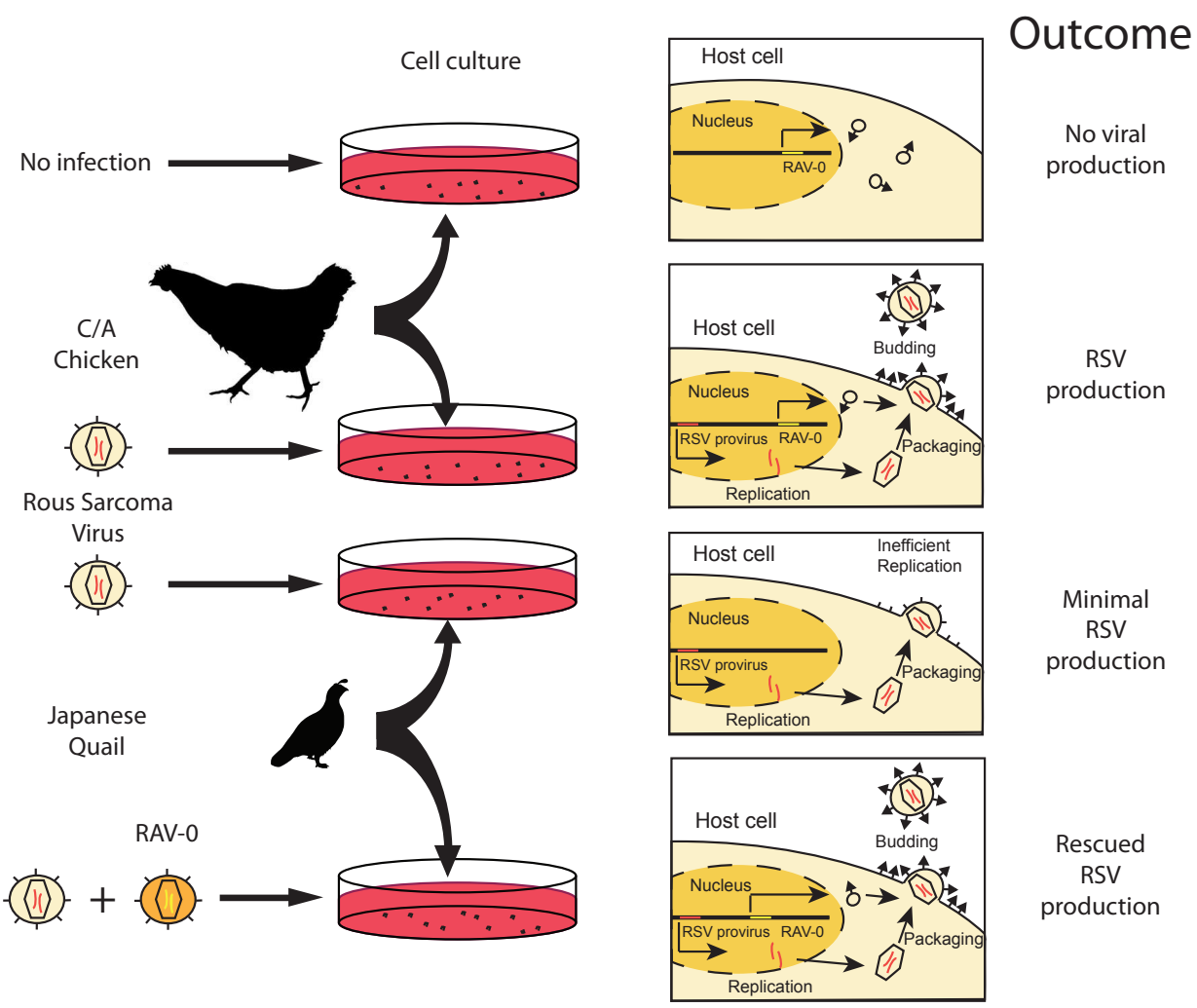

Figure 1.2: Rous Sarcoma Virus (RSV) Associated Virus (0) (RAV-0) is an endogenous retrovirus that does not produce infectious virus. In C/A strain chickens harboring RAV-0, infections with RSV leads to replication of the virus. Japanese quail are susceptible to RSV infection, but they have minimal RSV replication. When Japanese quail are co-inoculated with RAV-0 and RSV, RAV-0 acts as a helper virus that rescues Japanese quail cells to produce replicate.

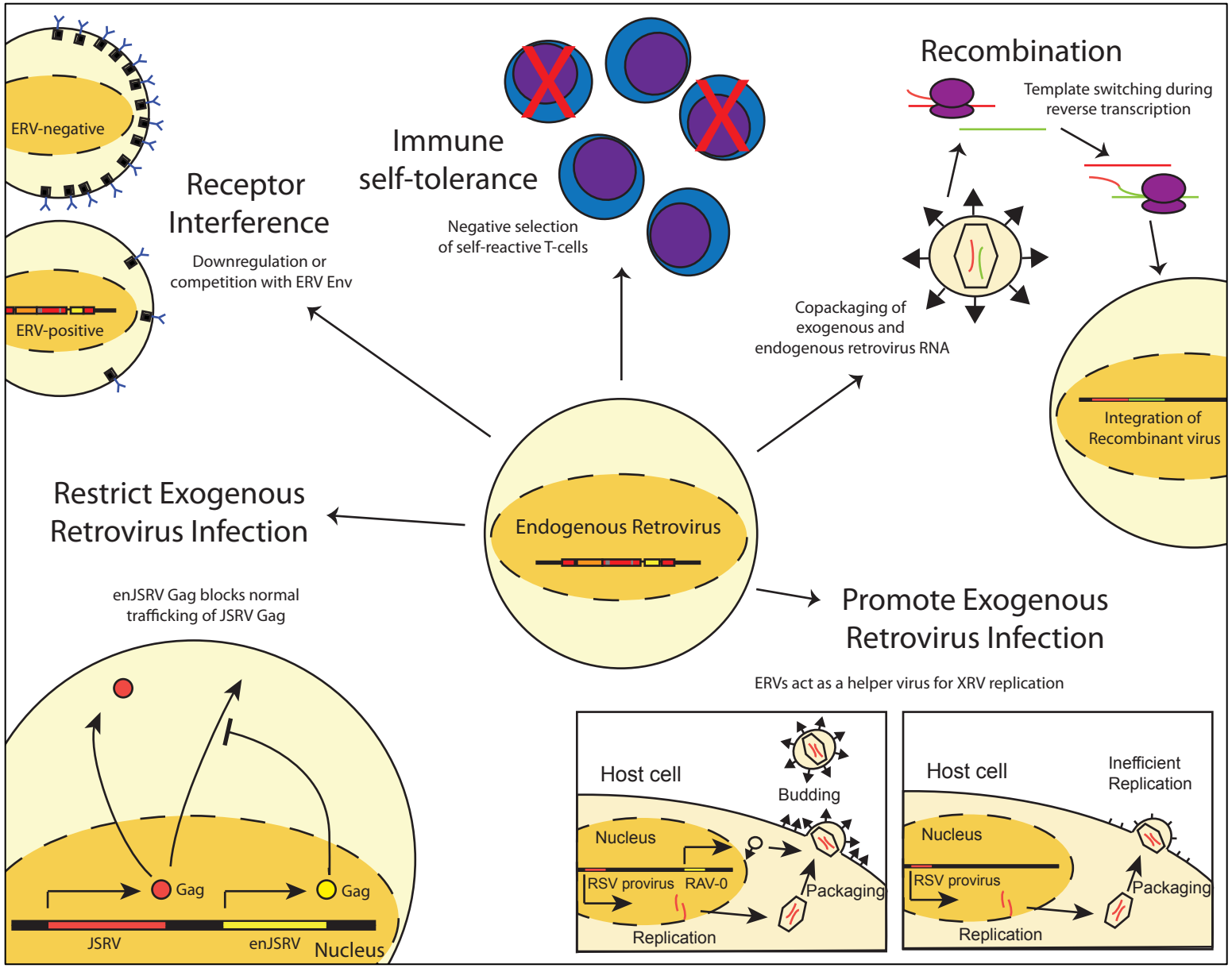
ERV-negative
ERV-positive
Receptor Interference
Downregulation or competition with ERV Env
Immune self-tolerance
Negative selection of self-reactive T-cells
Recombination
Template switching during reverse transcription
Copackaging of exogenous and endogenous retrovirus RNA
Integration of Recombinant virus
Endogenous Retrovirus
Restrict Exogenous Retrovirus Infection
enJSRV Gag blocks normal trafficking of JSRV Gag
Gag
Gag
JSRV
enJSRV
Nucleus
Promote Exogenous Retrovirus Infection
ERVs act as a helper virus for XRV replication
Host cell
Budding
Nucleus
RSV provirus
RAV-0
Packaging
Replication
Host cell
Inefficient Replication
Nucleus
RSV provirus
Packaging
Replication

Figure 1.3: Endogenous retroviruses have many functions that impact normal host cell biology. A number of these functions specifically interact with exogenous retroviruses. Major classes of interaction include receptor interference, immune self-tolerance, recombination, as well as the simultaneous action of restriction and promotion of exogenous retrovirus infection through different mechanisms. Above, particular examples are shown, but are not exhaustive as particular interactions virus-specific.

Table 1.1: Six endogenous/exogenous retroviral dyads have been identified. ERV = endogenous retrovirus; XRV = exogenous retrovirus; RSV = Rous sarcoma virus; ASLV = avian sarcoma leucosis virus; JSRV = Jaagsiekte sheep retrovirus; MMTV = mouse mammary tumor virus; MuLV = murine leukemia virus; FeLV = feline leukemia virus; KoRV = koala retrovirus; ND = not determined.

Virus	**Host**	**ERV terminology**	**Date of earliest ERV Integration**	**Endogenous copy number**	**Shared identity with XRV**	**Common diseases associated with XRV**
RSV/ ASLV	Galliform birds	endogenous ASLV	ND	average 5	>97% (*pol* and *env;*nt); 59% (*gag;*nt)	Sarcoma, leukosis
JSRV	Sheep, Goats	enJSRV	0.9-1.8 MYA	average 30	90-98% (AA)	Ovine pulmonary adenocarcinoma
MMTV	Mice	*Mtv*	ND	2-8	ND	Mammary adenoma
MuLV	Mice	*Emv, Xmv, Pmv*	~1 MYA	>50	100%* (nt)	Leukemia
FeLV	Felid cats	enFeLV	3-4 MYA	8-24	86% (nt)	Leukemia, lymphoma, anemia, immune suppression
KoRV	Koalas	KoRV-A	22,200-49,900	0-165	ND	Associations with neoplasia and secondary, opportunistic infections, including Chlamydiosis

Table 1.2: Endogenous/exogenous retroviral dyads interact in five main categories. The specifics of interactions are specific to the respective couplets. ND = not determined

Virus	**ERV terminology**	**Immunological self-tolerance**	**Recombination**	**Receptor interference**	**Restrict exogenous infection**	**Promote exogenous infection**
RSV/ASLV	endogenous ASLV	Yes	Yes	Yes	Yes	Yes
JSRV	enJSRV	Yes	ND	Yes	Yes	ND
MMTV	*Mtv*	Yes	ND	ND	Yes	Yes
MuLV	*Emv, Xmv, Pmv*	ND	Yes	ND	Yes	Yes
FeLV	enFeLV	ND	Yes	Yes	Yes	Yes
KoRV	KoRV-A	Yes	Yes	ND	ND	ND

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CHAPTER TWO

A Retrospective Examination of Feline Leukemia Subgroup Characterization: Viral Interference Assays to Deep Sequencing

INTRODUCTION

In the early 1960's, William Jarrett described feline leukemia virus (FeLV) as the infectious agent responsible for approximately half of observed cases of feline leukemia and lymphoma (Jarrett et al., 1964). The discovery of this pathogenic gammaretrovirus launched the field of feline retrovirology and discoveries relating to mechanisms of retroviral-induced cancers and oncogenes (Benveniste et al., 1975; Willett and Hosie, 2013). FeLV was historically a common domestic cat pathogen, and remains one of the few retroviral diseases for which there is an effective vaccine (Hoover et al., 1996; Pedersen et al., 1979; Willett and Hosie, 2013). As the incidence of FeLV decreased via effective quarantine and vaccination procedures, and with the discovery of feline and simian immunodeficiency viruses as alternate and more analogous models for HIV research, studies of FeLV biology and pathogenesis diminished. Therefore, most of the significant FeLV literature was generated before the development of 'modern' molecular techniques. In this retrospective, we review the traditional assays used to establish classical virus subgroups, examine how modern molecular techniques may be used to re-evaluate FeLV subgroup classification schemes, and provide new information to unravel interactions between exogenous and endogenous retroviruses.

FeLV GENOME ORGANIZATION

The genome structure of retroviruses includes three genes flanked by un-translated regulatory sequences known as long terminal repeats (LTR). G*ag* encodes group-specific capsid antigens, *pol* encodes protease, integrase, and reverse transcriptase enzymes, and *env* encodes the envelope proteins (Coffin et al., 1992). FeLV is approximately 8.4-kb in length and lacks accessory genes characteristic of complex feline retroviruses such as feline immunodeficiency virus (FIV) and feline foamy virus (FFV,

also referred to as feline spumavirus, FSV). FeLV contains two reading frames, one for *gag* and *pol* genes and a second that encodes the *env* transcript (Figure 1) (Willett and Hosie, 2013).

ENDOGENOUS FeLV (enFeLV)

As part of the retroviral infection cycle, viral RNA is reverse transcribed into DNA, which enters the nucleus and integrates within the host genome. This process leads to an integrated provirus in host cell DNA, a hallmark of retroviral infection that is a required component of the viral lifecycle. If integration occurs in a germ cell, the provirus can be transmitted vertically through simple Mendelian inheritance (Lavialle et al., 2013). As retrotransposable elements, endogenized retroviruses have duplicate flanking LTRs, and thus can be excised and relocate to other areas of the genome via recombination. Endogenized viruses may acquire mutations that impair productive viral replication, yet remain as endogenous genomic elements fixed in the host genome (Boeke and Stoye, 1997).

enFeLV appears to have invaded the feline genome prior to the speciation of the *Felis* genus (Polani et al., 2010). While enFeLVs do not induce disease in the host, they are highly relevant to domestic cat FeLV biology. enFeLV is expressed in many tissue types and is associated with FeLV infection (Krunic et al., 2015; McDougall et al., 1994; Tandon et al., 2008a; Tandon et al., 2008b). enFeLV integration site and copy numbers vary among individual cats (8-12 copies per haploid genome; up to 19 per diploid genome) due to viral transposition events and multiple independent integrations (Boeke and Stoye, 1997; Koshy et al., 1980; Polani et al., 2010; Roca et al., 2005; Roca et al., 2004). Increased enFeLV proviral copies have been correlated with both increased (Tandon et al., 2008a; Tandon et al., 2008b) and decreased (Powers et al.) susceptibility to FeLV infection, but not with disease progression (Tandon et al., 2008a). Endogenous and exogenous FeLVs are approximately 86% similar at the nucleotide level. Differences between enFeLV and exFeLV occur in *gag* and *env*, and feature insertions and deletions (INDELs), frameshifts, nonsense mutations, and changes to the unique 3' regions of the LTR (Figure 1) (Willett and Hosie, 2013). As noted below, enFeLV recombination with exFeLV results in novel FeLV subgroups (Soe et al., 1985), though the relationship between enFeLV and exFeLV infection has not been

extensively studied. Because most felid species do not harbor enFeLV, naturally occurring FeLV infections in non-domestic felids provide an opportunity to interrogate protection or promotion of exogenous FeLV by enFeLV in a biologically relevant system.

EXOGENOUS FeLV (exFeLV)

It is postulated that FeLV arose from a rodent-derived virus that evolved to infect cats as a consequence of predator/prey relationship between cats and mice (Benveniste et al., 1975). Exogenous (horizontally transmissible/infectious) FeLVs have been classified as subgroups based upon functional and genetic relatedness. The first three FeLV subgroups identified (FeLV-A, B, and C) were characterized using viral interference (VI) assays, and eventually were associated with subgroup-specific clinical phenotypes by O. Jarrett and colleagues (Jarrett, 1992; Sarma and Log, 1971, 1973). Definition of FeLV subgroups was an early area of intense FeLV study because of their relation to differences in disease progression and prognosis. FeLV-A is the most common horizontally transmitted subgroup (Jarrett et al., 1978; Jarrett and Russel, 1978). While FeLV-A has been reported to be less pathogenic than other FeLV subgroups, it has been associated with macrocytic anemia, immunosuppression, and lymphoma (Hartmann, 2012; Willett and Hosie, 2013). FeLV-B, a recombinant of FeLV-A with endogenous FeLV (enFeLV), has been reported to occur in approximately half of cats infected with FeLV-A. It arises by recombination between FeLV-A and enFeLV subsequent to co-packaging of expressed enFeLV and exFeLV transcripts into a single virion, followed by strand displacement during reverse transcription (Roy-Burman, 1996; Stewart et al., 2011; Stewart et al., 1986). FeLV-B is tumorigenic (Hartmann, 2012), and is considered to be incapable of horizontal transmission unless it is co-transmitted with FeLV-A (Sarma and Log, 1973), with rare exception (Silva et al., 2016; Stewart et al., 2013). FeLV-C is a less common subgroup that arises from *de novo* mutations in *env* of FeLV-A and has been associated with the development of aplastic anemia (Abkowitz et al., 1987; Hoover et al., 1974; Mackey et al., 1975; Onions et al., 1982; Overbaugh et al., 1988; Riedel et al., 1986; Willett and Hosie, 2013).

VIRAL INTERFERENCE ASSAYS (VI)

Viral interference assays test the ability of one viral strain to limit infection with a second viral isolate. Viral interference occurs via both intrinsic and extrinsic mechanisms resulting from cellular pathways that are perturbed during viral infection. Extrinsic VI is caused by competitive blockage of virus receptor by proteins or other viruses that bind and occlude receptor-mediated entry for subsequent viruses. Intrinsic VI refers to multiple processes including intra-cellular receptor fatigue (Breiner et al., 2001; McDougall et al., 1994; Piguet et al., 1999; Rasmussen, 1997), interferon-mediated interference in response to viral genetic material (Haller et al., 2006), and superinfection exclusion (Folimonova, 2012).

Viral interference assays were used to distinguish and initially define FeLV subgroups A, B, and C, presumably via intrinsic mechanisms. FeLV viruses that "interfere" with one another (i.e., virus A precludes superinfection with virus B) were tested by a classical method to identify viral groups of the same subgroup (which interfere) versus viruses of different subgroups (which do not interfere) (Marcus and Carver, 1967; Rott et al., 1972). In 1971, Sarma and Log used interference assays to establish the first three recognized FeLV subgroups: A, B, and C (Figure 2) (Sarma and Log, 1971). Focus-forming FeLV/murine sarcoma virus (MSV) pseudotypes (viral chimeric constructs in which MSV envelope proteins have been replaced by FeLV *env*) were produced by rescue of 9 natural tumorigenic FeLV isolates following co-culture on Harvey MSV-infected hamster tumor cells and feline embryonic fibroblasts. Subsequent in vitro infection of feline embryo fibroblasts with one subgroup resulted in the blockage of the corresponding pseudotype. Cell cultures were considered to demonstrate viral interference if a 2-log drop in focus forming titer was measured. For example, when feline embryo fibroblast cultures were infected with FeLV-A, they were still susceptible to FeLV-B and C pseudotypes (i.e., foci were present following secondary infection). Additionally, cells infected with FeLV-C were susceptible to FeLV-B pseudotype infection, and vice versa (Figure 2). These experiments led to the conclusion that FeLV-A, B and C were genetically different and capable of superinfection in cells.

Curiously, primary infection with FeLV-B or FeLV-C virus blocked subsequent infection of FeLV-A pseudotype. This unexpected display of viral interference between different strains subgroups provided evidence for co-infection between FeLV-A and other. This led to the hypothesis that FeLV-A is a necessary precursor for the development of more pathogenic FeLV subgroups and is an essential helper virus for other subgroups. Subgroups were further described by demonstrating that neutralizing antibodies raised in goats and cats inoculated with different strains demonstrated subgroup neutralizing specificity, further elucidating variation among subgroups (Russell and Jarrett, 1978). Using this criterion, FeLV-A was more monotypic compared to FeLV-B and C, which displayed more antigenic variation.

On a functional level, VI among FeLV subgroups may be explained by variation in receptor use (extrinsic interference). FeLV-A uses thiamine transporter receptors (ThTR-1) (Mendoza et al., 2006) while FeLV-B uses a common retroviral entry receptor, the phosphate transporter receptors (PiT-1/2) (Anderson et al., 2001; Johann et al., 1992; O'Hara et al., 1990; Takeuchi et al., 1992). FeLV-A env would bind ThTR-1, which would not preclude binding to PiT-1/2, but cells infected with FeLV-B would not be permissive to an additional FeLV-A infection as FeLV-B infections almost always involve a FeLV-A co-infection. FeLV-C uses a heme exporter receptor (FLVCR-1/2) along with ThTR-1/2 (Quigley et al., 2000; Shalev et al., 2009; Tailor et al., 1999).

SANGER SEQUENCING

Now a fundamental technique in molecular biology, Sanger sequencing was developed in 1977, after FeLV was discovered and classified by VI assays (Sanger et al., 1977; Sarma and Log, 1971, 1973). Sanger sequencing introduced nucleotide analysis allowing researchers to understand and associate FeLV genetic sequences with functional proteins (Tailor and Kabat, 1997). Additionally, other FeLV subgroups marked by relatively minor genetic variations were identified , making subgroup identification more complicated.

In 1980, Rosenberg et al. conducted a sequence-level comparative analysis of FeLV-A, B, and C. Homology indices based on 2D PAGE fingerprinting were low among all subgroups (37-66%)

(Rosenberg et al., 1980). Modern sequencing technologies have allowed full genome analyses of FeLV, and documented homology among all subgroups and enFeLV by pairwise comparison. Figure 3 illustrates strain similarities using SDTv1.2 nucleotide pairwise comparison tool following MAFFT multiple sequence alignment (Muhire et al., 2014). FeLV-A displays the strongest sequence conservation among distinct FeLV-A isolates, with some genes having 98% homology (Boomer et al., 1994; Donahue et al., 1988). Other subgroups are less well conserved. For instance, FeLV-B was first characterized as having up to ten variable regions with respect to FeLV-A (Nunberg et al., 1984; Riedel et al., 1986; Riedel et al., 1988; Roy-Burman, 1996) (*J. I. M., unpublished results*). The sequences of the variable region depend on the enFeLV source. enFeLVs have not been rigorously examined at the nucleotide level; as a result few FeLV-B sequences have been recorded to allow for detailed nucleotide comparisons (Boomer et al., 1997; Borjatsch et al., 1992; Miyake et al., 2016; Nunberg et al., 1984; Riedel et al., 1986; Riedel et al., 1988; Rigby et al., 1992; Rohn et al., 1998; Stewart et al., 1986; Sugai et al., 2001; Tailor and Kabat, 1997). Variable regions 1-5 (*vr1-5*) and potentially the C-terminus domain are believed to be responsible for altering cellular tropism due to changes in the receptor binding protein (gp70) based upon phylogenetic analysis (Faix et al., 2002; Riedel et al., 1988; Stewart et al., 1986). Few studies have been performed to document consequences of amino acid variation in other variable regions. Alignment and comparative analyses of enFeLV, FeLV-A and FeLV-B sequences identify a relatively conserved 5' recombination site in the 5' gp70 gene. A 3' recombination site region is also evident, but is more variable (Boomer et al., 1994; Stewart et al., 2011; Watanabe et al., 2013). Variation in recombination sites between enFeLV and exFeLV results in nucleotide divergence among FeLV-B genotypes, particularly in the envelope gene. However, FeLV-B's still share significant pairwise identity to the closely related FeLV-A's. Work from the laboratories of Roy-Burman and Overbaugh examining exFeLV/enFeLV recombination during *in vitro* infections has revealed that replication efficiency and cellular tropism depends on the length and region of the enFeLV sequence incorporated into the FeLV-B recombinant (Pandey et al., 1991). Amino acid changes localized to two variable regions (VRA and VRB) mediate the ability of FeLV-B to bind to receptors Pit1 and/or Pit 2 (Boomer et al., 1997; Sugai et al., 2001). Aside from changes to the *env* gene,

FeLV-B recombinants have been described that incorporate enFeLV sequences in the LTR region and *gag* gene (Tzavaras et al., 1990). Curiously, while enFeLV is seen as a necessary progenitor for the generation of FeLV-B, it has also been posited that truncated enFeLV Env may act to interfere with FeLV-B infection (McDougall et al., 1994).

Sequence analysis of FeLV-C linked genotypic determinants to disease phenotypes (Riedel et al., 1986). Changes in the FeLV-C 3' *pol* and 5' *env* gene are associated with aplastic anemia and expand the host range to other species in cell culture (Riedel et al., 1988). Naturally occurring FeLV-C isolates demonstrate that FeLV-C is the result of amino acid changes in the N-terminal portion of the surface protein. Further studies indicated that an 886-bp fragment from FeLV-C encompassing the 3' end of *pol* (73 amino acids) and the 5' end of *env* (241 amino acids) to a recipient FeLV-A were necessary to confer the fatal aplastic anemia phenotype (Riedel et al., 1988). Subsequent analysis indicated that a three-codon deletion within the first variable region of the *vr1* of the 5' *env* gene and nine adjacent substitutions may be sufficient to confer virulent phenotype (Borjatsch et al., 1992; Rigby et al., 1992). These findings suggest precise mutations at specific loci may dictate disease phenotypes typically ascribed to FeLV-C.

FeLV-61C (aka FeLV-T), is a T-cytopathic FeLV subgroup capable of forming syncytia in 3201 cells, was first isolated in a natural thymic lymphoma (Mullins et al., 1989). FeLV-T induces a fatal immunosuppressive disorder described as FeLV-FAIDS (72). The subgroup was characterized following experimental infections of a domestic cats with a transmissible FeLV clone, 61E (35, 72, 73).. An infected cat subsequently developed thymic lymphoma, atypical of FeLV-A infection, and tissues were analyzed for mutations underlying this phenotype (35). Sequence analysis revealed a variant of primary FeLV-A *env* containing a 6-amino acid insertion and 6-amino acid deletion (71). Another FeLV variant with a 4-amino acid insertion, (81T), was shown to be sufficient to induce the FeLV-T phenotype (Rohn et al., 1998). This variant, like 61C, was replication-defective (Gwynn et al., 2000). Chimeras generated from 61E and 81T generated tissue culture-adapted isolates with compensatory mutations at positions 7 and 375, rescuing the Env processing ability. These changes both occur outside of the receptor-binding domain (Gwynn et al., 2000). Further research documented that FeLV-T is incapable of membrane fusion

to its receptor (Pit1) due to a histidine-aspartate substitution at the N-terminus (Sakaguchi et al., 2015). Infection is possible only in the presence of FeLIX, a truncated envelope protein constitutively produced by enFeLV, which shares greater than 90% identity to FeLV-B *env* (Anderson et al., 2000). Ultimately, the progressive FeLV-FAIDS disease progression and augmented cellular tropism led to classification the FeLV-T subgroup (Donahue et al., 1991; Rohn et al., 1998).

In the late 1980's, Levesque et al. examined naturally occurring FeLV from a group of cats experiencing lymphomas. One animal had developed a multicentric lymphoma that was non-B-cell non-T-cell in origin (Levesque et al., 1990). LTR recombinants of FeLV-945 and a closely related retrovirus, Moloney murine leukemia virus, were identified in tumor tissue (Starkey et al., 1998). Variant FeLV-945 was shown to have a specific 21-bp tandem triplication repeatedly identified in independent multicentric lymphomas, conferring a replicative advantage in feline cells (Chandhasin et al., 2004; Levy, 2008; Prabhu et al., 1999).

The two most recent additions to the FeLV subgroup family include the less characterized FeLV subgroups D and TG35. FeLV-D was identified concurrent with the discovery of a novel domestic cat endogenous retrovirus (ERV-DC) that is divergent from enFeLV (Anai et al., 2012). Transduction of the ERV-DC *env* gene into FeLV produced FeLV-D that displayed novel receptor interference patterns (Ito et al., 2013). As has been hypothesized with FeLV-B, FeLV-D appears to be restricted by an ERV-DC envelope-like antiretroviral factor termed, Refrex-1 (Ito et al., 2015; Ito et al., 2013). FeLV-TG35 was identified in a 1-year-old castrated male cat. One of several *env* clones (TG35-2) harbored a 7- amino acid substitution and two amino acid insertions in the *vr1*. Although the sequence bore resemblance to FeLV-A, interference assays confirmed that TG35-2 Env targeted a different receptor, potentially constituting a new subgroup (Miyake et al., 2016).

This review of novel FeLV variants and subgroups demonstrates a wide range of sequence heterogeneity. Some subgroups represent infrequent point mutations, while others represent recombination events resulting in substitution of nearly 30% of exFeLV genome. Determining whether particular isolates are new subgroups vs. variants is reminiscent of the splitter-lumper debates that are

innate to taxonomy, systematics, and nosology (Mandy and Charman, 2012; McKusick, 1969; Senn et al., 2014; Williams and Moore, 2015). Since not all FeLV sequenced variants have been definitively associated with disease, it is also unclear if some sequenced isolates represent truncated defective viruses that are apathogenic. The presence of variable enFeLV proviral copy number and genotype provide a rich potential for the generation of many new FeLV variants during the course of infection.

POLYMERASE CHAIN REACTION

The development of PCR in the early 1980's provided scientists with the ability to directly target specific nucleic acid sequences for amplification and detection, either by visual or digital optic means (Mullis et al., 1986). The specificity of a PCR assay depends on the primers used to discriminate between targets. The presence of enFeLV in all domestic cats has added additional challenges to the understanding of FeLV biology. EnFeLV can exist as nearly full length pseudogenomes or may be present as a small fraction of the genome consisting solo LTRs. This factor, coupled with the significant homology between enFeLV and exFeLV genomes makes PCR differentiation of these two forms challenging. Regions of relatively high sequence heterogeneity in the *env* gene and LTR sequences have been exploited to develop PCR primer targets to distinguish between enFeLV and exFeLV subgroups (Polani et al., 2010; Torres et al., 2005). This has allowed investigators to begin to interrogate interactions between the enFeLV genotype and exFeLV susceptibility and disease outcome (Tandon et al., 2008b). Additionally, infection outcome categories previously characterized by antigen detection have been re-examined using PCR focusing on the differences in proviral load and viremia (Hofmann-Lehmann et al., 2008; Rojko et al., 1979; Torres et al., 2005). This advance allowed for the definition of 4 different viral outcomes based upon viral load: progressive, regressive, latent, and abortive (Hartmann, 2012; Hofmann-Lehmann et al., 2008; Torres et al., 2005). Determination of proviral and viral loads and correlation with FeLV subgroups and tissue tropism will further help to understand determinants of FeLV pathogenesis.

NEXT GENERATION SEQUENCING…AND BEYOND

Viral infections typically result in populations of viral quasi-species representing a vast amount of diversity (Domingo et al., 1978). Despite its relatively slow mutation rate compared to other retroviruses, multiple FeLV variants can be detected within a single infected cat (Coffin, 1992). Next generation sequencing (NGS) can be implemented in FeLV research to examine within host or within population viral diversity, enFeLV and exFeLV integration sites, and physiological responses to infection, which have formerly been inferred using indirect genetic analysis (Ciuffi et al., 2016; Gogol-Döring et al., 2016; Jackson et al., 2016; Muzzey et al., 2015). As of this writing, only one group has used NGS as a methodology for examining FeLV (Krunic et al., 2015). In this RNA-seq study, Krunic *et al*. measured a 3.4-fold decrease in enFeLV expression in feline lymphomas compared to case controls. NGS methods could allow for re-examination of enFeLV infection interactions in the presence and absence of exogenous (Busch et al., 1983; Sheets et al., 1993).

While NGS will open a new frontier for FeLV studies, significant challenges are inherent in enFeLV genotypic analysis. Assembling exogenous FeLV provirus are complicated by the presence of enFeLV, given the high homology between the two forms of the virus and variation in insertion sites within the feline genome. These difficulties will likely be overcome by advances in analytic analyses, which are occurring rapidly

CONCLUDING REMARKS

The history and biology of FeLV infection has been enriched with the introduction of modern sequencing methods. Multiple FeLV subgroups and the virus' propensity to interact with endogenous elements of the feline genome provide unique viral replication and transmission attributes that significantly impact disease outcomes. Viral interference assays initially determined the virus biological activity and co-infection profiles of FeLV, and predicted genomic changes that were discovered years later *with remarkable precision*. Sanger sequencing has allowed partial resolution of genotypic characterization of subgroups, resulting in a greater understanding of FeLV diversity. PCR has allowed further dissection of viral replication kinetics during infection, and next generation sequencing provides a

future landscape to derive additional information about this interesting virus and its interaction with host genomic elements. Despite the introduction of each new technology, classical techniques continue to identify historic and novel subgroups. Questions that remain include: How should we regard FeLV subgroups, and how should they be classified? Should a minimum sequence length or disease outcome define FeLV subgroups, or should subgroups be defined based on cellular tropism and demonstrated ability to replicate *in vitro* or *in vivo*? What are the molecular mechanisms and genotypic correlates that underlie disease phenotypes and outcomes? How does enFeLV genotype influence exFeLV susceptibility and disease outcome? This unique virus will continue to be an important pathogen to both domestic and wild felids. Naturally occurring infections can provide an interesting basis for examination of interactions between endogenous elements and exogenous viral agents in mammalian hosts.

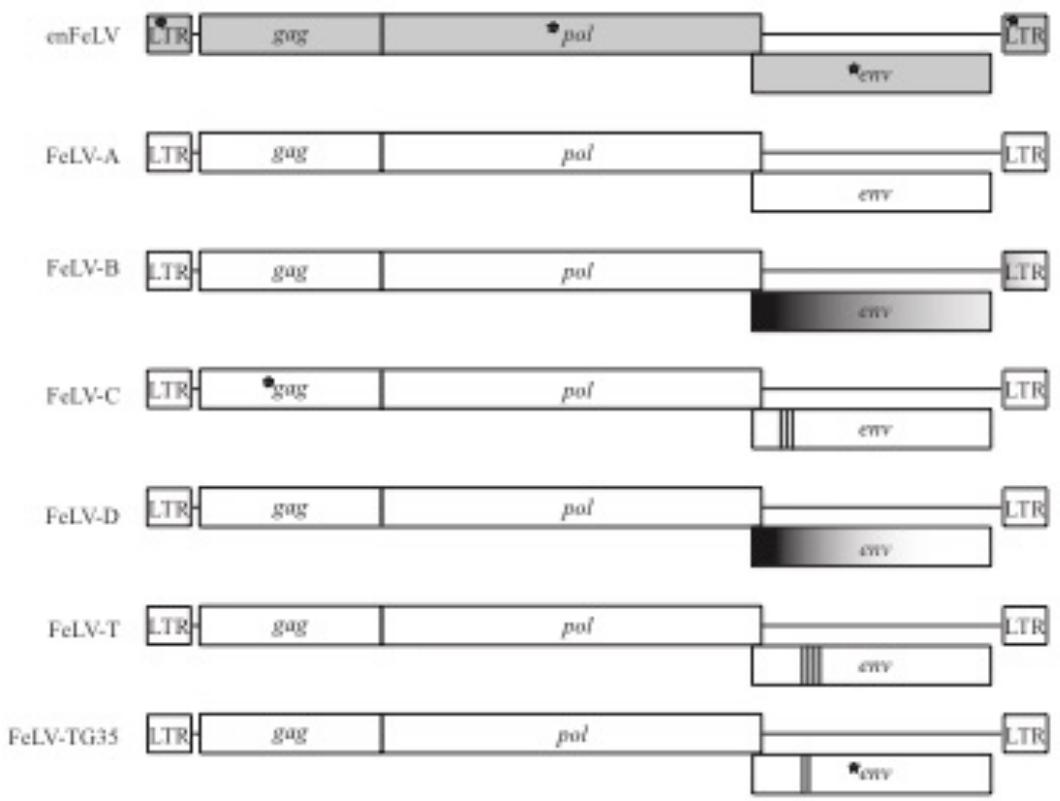

Figure 2.1: Genomic map of FeLV subgroups. Six different FeLV subgroups have been associated with different disease outcomes that differ genetically and biologically from endogenous FeLV (enFeLV). EnFeLV is the most genetically distinct from FeLV-A, with nucleotide differences noted in LTR, *gag*, and *env*. FeLV-B is formed by recombination of the enFeLV *env*-LTR with FeLV-A. The 5' recombination site is more conserved than the 3' site. FeLV-C, T, and TG35 have focal insertions, substitution, and deletions within the parent FeLV-A virus at different regions. Insertions are most often localized to the 5' *env* and are demarcated here by bold vertical bars, with each line denoting a minimum of one amino acid insertion. Stars denote presence of. SNPS that are highly concentrated in the respective genes between FeLV-A and other subgroups. FeLV-D displays a recombination event with another domestic cat endogenous virus (ERV-DC; for simplicity, we have not indicated ERV-DC here).

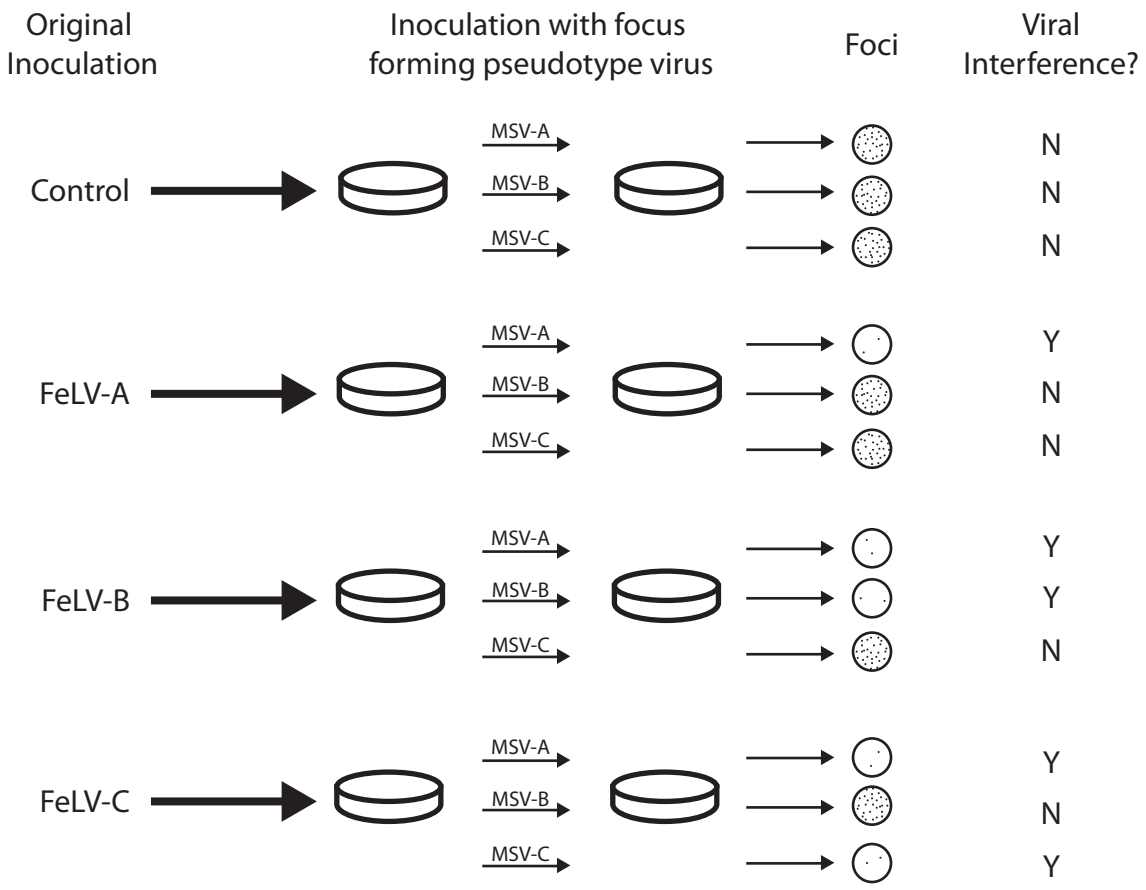

Figure 2.2: Predicted outcomes of FeLV viral interference as defined by Sarma and Log (1971). FeLV subgroups were first identified by ability or inability of virus types to infect Murine sarcoma virus (MSV)-infected hamster cells (Sarma and Log, 1971).. Focus-forming pseudotypes (chimeras with the ability to form plaques) were plated on previously infected cell cultures. Cultures with a 2-log reduction in focus-forming units were considered to demonstrate viral interference.

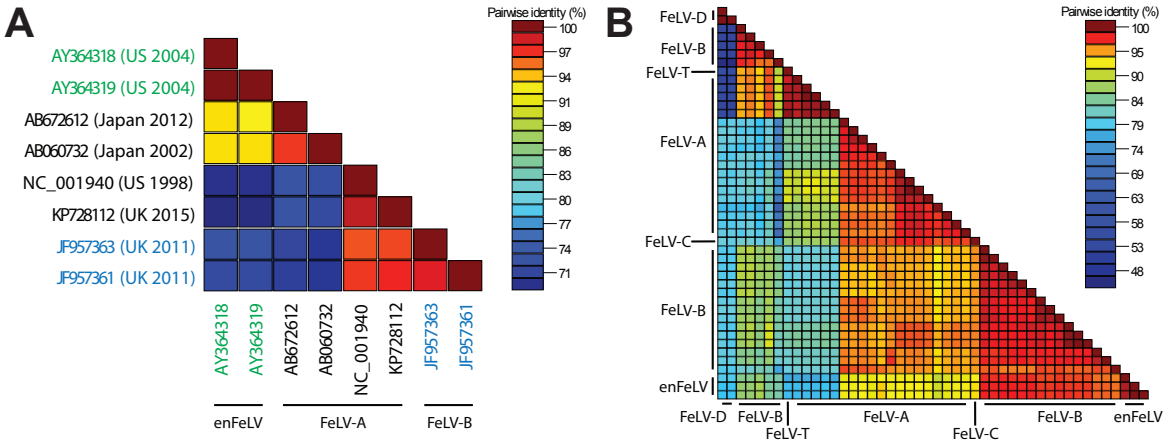

Figure 3. Pairwise identity across FeLV subytpes. A) Full genomes of enFeLV (green font), FeLV-A (black font), and FeLV-B (blue font) document discrimination of two major groups (indicated by blue/green grid and yellow/red grid). Pairwise identify is indicated by color scale of intersecting grid blocks. FeLV-A is highly conserved (>94% pairwise identity), though two subgroups are indicated by red versus yellow-orange grid colors. Isolates demonstrate great variation between clades (70-77% pairwise identity with highest conservation in *gag* and *pol*), although genetic similarity is not entirely driven by subgroup; B) Pairwise identity of the *env* genes demonstrates that this region is most divergent among FeLV subgroups. Sequence accession numbers used for analysis: enFeLV – AY364318-9; FeLV-A – AB060732, AB635483, AB635500, AB635510, AB635516, AB672612, EU359303-6, EU359308-9, KP728112, M12500, M18247-8, M89997; FeLV-B – AB635492, AB635494, AB635499, AB635502, AB635506, AB635512, AB635517, AB635526, AB635579, AB635581, AB635638, AF403716, J03448, JF957361, JF957363 K01208-9, V01172, X00188; FeLV-C – M14331; FeLV-D – AB673426, AB673432; FeLV-T – M18246.

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CHAPTER THREE

Multiple Introductions of Domestic Cat (*Felis catus*) Feline Leukemia Virus in Endangered Florida Panthers (*Puma concolor coryi*)

INTRODUCTION

Feline leukemia virus (FeLV) is a common pathogenic infectious disease responsible for significant mortalities in domestic cats, particularly prior to the development of effective vaccines in the 1980s (Willett and Hosie, 2013). Subgroup FeLV-A, which is replication competent and horizontally transmissible, is responsible for most infections (Jarrett and Russel, 1978; Willett and Hosie, 2013). Other FeLV subgroups (B, C, D, E, and T) arise following recombination or through mutation (Chiu et al., 2018). FeLV causes immunosuppressive, neoplastic, and hematopoietic disorders that correlate with FeLV subgroups (Hartmann, 2012; Mackey et al., 1975; Mullins et al., 1989). Virulent FeLV-B, the most common novel variant, arises as a result of recombination between FeLV-A and endogenous FeLV (enFeLV) present in the domestic cat genome, resulting in altered cellular tropism (O'Hara et al., 1990; Roy-Burman, 1996; Stewart et al., 1986; Takeuchi et al., 1992; Willett and Hosie, 2013). FeLV-B horizontal transmission is rare in domestic cats and is thought to require co-transmission with FeLV-A as a helper virus due to its replication-defective nature (Hoover and Mullins, 1991; Stewart et al., 2013).

FeLV prevalence in domestic cats is variable, with reported ranges from 3-18% (Bandecchi et al., 2006; Gleich et al., 2009; Muirden, 2002; Yilmaz and Ilgaz, 2000). FeLV has the capacity to infect non-domestic species including, but not limited to jaguars, bobcats, the critically endangered Iberian lynx, and pumas, most notably the endangered puma subspecies, the Florida panther (*Puma concolor coryi*) (Cunningham et al., 2008; Filoni et al., 2012; Luaces et al., 2008; Silva et al., 2016; Sleeman et al., 2001). In all non-*Felis spp*. FeLV cases, the source was believed to be domestic cats, which serve as the dominant primary host. The *Felis* genus is the only taxon known to harbor enFeLV (Polani et al., 2010). The presence of FeLV genetic sequences in the germline results in recombination between exogenous

FeLV and FeLV-A during domestic cat infections, resulting in emergence of more deleterious subgroups (i.e. FeLV-B) that are not considered to be replication-competent in the absence of co-infection with FeLV-A (Stewart et al., 2013). It is assumed that felids belonging to genera other than *Felis* are only infected with FeLV-A since they do not harbor enFeLV genomes.

FeLV outbreaks have caused concern in endangered felids that suffer from population bottlenecks, as the species may be more vulnerable to infection due to reduced genetic diversity. For example, 21% of Iberian lynx sampled between 2003 and 2007 were FeLV positive; six died from FeLV-related disease (Meli et al., 2009). Between 2001-2004, an FeLV outbreak was documented in Florida panthers (Cunningham et al., 2008). Ten Florida panthers were FeLV PCR-positive, with 5 of these also diagnosed as antigen ELISA-positive. Three mortalities were attributed to FeLV-related disease (Brown et al., 2008; Cunningham et al., 2008). Phylogenetic analysis of a region of the FeLV *env* gene during this outbreak indicated a single circulating FeLV strain, likely following introduction of the virus from a domestic cat (Brown et al., 2008). The Florida Fish and Wildlife Conservation Commission (FFWCC) attempted to contain the FeLV outbreak by implementing a vaccination campaign spanning 2003-2007 (Cunningham et al., 2008).

Between August 2004 and November 2010, approximately 125 live-captured or necropsied panthers were tested for FeLV, and no additional cases were diagnosed (FFWCC, unpublished data). Since December 2010, however, a total of 6 Florida panthers found dead tested positive for FeLV antigenemia. These cases are separated in both time and space from the historic outbreak (Figure 1). Four likely scenarios exist to explain the epidemic curve: (1) absence of FeLV (2004-2010) may have been the result of a complete eradication of the first outbreak virus, followed by the introduction of another strain from domestic cats (Figure 2A); (2) contemporary cases may have arisen from infection that persisted but was unrecognized for 6 years (Figure 2B); (3) new cases may have resulted from a combination of scenarios 1 and 2 (Figure 2C); or, (4) new cases may be explained by the introduction of multiple strains (Figure 2D). In this report, we examine the genetic relatedness between new FeLV isolates and cases described prior to 2004 to: (1) establish whether recent cases represent a new outbreak or continuation of

the prior infection; (2) determine the genetic relationship between Florida panther and Florida domestic cat FeLV strains; and, (3) gain insight to the pathogen/host interactions to better inform management practices and reduce risk of FeLV spillover from domestic cats to endangered felid populations.

METHODS

Sample collection and processing

FFWCC routinely screens samples collected from Florida Panthers for FeLV antigenemia and FIV antibodies using commercially available test kits (SNAP® Combo FeLV/FIV test, IDEXX Laboratories, Westbrook, Maine). Surveillance includes animals reported dead or those live captured as part of ongoing health monitoring and population management efforts. Confidence intervals of prevalence for both outbreak periods and the intervening quiescent period were calculated using the Wilson score method without continuity correction (Newcombe, 1998). From 2010-2016, six of the 214 (2.8%) Florida panthers (*P. concolor coryi;* UCF149, UCFP 228, FP231, UCFP241, UCFP269, UCFP275) (Table 1) reported dead to the FFWCC tested positive for FeLV antigen during standard postmortem testing on heart, chest, or venous blood collected at necropsy. Lymphoid tissues from FeLV-positive and negative controls (i.e. bone marrow, lymph node, spleen, and thymus) in addition to a fibroblast tissue culture from one panther (UCFP241R1) were harvested in Florida, stored at -80˚C, and shipped to Colorado State University Feline Retrovirus Research Laboratory for additional testing. Genomic DNA was isolated from tissues by bead-beater disruption and phenol-chloroform extraction adapted from Fan and Gulley (Fan and Gulley, 2001). Approximately 100mg of each tissue was fractionated using 1.4 mm ceramic spheres in a Fastprep-24 tissue homogenizer (MP Biomedicals Inc). 3M SDS was added to the tissue homogenates at a final concentration of 10% and incubated at 37˚C overnight. Cell lysates were washed twice with phenol chloroform. Extracted DNA was concentrated by ethanol precipitation, pelleted, dried, and re-suspended in TE buffer.

In addition to tissues from the contemporary outbreak, DNA from three historically infected (2002-2004; FFWCC) FeLV-infected *P. concolor coryi* (FP115, FP122, FP132), and four Florida domestic cats (x1608, x1613, x2653, x2655) collected between 2008-2018, were analyzed for FeLV

genomes (Table 1). Florida panther samples were obtained as previously described (Cunningham et al., 2008). Domestic cat samples were remnants of archival samples from animals presented to shelters (Carver et al., 2016) or provided by veterinary clinics to FFWCC. An additional FeLV-positive DNA blood sample obtained from a domestic cat (x2512) was included for analysis in this study (Table 1) (Powers et al., 2018).

FeLV genome recovery and analyses

Full FeLV proviral genomes (8,448 bp) were sequenced from four domestic cats (one from Maryland and three from Florida), 4 of 6 contemporary Florida panthers (2010-2016), and 1 historic Florida panther (2001-2004, Table 1). Full FeLV genomes were sequenced by generating two 5-kb fragments spanning the ~8.4 kb genome, overlapping by 1.5 kilobases. PCR reactions contained 500 nM of each primer, HiFi Kapa polymerase (Kapa Biosystems), and 50-200 ng DNA template with. PCR primer and cycling conditions listed in Table 2. FeLV status was confirmed in our laboratory by FeLV-PCR and antigen ELISA tests using established protocols (Chandhasin et al., 2005; Powers et al., 2018).

PCR products were extracted from a 0.7% agarose gel, purified using a MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology, Korea), and cloned into a pJET 1.2 blunt vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Plasmids were transformed into XL1-Blue *E. coli* competent cells (Agilent). Positive clones were prepped using DNA-spin Plasmid Purification Kit (iNtRON Biotechnology, Korea) and plasmids Sanger sequenced using primer walking (Quintarabio, San Francisco, CA). Chromatograms were verified visually to ensure that bases were scored correctly. Full genomes were assembled using *de novo* assembly in Geneious 7.0.6.

Due to sample autolysis, full FeLV genomes from 4 panthers (two contemporary: UCFP228 and UCFP269, and two historic: FP115 and FP132) and one Florida domestic cat (x1608) were not recoverable. Partial genome sequencing was accomplished using 3 fragments in *gag* and *env* genes (Table 1). Forward and reverse primers were developed to sequence a 115-bp LTR-*gag* fragment, a 98-bp *gag* fragment, and a 121-bp *env* (Table 2).

FeLV-B screening assay

Sequence analysis of FeLV Florida panther UCFP241 full genomes revealed the presence of FeLV-B. Using FeLV-A, B and enFeLV sequences available in Genbank (Benson et al., 2013), a specific FeLV-B PCR assay was designed and used to screen all panther samples for the presence FeLV-B (Powers et al., 2018).

Phylogenetic analysis

Full genome and partial *env* (1-1294 nt) sequence datasets were analyzed separately. Florida panther FeLV (FeLV-Pco) and domestic cat FeLV (FeLV-Fca) sequence identity was compared using SDTv1.2 nucleotide pairwise comparison tool (Muhire et al., 2014). A partial *env* tree was drawn to include as many GenBank FeLV sequences as possible. The three datasets – full genome, concatenated partial (three small segments within the LTR-gag, gag and env regions from FP115, UCFP228, UCFP269, and x1608 along with those comparable regions from the FeLV full genomes available) and *env* – were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) in MEGA 5.3 and open-reading frames were manually checked (Edgar, 2004; Tamura et al., 2011). To investigate phylogeny, a midpoint-rooted maximum likelihood nucleotide tree for the full genome and concatenated partial dataset were constructed using 1,000 bootstrap replicates. Best fit substitution models were determined using jModelTest (Posada, 2009) in MEGA 5.3 and phylogenetic trees constructed in PhyML implemented in SeaView4 (Guindon et al., 2010); for the full genome dataset TN93 + G model (Tavaré, 1986) and the concatenated partial sequencing nucleotide K2 + G model (Kimura, 1980). A neighbor-joining tree was constructed for the *env* dataset using SeaView4 with a Jukes-Cantor substitution model and rooted with enFeLV and FeLV-B *env* genetic sequences (Jukes and Cantor, 1969). Recombination was not removed from *env* sequences prior to constructing the tree in order to clearly demonstrate the phylogenetic relationship of FeLV-B in relation to enFeLV and FeLV-A. For all phylogenetic trees, branches with support <60% were collapsed. Full genomes from UCFP149, UCFP149R1, UCFP231, UCFP275, x1613, and partial sequences (LTR-*gag*, *gag* and *env*) from FP115, FP132 (3,688 to 8,396 nt), UCFP228, UCFP269, and x1608 are available in Genbank (Benson et al., 2013) (Table 1). Pairwise identity of full genomes were compared for FeLV-Pco, FeLV-Fca, and FeLV-B (Accession numbers:

JF957361, JF957363; isolated from the UK) using Sequence Demarcation Tool v1.2 (Muhire et al., 2014).

RESULTS

FeLV diagnosis and case attributes

Prevalence and 95% confidence intervals for FeLV diagnosed in Florida Panthers during three time periods is recorded in Table 3. FeLV was first detected in the Florida panther in 2001 (Cunningham et al., 2008; Roelke et al., 1993). This outbreak affected panthers residing mainly in protected areas (Figure 1) including Florida Panther National Wildlife Refuge, Big Cypress Swamp, and Okaloacoochee Slough State Forest (Brown et al., 2008). Between 2001-2004, approximately 131 individual animals were tested for FeLV, and 5 (3.82%) were diagnosed as FeLV antigen positive (Cunningham et al., 2008). Between 2004 and 2010, approximately 125 animals were negative. The first FeLV case in Florida panthers following the initial outbreak was documented in a road-killed panther in December 2010. Between 2014 and 2016, 5 additional FeLV-positive free-ranging Florida panthers were identified west of the historic outbreak in more populated areas (Figure 1b). One of these panthers (UCFP231) may have succumbed to FeLV as the primary cause of death. Cause of death for the other five panthers was vehicle collision. Contemporary cases diagnosed between 2010-2016 (6 of 184) primarily represented animals hit by vehicles in human populated areas (Figure 1). Prevalence (3.26%) was similar to prevalence detected in 2001-2004 (Table 3). Lymphadenopathy was documented in two of the six animals (UCFP149, UCFP275). UCFP149 had a linear stomach ulcer. The other animals were too autolyzed to identify gross abnormalities. Confirmatory FeLV PCR and antigen ELISA tests re-established in positive and negative diagnoses the contemporary outbreak.

Identification of recombinant subgroup FeLV-B in Florida panthers

A clone sequenced from sample UCFP241 was identified as the recombinant subgroup FeLV-B. An *env* alignment of FeLV-Pco, FeLV-Fca, and enFeLV (Figure 3) confirmed an infection of FeLV-B resulting from a previously reported recombination event between FeLV-A and enFeLV. Multiple clones

were sequenced to confirm presence of FeLV-B. All other Florida panther samples were screened for FeLV-B by PCR (Powers et al., 2018), but were negative (data not shown).

Pairwise comparison and phylogenetic analysis

FeLV-Pco is ~75% identical at the nucleotide level to previously published FeLV-Fca and ~94-98% identical at the nucleotide level to FeLV isolated from Florida domestic cats (x1613, x2653, x2655). The Florida panther FeLV-B full genome sequenced from x2272 is ~75% identical to previously sequenced FeLV-B and 95-97% identical to FeLV-Pco subgroup A viruses (Figure 4).

i. Full genome phylogeny.

The maximum likelihood tree of full FeLV-Pco and FeLV-Fca genomes documents two monophyletic linages: (1) a lineage comprised of FeLV isolates recovered from Florida – both FeLV-Fca and FeLV-Pco and (2) a lineage comprised of previously published FeLV-Fca isolates from other geographically distinct locations in the UK and the US, including one FeLV-Fca isolate from Florida. FeLV-Fca isolate (x2655) from Florida groups basal to the two FeLV-Pco clades, but within the larger Florida FeLV linage. FeLV-Fca isolate x1613 is basal to the clade that includes all historic and related contemporary isolates. FeLV-Fca isolate x2512 was recovered as part of this study originating from Maryland, and groups with FeLV-Fca isolate x2653 as part of a relatively homogenous US/UK clade (Figure 5a).

FeLV-Pco isolates fall into at least two clades. Two isolates from the contemporary outbreak are monophyletic (UCFP231 and UCFP241) and are referred to here as “contemporary FeLV-Pco.” The other FeLV-Pco clade contains two FeLV-Pco from the contemporary outbreak (UCFP149, UCFP275) and is most closely related to the historic FeLV-Pco isolate FP122; this group is therefore referred to here as “historic FeLV-Pco.”

ii. Partial genotype phylogeny.

The partial genotyping FeLV-Pco sequences documented 10 single nucleotide polymorphisms (SNPs) and a nine-nucleotide insertion in the un-translated LTR-*gag* region, three SNPs in the *gag* region, and eight SNPs in the *env* region. Phylogenetic analysis of the short concatenated sequence supported

similar relationships established by full genome nucleotide trees (Figure 5b). An additional historic FeLV-Pco (FP115) is most related to other historic FeLV-Pco isolates. FeLV-Pco from a contemporary outbreak sample (UCFP228) also falls in the historic FeLV-Pco clade. Five clones sequenced from the *env* portion of FeLV-Pco UCFP269 display two genotypes. Concatenated sequences from UCFP269 displayed a paraphyletic relationship (Figure 5b). Both isolates clustered outside the historic and contemporary FeLV-Pco strain clades (Figure 5b). FeLV-Fca (x1608) groups within the major Florida FeLV clade (Figure 5b).

iii. *env* phylogeny.

Phylogenetic relationships established by full genome and concatenated partial sequence trees were supported by the *env* neighbor-joining tree (Figure 6). *Env* phylogeny clearly shows differentiation between FeLV-A/FeLV-B/enFeLV subgroups. FeLV-B and enFeLV subgroups cluster together, while FeLV-A remained a monophyletic group. All FeLV-Pco sequences detected during the historic outbreak are monophyletic (Brown et al., 2008). Two *env* sequences described here are closely related to previous sequences reported from the same individual. FP132, an isolate from a panther sample obtained in 2004 has two SNPs; FP122, also obtained in 2004 has 5 SNPs. FeLV-Pco sequences clustered into two clades, supporting previously identified relationships. One FeLV-Pco clone (UCFP241B) clusters in the FeLV-B clade.

DISCUSSION

Two distinct FeLV outbreaks were recorded in the Florida panther population between 2001-2016 (Table 3). Phylogenetic data from both outbreaks documents that FeLV-Pco resulted from initial spillover from domestic cats, presumably during a predatory event (Brown et al., 2008; Roelke et al., 1993). While FeLV prevalence in domestic cats is relatively low (~4%) in Florida (Lee et al., 2002), it has been reported that domestic cats make up ~5% of Florida panther diet (Caudill et al., Submitted). The relatively high number of domestic cats consumed, particularly in panther habitat proximal to human development, presents opportunities for FeLV spillover from cats to panthers (Lee et al., 2002).

Genomic analysis of contemporary FeLV-Pco identified three independent isolates (UCFP149, UCFP149R1, UCFP275) that were genetically similar to historic FeLV-Pco (FP122, Figure 5A). Analysis additionally detected FeLV in a paraphyletic clade that is similar to, but distinct from, historic FeLV-Pco (>97.0% nucleotide identity to FP122; Figure 4). This genotype was detected in two panthers sampled in 2015 (UCFP231, UCFP241) (Figure 5A). A third FeLV strain may be present in contemporary samples, represented by partial FeLV sequences derived from one panther (UCFP269, Figure 5B). Three Florida FeLV-Fca isolates (x1613, x2655, x1608) clustered with Florida panther genotypes. One Florida FeLV-Fca (x2653) was significantly divergent to other Florida FeLV-Fca, resembling previously characterized FeLV-61E and domestic cat FeLV isolates from the US and UK. Movement of domestic cats by owners likely results in mixing of FeLV strains beyond geographic sites.

Full genome and concatenated partial genome trees demonstrate that domestic cat FeLV strains are situated basal to FeLV-Pco, providing evidence of a domestic cat origin of the panther FeLV infections. Genetic distances between these Florida FeLV-Fca isolates and non-Florida FeLV-Fca indicate a more distant evolutionary relationship between domestic cat strains compared to Florida FeLV-Fca and FeLV-Pco (Figure 5), suggesting minimal species adaptation is required for cross-species transmission between cats and panthers. Additional FeLV full genome samples would enable a Bayesian ancestral reconstruction analyses to further resolve FeLV isolate ancestry.

Full genome phylogenetic analysis supports both “combination” and “hybrid” panther FeLV re-emergence hypotheses (Figure 2C-D). Assuming that the Florida Panther population was approximately 300 individuals between 2004-2010, sampling 125 of these individuals with no FeLV detected provides a >95% confidence interval that FeLV prevalence was 3% or lower. Test results during both outbreak periods indicated ~3% FeLV prevalence, signifying that control measures initiated during the historic outbreak were successful in at least controlling, if not eliminating, additional panther FeLV infections for several years. Contemporary FeLV in Florida panthers was identified near human population centers where exposure to feral domestic cats would be more likely to occur (Aguilar and Farnworth, 2013). While it is feasible that each contemporary case represented an individual exposure to a different

domestic cat, panther to panther transmission cannot be excluded, particularly for cases that occurred around the same time period and displayed similar genotypes (i.e. UCFP231 and UCFP241 sampled in 2015; UCFP228 and UCFP275 sampled in 2014 and 2016, respectively).

In addition to the common horizontally transmissible FeLV subgroup (FeLV-A), we recovered and sequenced an oncogenic FeLV subgroup (FeLV-B) from tissues from a contemporary Florida panther (UCFP241B; Figure 5b, Sup Fig 1). This subgroup is a recombinant of FeLV-A and enFeLV, an endogenous retrovirus harbored only by members of the *Felis* genus. Identification of FeLV-B infection in a Florida panther is only possible as a result of horizontal transmission of FeLV from a domestic cat, as panthers lack enFeLV to support recombination (Polani et al., 2010). FeLV-B is common in domestic cats; recombination occurs in approximately 33-68% of cats infected with FeLV-A (Jarrett et al., 1978), presumably via independent recombination events that occur *de novo* following infection of domestic cats with FeLV-A. FeLV-B horizontal transmission has only been described on three prior occasions (Stewart et al., 2011). One study reports that FeLV-B was detected in a jaguar (*Panthera onca*); however, this analysis was based upon sequence amplified from a 232-bp region of the LTR (Silva et al., 2016; Stewart et al., 2013). Here we have clearly documented a full FeLV-B genomic sequence in an endangered non-*Felis* species. This is of concern, as FeLV-B is oncogenic and associated with increased morbidity and mortality in domestic cats (Hartmann, 2012; Onions et al., 1980; Tzavaras et al., 1990). As non-*Felis spp.* lack enFeLV, they may be more vulnerable to an adapted FeLV-B that is readily horizontally transmitted between individuals; thus spillover of FeLV-B from domestic cats co-infected with this recombinant strain could therefore represent a more significant risk to vulnerable non-domestic cat populations.

Besides individual and population health effects, a potential outcome of FeLV infection in non-domestic felids is germline infection leading to endogenization. Early endogenization results in an infection where the virus has yet to accumulate mutations rendering the endogenous retrovirus defective; therefore, at this stage the virus may be passed horizontally to other animals (Lober et al., 2018). Early endogenization may result in decreased fitness, as exemplified by endogenization of koala endogenous retrovirus (KoRV-A, (Avila-Arcos et al., 2013; Lober et al., 2018). Infection with KoRV-A has also led

to higher incidence of secondary infections like chlamydiosis and neoplasias (Tarlinton et al., 2005). Therefore, FeLV infection of panthers and other non-*Felis* species is a greater concern for long-term population effects.

Our study demonstrates that even with efforts to control FeLV in an intensively managed population, FeLV remains a risk to Florida panthers, particularly for animals inhabiting areas near urban centers. Moss *et al* (2016) has reported that the proportion of diet consisting of domestic animals is increasing in Colorado pumas concurrent with puma co-localization in human habitats (Moss et al., 2016). This trend likely exists in Florida.

This report highlights the importance of continued surveillance of Florida panthers for exposure to FeLV as an important risk management strategy (Caudill et al., Submitted). Annual sampling of a proportion of the Florida panther population that is sufficient to detect 3% FeLV incidence with relative certainty, and increased vaccination of panthers and domestic cats along sites of potential interaction, are recommended measures to protect against future outbreaks.

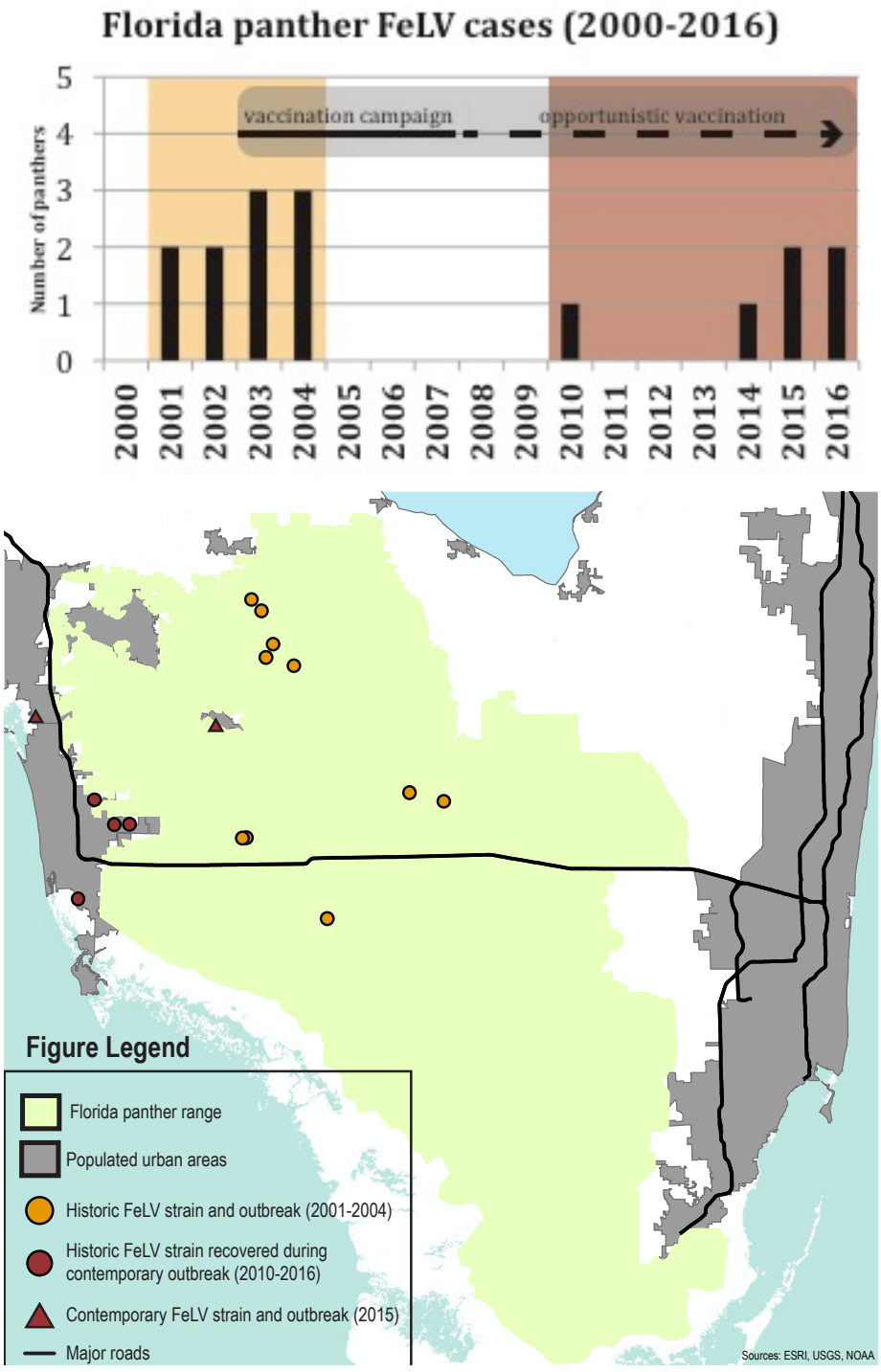

Figure 3.1: Temporally and spatially distinct FeLV cases in endangered Florida Panthers. A) Incidence of FeLV in live caught and necropsied Florida panthers. Recaptured panthers are not represented. Different colors indicate 1st (yellow) and 2nd (red) outbreak events. A vaccination campaign began in 2003 and efforts to actively vaccinate panthers continued until 2007; opportunistic vaccination has continued opportunistically since the campaign. B) Distribution of historic and contemporary Florida panther FeLV cases.

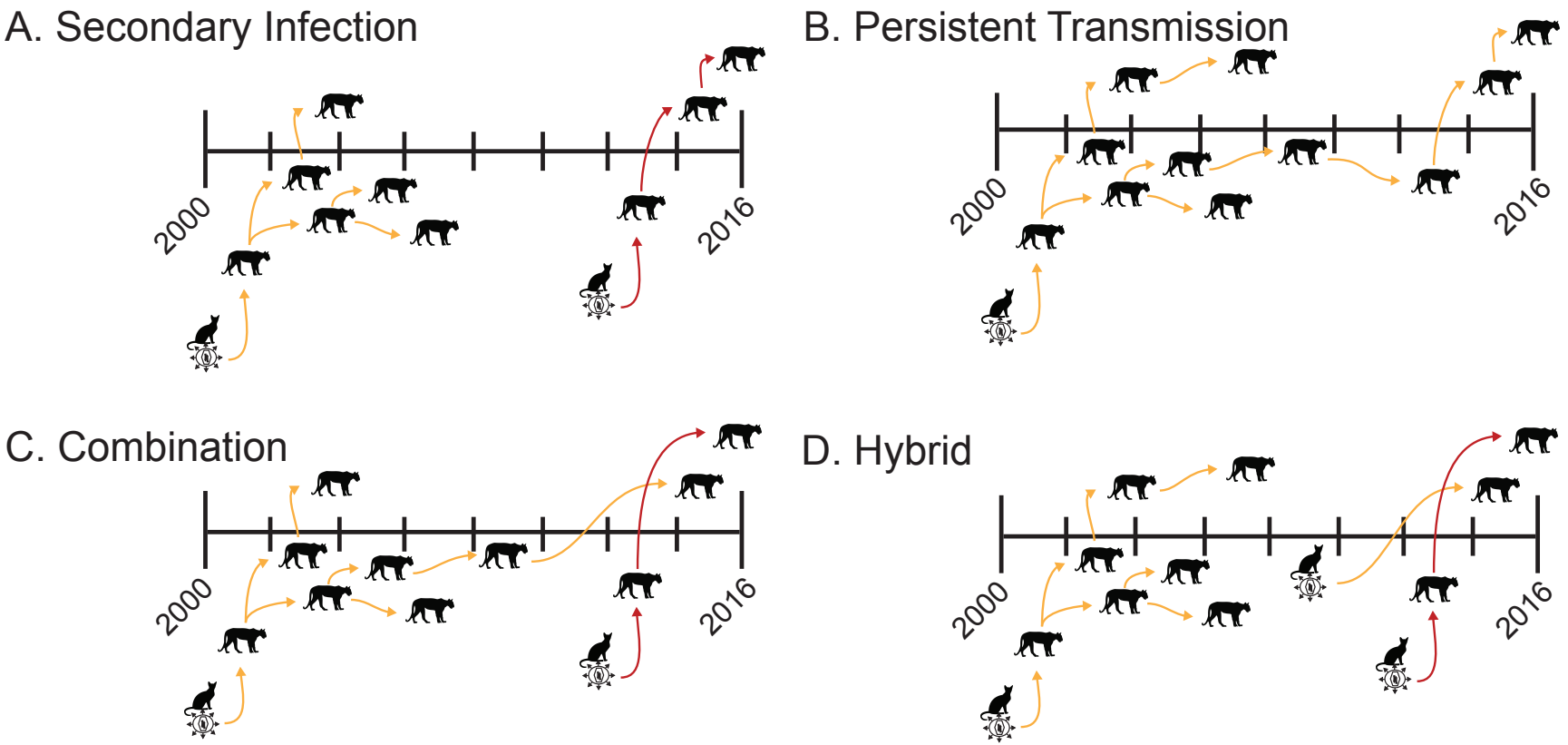

Figure 2. Hypotheses for FeLV outbreaks represent A) two separate viral strains, B) persistent transmission of on FeLV strain, or C) a combination of the two scenarios. Transmission of different viral strains are indicated by either orange or red arrows.

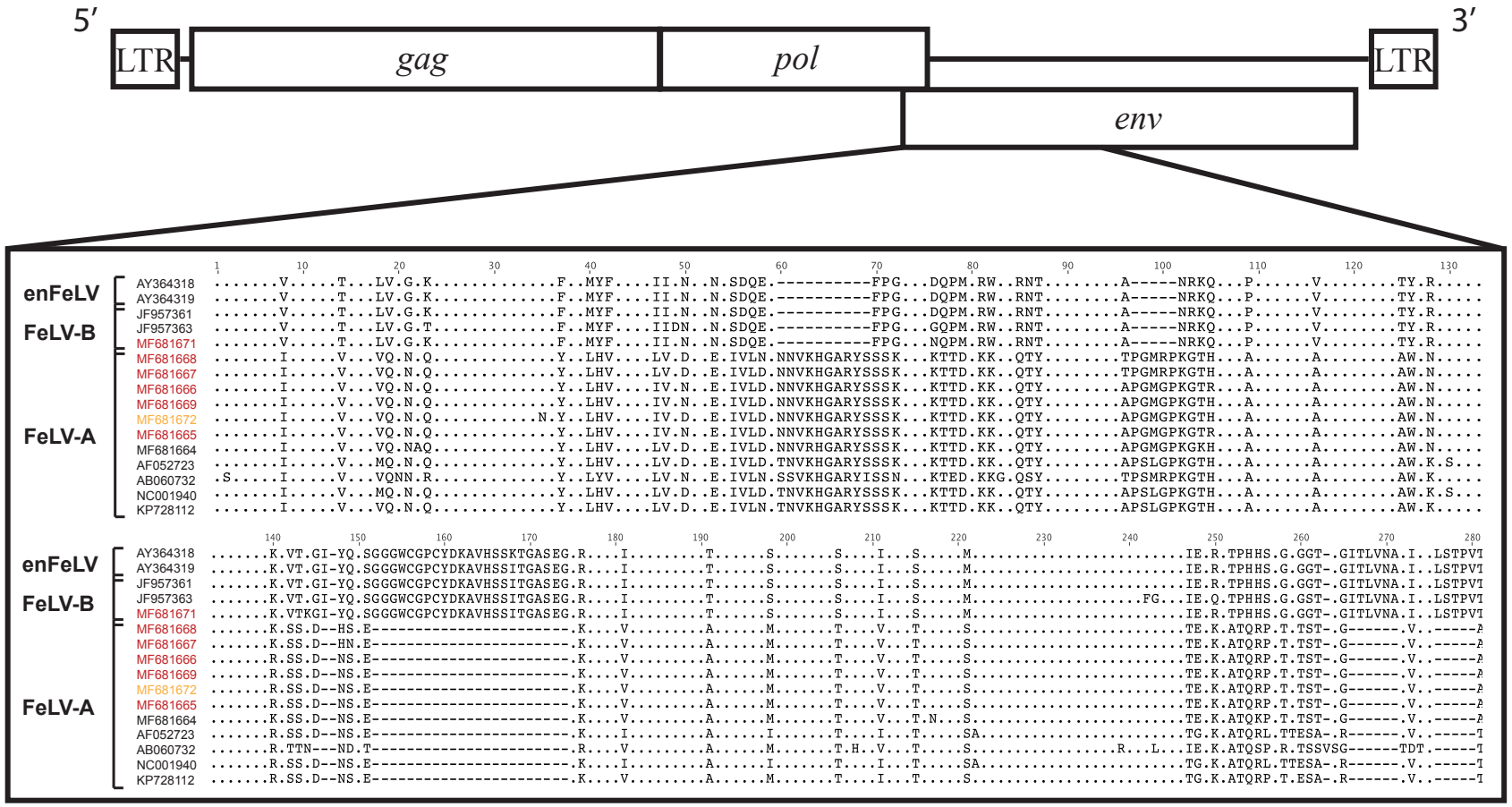

Figure 3.3: FeLV Env alignment of FeLV-A, FeLV-B, and enFeLV from previously published sequences and Florida panthers support the identification of FeLV-B in one Florida panther (MF681671).

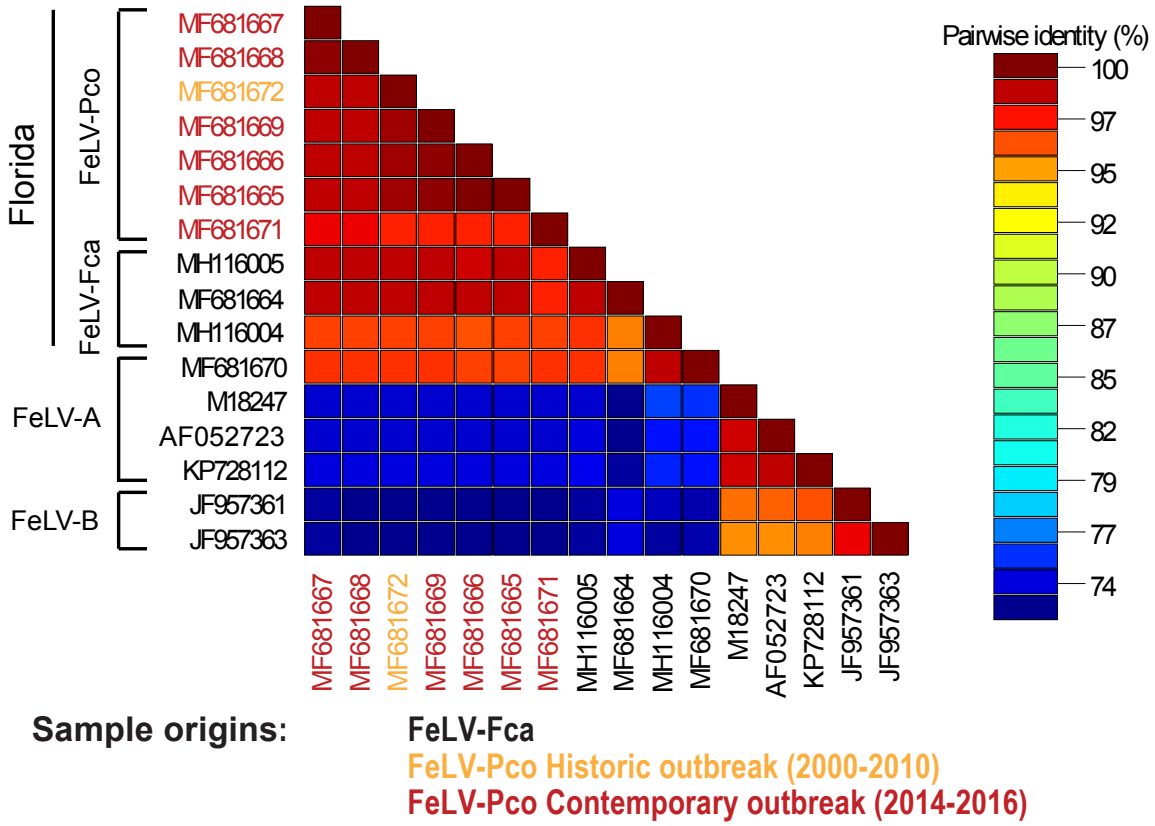

Figure 3.4: Heat map of FeLV-Fca and FeLV-Pco shows high homology of FeLV in the Florida panthers and Florida domestic cats. Previously published FeLV-A displayed two groups of related viruses.

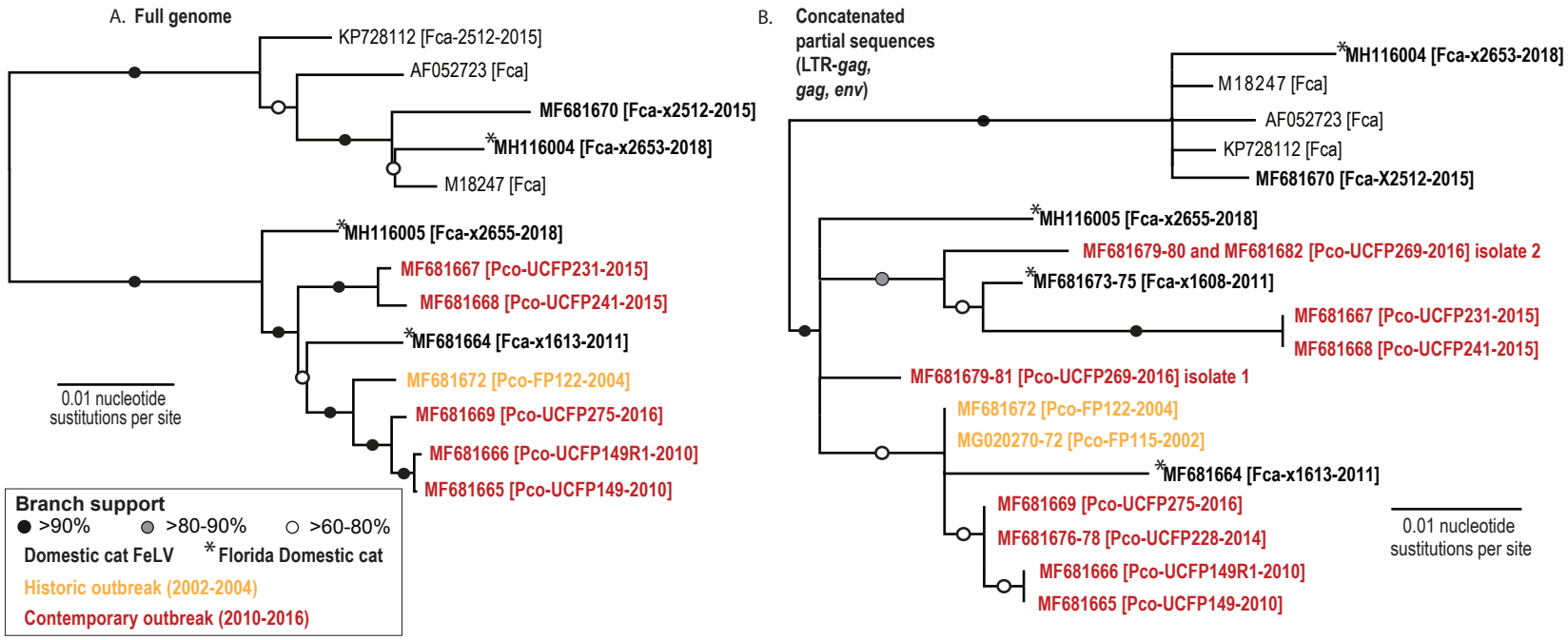

Figure 3.5: Maximum likelihood phylogenetic trees document two distinct FeLV-Pco clades. A) Full genome phylogeny indicates Florida FeLV-A sequences are monophyletic. Historic and contemporary FeLV outbreak sequences reside in one clade, while a second clade consists solely of contemporary FeLV outbreak sequences. B) Genotyping sequence phylogeny generated from concatenating three ~100bp regions (LTR-*gag*, *gag* and *env*) compare full genome isolates demonstrated in (A) in addition to 4 individual sequences. Bolded identifiers represent isolates sequenced in this study.

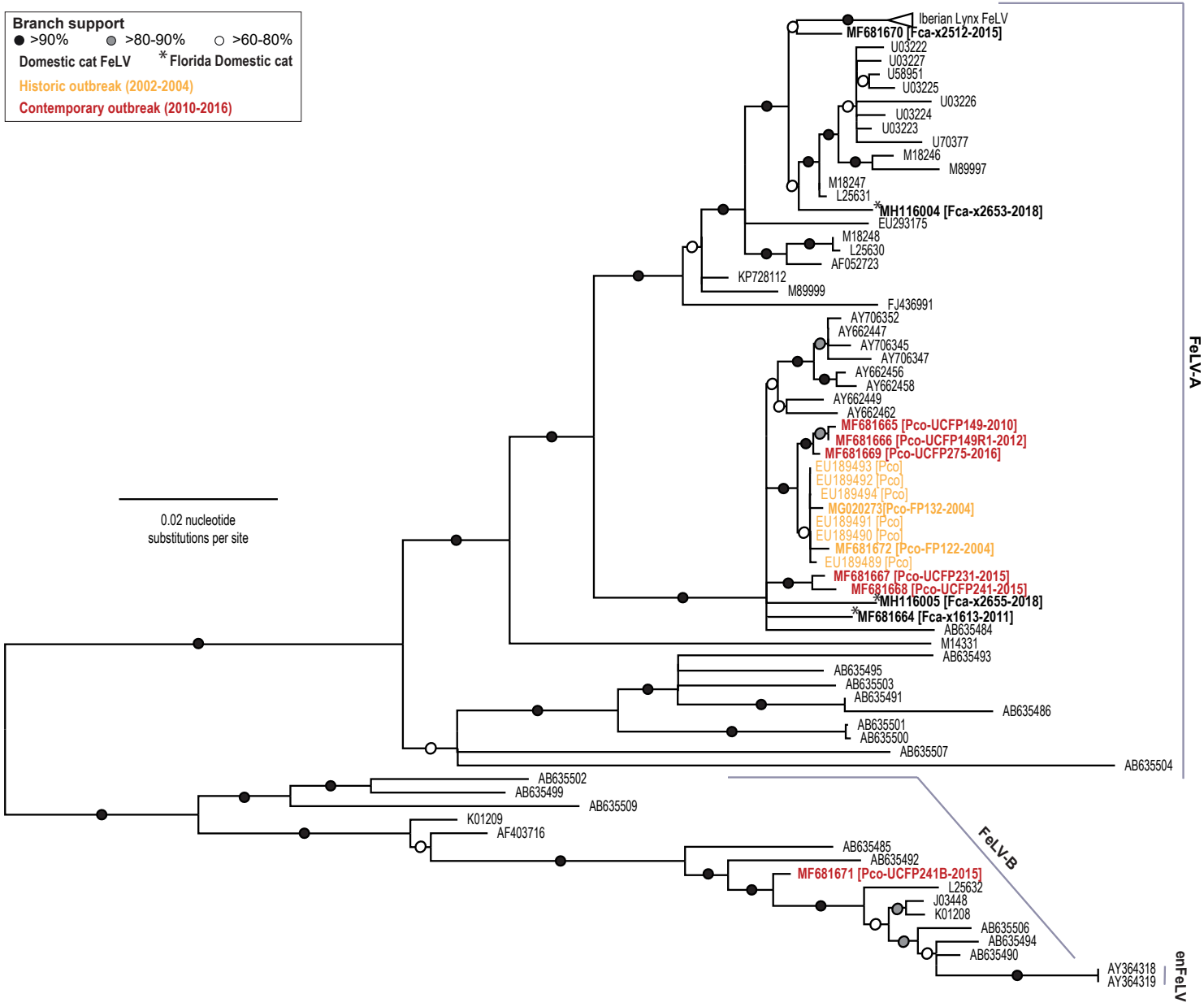
Branch support
>90%
>80-90%
>60-80%
Domestic cat FeLV
*Florida Domestic cat
Historic outbreak (2002-2004)
Contemporary outbreak (2010-2016)
0.02 nucleotide substitutions per site
Iberian Lynx FeLV
MF681670 [Fca-x2512-2015]
U03222
U03227
U58951
U03225
U03226
U03224
U03223
U70377
M18246
M89997
M18247
L25631
*MH116004 [Fca-x2653-2018]
EU293175
M18248
L25630
AF052723
KP728112
M89999
FJ436991
AY706352
AY662447
AY706345
AY706347
AY662456
AY662458
AY662449
AY662462
MF681665 [Pco-UCFP149-2010]
MF681666 [Pco-UCFP149R1-2012]
MF681669 [Pco-UCFP275-2016]
EU189493 [Pco]
EU189492 [Pco]
EU189494 [Pco]
MG020273[Pco-FP132-2004]
EU189491 [Pco]
EU189490 [Pco]
MF681672 [Pco-FP122-2004]
EU189489 [Pco]
MF681667 [Pco-UCFP231-2015]
MF681668 [Pco-UCFP241-2015]
*MH116005 [Fca-x2655-2018]
*MF681664 [Fca-x1613-2011]
AB635484
M14331
AB635493
AB635495
AB635503
AB635491
AB635486
AB635501
AB635500
AB635507
AB635504
AB635502
AB635499
AB635509
K01209
AF403716
AB635485
AB635492
MF681671 [Pco-UCFP241B-2015]
L25632
J03448
K01208
AB635506
AB635494
AB635490
AY364318
AY364319
FeLV-A
FeLV-B
enFeLV

Figure 3.6: *Env* phylogenies support relationships established in the full genome tree and document FeLV-B-Pco relationship to other known recombinant viruses. *env* tree displays FeLV-A, FeLV-B, and enFeLV sequences (neighbor-joining analysis). One Florida panther sequence (MF681671) can be found in the FeLV-B cluster, identifying it as the recombinant subgroup. Identifiers in bold represent sequences recovered in this study.

Table 3.1: Florida panther sample identification table including demographic information and regions of the genome sequenced.

Field sample ID	Lab Sample ID	Host Species	Collection date	Location	Full genome	Partial Regions (LTR-gag, gag and env)	Accession number
FP115	x1755	*Puma concolor coryi*	**26-Nov-02**	**FL**		**X**	MG020270-MG020272
FP122	x1948	*P. concolor coryi*	**30-Jan-04**	**FL**	**X**	**X**	MF681672
FP132	x1955	*P. concolor coryi*	**17-Mar-04**	**FL**		**X (3,688 to 8,396 nt)**	MG020273
UCFP149	x2004	*P. concolor coryi*	**13-Dec-10**	**FL**	**X**	**X**	MF681665
UCFP149R1	x2004R1*				**X**	**X**	MF681666
UCFP228	x2271	*P. concolor coryi*	**28-Dec-14**	**FL**		**X**	MF681676-678
UCFP231	x2270	*P. concolor coryi*	**20-Jan-15**	**FL**	**X**	**X**	MF681667
UCFP241	x2272	*P. concolor coryi*	**30-Apr-15**	**FL**	**X**	**X**	MF681668 and MF681671 (FeLV-B)
UCFP269	x2274	*P. concolor coryi*	**18-Feb-16**	**FL**		**X**	MF681679-682, two *env* genotypes from one sample
UCFP275	x2273	*P. concolor coryi*	**6-Apr-16**	**FL**	**X**	**X**	MF681669
517278	x1608	*Felis catus*	**10-Nov-11**	**FL**		**X**	MF681673-675
517453	x1613	*F. catus*	**10-Nov-11**	**FL**	**X**	**X**	MF681664
BDX387	x2512	*F. catus*	**3-Jul-15**	**MD**	**X**	**X**	MF681670
	x2653	*F. catus*	**6-Jan-18**	**FL**	**X**	**X**	MH116004
	x2655	*F. catus*	**31-Jan-18**	**FL**	**X**	**X**	MH116005

NB: * recaptured puma x2004. FL=Florida and MD=Maryland
FP = Florida panther
UCFP = Un-collared FP

Table 3.2: Primer sequences used for each species and locus. The first half-genome primer set for FeLV sequencing (F/5.2R) spanning 5'-LTR/3'-pol were the same for the FeLV-positive Florida panthers and domestic cats. Reverse half-genome primers were designed to avoid amplification of domestic cat enFeLV and therefore differed for Florida panthers and domestic cats.

Region	**Sequence**	**Species**	**Cycling conditions**
Full Genome (1^{st} half)			95˚C : 3 min;
Forward	5'-TGAAAGACCCCCTACCCCAAAATTTAGCC-3'	*Puma concolor coryi/ Felis catus*	
Reverse	5'-GCGGGTCCATTATCTGAACCCAATACC-3'	*P. concolor coryi/ F. catus*	30 cycles of
			98˚C: 20 sec;
Full Genome			60˚C: 15 sec;
(2^{nd} half)			72˚C: 2 min 40 sec;
Forward	5'-GAGTTCCTTGGAACTGCAGGTTACTGCC-3'	*P. concolor coryi/ F. catus*	
Reverse	5'-TGAAAGACCCCTGAACTAGGTCTTCCTCG-3'	*P. concolor coryi*	72˚C: 2 min 40 sec
Reverse 2	5'-GCTGGCAGTGGCCTTGAAACTTCTG-3'	*F. catus*	
FeLV-B *env*			
Forward	5'-CAGATCAGGAACCATTCCCAGG- 3',	*P. concolor coryi*	
Reverse	5'-CCTCTAACTTCCTTGTATCTCATGG -3'	*P. concolor coryi*	[28] *Powers et al., 2018*
LTR-*gag*			
Forward	5'-CGCAACCCTGGAAGACGTTCCA-3'	*P. concolor coryi/ F. catus*	
Reverse	5'-TCGTCTCCGATCAACACCCTGTATTCA -3'	*P. concolor coryi/ F. catus*	95˚C : 3 min;
gag			30 cycles of
Forward	5'-GGACCTTATGGACACCCCGACCAA -3'	*P. concolor coryi/ F. catus*	98˚C: 20 sec;
Reverse	5'-GGAGGGGGTAGGAACGGACGAA-3'	*P. concolor coryi/ F. catus*	60˚C: 15 sec;
			72˚C: 15 sec;
env			
Forward	5'-CCTTTTACGTCTGCCCAGGGCAT-3'	*P. concolor coryi/ F. catus*	72˚C: 30 sec
Reverse	5'-TTCCACCAAGCTTCTCCTGTGGTCT -3'	*P. concolor coryi/ F. catus*	

Table 3.3: Two independent FeLV outbreaks have been detected in Florida Panthers. Prevalence and 95% confidence intervals indicated for historic and contemporary FeLV cases detected in Florida panthers. Animals were tested using a commercially available test that detects FeLV antigenemia. Animals tested prior to 2001 were uniformly negative.

Time frame	Number positive individuals	Prevalence	95% CI
2001-2004	5/131	3.82%	1.64 – 8.62
2004-2010	0/125	0%	0 – 2.98
2010-2016	6/184	3.26%	1.50 – 6.93

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CHAPTER FOUR

Comparisons of feline leukemia virus (FeLV) Susceptibility of puma (*Puma concolor*) and domestic cat (*Felis catus*) cells in relation to endogenous FeLV elements

INTRODUCTION

The vast majority of vertebrate genomes, including up to 8% of the human genome, harbor fossils of ancient viral infections made up predominantly of retroviral genetic material (Bock and Stoye, 2000; Consortium, 2001; Katzourakis and Gifford, 2010). During infection, the retroviral RNA genome is reverse-transcribed to form double stranded DNA, which is in turn integrated into the host's genome (Coffin, 2004). While most of these infections target somatic cells, there have been numerous examples of the viruses infecting and integrating into the germ line (Xu et al., 2018). The consequences of viral integration into germ cells may be profound, and ultimately the virus has the potential to be vertically transmitted as permanent genetic elements (Greenwood et al., 2018). Fixation of the retroviral content in host genomes is a process termed endogenization and leads to new host genetic elements called endogenous retroviruses (ERVs) (Boeke and Stoye, 1997). ERVs in their early stages are believed to undergo massive changes during host cell transcription, and this foreign, potentially deleterious genetic material accumulates mutations and deletions that often render the newly endogenized virus defunct (Lober et al., 2018). While typically unable to produce infectious virions, many ERVs are still capable of undergoing transcription and may even produce functional viral proteins, which sometimes remarkably become functional in important physiologic, cellular, or biological processes (Knerr et al., 2004; Li and Karlsson, 2016). ERVs have been implicated in other biological processes such as placentation, oncogenesis, immune modulation, and infectious disease progression (Chuong, 2013; Crittenden et al., 1987; Umemura et al., 2000; Zeng et al., 2014). While many ERVs do not have remaining exogenous retroviral counterparts that continue to function as horizontally transmitted viruses, several well-studied examples of ERV-exogenous virus pairings are reviewed in the introductory chapter of this dissertation.

This chapter more fully explores the implications of endogenous-exogenous FeLV interactions in feline hosts relating to outcomes of exogenous viral infection.

Feline leukemia virus is a common domestic cat virus endemic in several *Felis* species (Polani et al., 2010; Willett and Hosie, 2013). Despite control of the virus following the development of an effective vaccine, the virus is still maintained in multi-cat households and feral cat populations (Burling et al., 2017; Powers et al., 2018) at variable prevalence across the world. FeLV-A is a subgroup of exogenous FeLV (exFeLV) that is most commonly horizontally transmitted between domestic cats (Jarrett et al., 1978). Interestingly, FeLV has endogenized in domestic cats and related species, (Polani et al., 2010), an event that is believed to have originated in a basal species prior to the speciation of *Felis* spp.

FeLV is not endemic in the majority of wild non-*Felis* species, but epizootic outbreaks have been documented following introduction from domestic cats into susceptible populations (Bandecchi et al., 1992; Chiu et al., 2019; Gleich et al., 2009; Luria et al., 2004; Meli et al., 2009; Muirden, 2002; Yilmaz and Ilgaz, 2000). Many felid species implicated in these outbreaks, including the North American puma (*Puma concolor*) do not have enFeLV present in their genome, though they remain susceptible to exogenous infection (Polani et al., 2010).

enFeLV is approximately 86% homologous to exogenous FeLV-A at the nucleotide level (Chiu et al., 2018). A domestic cat infected with FeLV-A can undergo recombination with enFeLV to form an oncogenic variant (FeLV-B), for which the virus was named (Stewart et al., 1986). This recombination between endogenous and exogenous retroviruses is not unique to FeLV, and has been documented in many endogenous and exogenous retrovirus pairs (seen introductory chapter, as well as (Bamunusinghe et al., 2017; Vogt and Friis, 1971).

One mechanism by which endogenous retroviruses may interact with exogenous retroviral infection includes the co-option of endogenous retrovirus long terminal repeats (LTRs), which harbor enhancer and promoter regions to stimulate host gene transcription. Endogenous retrovirus genome structures consist of protein encoding genes (*gag*, *pol*, and *env* are the minimum requirements for infectious retroviruses) capped on both ends by LTRs oriented in the same direction (Coffin, 2004). The

duplicate LTRs allow the virus to excise its genome from the cell and transpose itself in other loci of the host genome (Bock and Stoye, 2000). This action of retrotransposition typically results in a solo-LTR left behind in the original location (Vitte and Panaud, 2003). In the case of murine leukemia virus (MuLV), endogenous MuLV-LTRs have been co-opted to transcribe host antiviral genes including, but not limited to APOBEC3, a potent host retroviral restriction factor (Sanville et al., 2010).

FeLV represents an endogenous-exogenous retroviral system that has perhaps been best studied with regard to disease biology and outcome during naturally occurring infections in an outbred, highly dispersed mammalian host, and thus evaluation of this system provides opportunities to better understand ERV-exogenous viral interactions that are highly relevant to virus and host evolution and ecology. While enFeLV is known to recombine with the most common exogenous subgroup (FeLV-A) to form an oncogenic variant (FeLV-B) (Chiu et al., 2018), enFeLV impact on FeLV-A infection remains understudied. We know that in wild felid FeLV epizootics, the viral infection may cause significant disease, frequently leading to serious disease in non-domestic cats (Cunningham et al., 2008; Luaces et al., 2008). Furthermore, a significant proportion of FeLV-A infection in adult cats less frequently develop to progressive infections, but the proportion of wild cats that develop progressive versus lesser forms of infection is yet unknown. FeLV-B, thought to be incapable of horizontal transmission with rare exception, was recently documented in a Florida panther (*Puma concolor coryi*) (Chiu et al., 2019; Stewart et al., 2013). enFeLV was also recently associated with infection outcomes following a natural FeLV outbreak in a multi-cat household, indicating a possible protective capacity of enFeLV-LTRs (Powers et al., 2018). Given the wealth of literature showing different interactions between endogenous and exogenous retroviruses, we hypothesize that the presence of genomic enFeLV protects against virulent exFeLV infection. Thus in this chapter we evaluate FeLV infection of puma (*P. concolor*) and domestic cat cells *in vitro* and *in situ* to examine the susceptibility of endemic and novel hosts to FeLV infection with respect to enFeLV.

METHODS

Sample Acquisition

Full skin thickness biopsies from puma pinnas were collected by the Colorado Parks and Wildlife under their IACUC protocols. Abdominal skin samples were opportunistically collected during necropsies performed at the Colorado State University College of Veterinary Medicine. Primary fibroblasts were isolated as previously described (Vangipuram et al., 2013) and cultured in 20% FBS-supplemented DMEM high glucose media and 1x antibiotic-antimycotic solution (Gibco, penicillin/streptomycin/fungizone). Primary cultures were expanded for at most four passages before being frozen in (20% DMSO, 10% FBS, 70% serum free DMEM) using a Mr. Frosty freezing container (Nalgene) and stored at -80˚C.

Blood was drawn from specific pathogen free domestic cats housed at Colorado State University (IACUC protocol #16-6390A). Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by ficoll-gradient centrifugation. PBMCs were cultured in 20% FBS-supplemented RPMI media supplemented with 100 ng/mL IL-2 (Sigma, USA) and 50 ng/mL concanavalin A (Sigma, USA). Primary PBMC cultures were expanded for 2 passages before being directly infected.

Bone marrow, thymus, spleen, and lymph node from naturally FeLV-infected domestic cats (n=6) and Florida panthers (*P. concolor coryi*, n=11) were collected and shipped to the Colorado State University Feline Retrovirus Research Laboratory for additional testing. Panther tissues were collected by the Florida Fish and Wildlife Conservation Commission from animals that had succumbed to FeLV following the introduction of the virus in two epizootics (Chiu et al., 2019; Cunningham et al., 2008). Domestic cat tissues were obtained from cats with terminal FeLV disease graciously provided by Animal Protective League (Springfield, IL), a shelter for injured and ill animals that houses FeLV infected cats. Puma and domestic cat lymphoid tissues were prepared for ELISA and qPCR assays described below. DNA was extracted by bead-beater disruption and phenol-chloroform extraction using previously reported methods (Chiu et al., 2019) to measure FeLV proviral load. Tissues were homogenized by bead-beater disruption (6.0 m/s for 60 seconds) in PBS with protease inhibitor (Pierce, Waltham, MA). Homogenates were diluted to 1% and 0.1% tissue for p27 capsid antigen quantification.

3’-azido-3’-deoxythymidine (AZT) treatment

Feline foamy virus (FFV) is a common virus that infects domestic and wild felids (Kechejian et al., *in revision-a;* Kechejian et al., *in revision-b;* Winkler et al., 1999). One sample of primary puma fibroblasts developed cytopathic effects characteristic of FFV in culture, which was confirmed by qPCR using previously reported methods (Ledesma-Feliciano et al., 2018). Because we were unable to recruit large number of puma fibroblast samples, this culture was treated prophylactically with the anti-retroviral drug, AZT (100 ug/ml; Sigma) per manufacturer's direction until infection CPE subsided. Cells were passaged two times for approximately 10 days in media without AZT washout prior to infection.

Virus titration

Crandell Reese feline kidney cells (CrFK) were plated at a density of 1,250 cells per 0.32cm^2 in a flat-bottomed 96-well plate. FeLV-61E was obtained (gift of Edward Hoover and Candace Mathiason) and CrFK cells were infected in quintuplicate following a ten-fold dilution series. Cells were washed with sterile PBS, were given fresh media, and were incubated with 5% CO_2 at 37˚C for ten days. Titration was repeated three times. FeLV antigen ELISA detection, described below, was used to detect viral capsid antigen p27 in the supernatant. The quantity of virus necessary to infect 50% of tissue cultures ($TCID_{50)}$ was calculated by previously published methods (Hierholzer and Killington, 1996).

enFeLV and exFeLV quantification

Two measures of enFeLV were quantified: *env*, used as a proxy for full-length endogenous FeLV, and LTR, used as a measure of full length enFeLV as well as solo LTRs. Exogenous FeLV proviral DNA was measured by targeting exFeLV specific LTRs. enFeLV-*env* and LTR in addition to exFeLV primers and probes were previously designed and reactions were performed as described (Tandon et al., 2008) on a Biorad CFX96 thermocycler. Primer and probe sequences and qPCR thermocycling conditions are reported in Table 1. In order to determine enFeLV and exFeLV proviral load, quantified FeLV was normalized against host gene CCR5 (C-C chemokine receptor type 5; (Howard et al., 2010)) using delta cT method accounting for two CCR5 genes per cell (Pfaffl, 2006). Custom DNA oligos were synthetically constructed with target regions of enFeLV LTR and env, exFeLV, and CCR5 on one DNA construct for quantification (gBlock, IDT; Figure 1).

Viral infection

Low passage (fewer than 5 passages) primary fibroblast culture from pumas and domestic cats were cultured in 20% FBS-supplemented DMEM high glucose media. Cells were plated at a density of 50,000 cells per 2 cm^2 in a 24-well plate and infected with a multiplicity of infection (MOI) of 0.01 FeLV-61E in triplicate and cultured with 1.2mL media. 120 μL of supernatant was collected and stored at 80˚C at days 0, 1, 3, 5, 7, and 10 for detection of p27 ELISA. At days 5 and 10, cells were harvested to determine cellular viability based on cell number and percent mortality by counting cells stained with trypan blue (Gibco) on a hemocytometer. One domestic cat cell culture, one puma cell culture, and one CrFK cell culture infection were terminated at day 7 due to equipment failure. One puma primary cell culture triplicate infection was repeated twice. Domestic cat PBMCs were similarly cultured and infected, but in RPMI media as previously described (Riedhammer et al., 2014). PBMCs were plated at $1x10^6$ cells/mL and infected with an MOI of 0.01 FeLV-61E. Supernatant was sampled at days 0, 1, 3, 5, and 7 and tested for viral antigen using p27 ELISA. Fibroblasts were harvested at day 5 and end of study to enumerate cell viability and proviral copy number; PBMCs were harvested at day 5 to enumerate cell viability and proviral copy number.

Enzyme linked immunosorbent assay

FeLV capsid antigen p27 was measured by sandwich ELISA. Costar Immulon 2HB plates were coated with 600ug CM1 capture antibody (Custom Monoclonal, Inc., US) in 100uL 0.1M Carbonate buffer (7.5 g/L Sodium Bicarbonate, 2.0 g/L Sodium Carbonate, pH ~9.5) overnight at 4˚C. Plates were blocked with 200uL 2% BSA in TEN buffer for two hours. One hundred μL of samples buffered with 50 μL ELISA diluent were incubated for two hours on a plate shaker. Six hundred micrograms of biotinylated secondary antibody (CM2-B; Custom Monoclonal, Inc., US) was incubated in each well, followed by 1:4000 dilution of HRP-conjugated streptavidin (ThermoFisherScientific, MA). Each step following sample incubation was followed with 5x wash with TEN buffer (0.05M TRIS Base, 0.001M EDTA, 0.15M NaCl, pH 7.2-7.4) with 0.1% Tween. All incubations were performed at room temperature. p27 antigen was detected indirectly following the addition of 3, 3', 5, 5' tetramethyl benzidine (TMB)

substrate and peroxidase (Biolegend, San Diego, CA) at room temperature for 7.5 min before adding 2.5 N H_2SO_4 was quantified by Bioanalyzer at 450nm. Semi-purified FeLV p27 diluted in appropriate media (DMEM or RPMI) was used as a standard curve. Cutoff values for negative samples were three times the standard error over the average OD measured for control media samples.

Realtime FeLV PCR

Real time PCR was used to quantify exogenous FeLV provirus using previously described primers and probe (Table 1) (Torres *et al*., 2008). exFeLV qPCR reactions contain 400 nM of both primers, 80 nM probe, iTaq Universal Probe Supermix (Bio-Rad), water, and DNA template. CCR5 exists as two copies per cell. Puma-specific CCR5 primers and probes adapted from Howard *et al*. were used to normalize FeLV copy numbers per 10^6 cells (Table 1). The probe was labeled with FAM (6-carboxyfluorescein) reporter dye at the 5' end, ZEN (Integrated DNA Technologies (iDT), Coralville, Iowa) internal quencher and IABkFQ (Iowa Black Fluorescein Quencher; iDT, Coralville, Iowa) at the 3' end. CCR5 qPCR reactions contained 200nM forward primer, 500 nM reverse primer, and 200nM probe, iTaq Universal Probe Supermix (Bio-Rad), water, and DNA template. FeLV and CCR5 reactions were run simultaneously on the same plate on a Bio-rad CFX96 at 95˚C for 3 minutes, followed by 40 cycles of 95˚C for 5 seconds and 60˚C for 15 seconds. The limit of detection for this assay is ≥10 copies per reaction. Standards for this assay were created as custom synthetic oligos (gBlocks, iDT) containing a relevant fragment of the exogenous FeLV and CCR5 genes (Figure 1). Standard dilution and controls were run in duplicate and samples were run in triplicate.

RESULTS

Primary fibroblasts were cultured from 3 free-ranging puma ear punches (two kittens of unknown sex and one adult male) and 1 abdominal skin incision from >10 year old female captive puma. Primary fibroblasts were cultured from 7 domestic cats abdominal full skin biopsies from necropsied cats at Colorado State University (unknown sex and age).

Fibroblast infections

Exogenous FeLV proviral load was significantly and substantially greater in puma fibroblasts compared to domestic cat cells (Figure 2A). At days 5 and 10, puma cells were infected with approximately 1 proviral FeLV per cell; domestic cat cells infection consisted of 1 provirus per 10 cells. For comparison across all infections, CrFK infections were included. FeLV infections of CrFKs displayed reproducible levels of infections with tight interquartile ranges while domestic cat and puma fibroblast infections displayed greater variance (Figure 2A). Differences exhibited between puma and domestic cat cells were statistically significant by Kruskal-Wallis test (*Fca* D5 vs. *Pco* D5 = -29.8, $p<0.01$; *Fca* D10 vs. *Pco* D10 = -32.55, $p<0.01$). FeLV infection in puma and domestic cat fibroblasts similarly differed in viral antigen (Figure 2B). Puma generated FeLV antigen detected in the supernatant was greater than in domestic cat cultures beginning days 3 (Tukey's multiple comparison test; $p=0.0369$).

There was no difference between domestic cat and puma viable cell count on days 5 or 10, averaging $1.28x10^5$ cells per 2 cm^2 for domestic cats cells and $1.92x10^5$ cells per 2 cm^2 for puma cells (Figure 3A). CrFKs are smaller than both domestic cat and puma primary fibroblasts and therefore viable cell count was higher ($3.54\ x10^5$ cells per 2 cm^2 at day 5 and $4.13\ x10^5$ cells per 2 cm^2 at day 10). Percent mortality experienced between day 5 and 10 was 3.94±0.70% in domestic cat cells and 4.73%±1.45% in puma cells regardless of infection status (Figure 3C). There was a consistent trend for lower percent mortality at day 5 when cells first reached confluency. CrFKs experienced greater cell mortality over the course of the infection regardless of infection status (2.59% at day 5 and 14.26% at day 10).

Peripheral blood mononuclear cells

Exogenous FeLV proviral load in 6 domestic cat PBMCs was much lower than domestic cat fibroblast infections (Mann-Whitney U test, $p=0.0022$; Figure 2C). At day 5, domestic cat cells were infected at a maximum of $5x10^3$ proviral copies of FeLV per 10^6 cells. CrFK infections were included as positive controls, and as expected, CrFKs infection was more consistent as evidenced by tight interquartile ranges.

FeLV replication was not supported by domestic cat PBMC to the degree measured in fibroblast infections despite infection with a similar MOI (Figure 2D). FeLV replication peaked above negative

cutoff levels in samples from only two animals. PBMCs cell counts increased from $1x10^6$ cells/mL to $6.24x10^7$ cells/mL at day 5 and $10.28x10^7$ cells/mL at day 7(Figure 3B). Percent mortality at day 5 was 37.84.94±5.41% and 59.38%±18.35% at day 7 (Figure 3D).

enFeLV-exFeLV correlation

Domestic cat cells harbored more enFeLV LTRs than enFeLV *env* genes. Normalized LTR sequence copy numbers in domestic cat cells ranged from 32 and 122 copies per cell with an average of 67 copies per cell and one outlier harboring 397 copies (Figure 4). Copy numbers for *env*, were significantly lower, ranging from 9 to 14 copies per cell with an average of 11 copies and one outlier harboring 68 copies (Figure 4).

Variation in domestic cat fibroblasts enFeLV-LTR copy number correlated to FeLV replication (day 7, Pearson's correlation coefficient=-0.8943; $p<0.05$; Figure 5A), whereas variation in enFeLV-*env* did not (day 7, Pearson's correlation coefficient=-0.1071, $p=0.8397$; Figure 5B). Correlation was not detected between enFeLV-LTR or *env* copy number with regard to FeLV proviral integration in PBMCs (Data not shown).

Naturally infected animals

A variety of lymphoid tissues were available from FeLV-infected domestic cats; however, only a subset of tissues was available from FeLV-infected panthers (Table 2). Both tissue proviral load and tissue antigen load failed the Kolmogorov-Smirnov test for log-normality. FeLV infection measured by proviral was greater in domestic cat compared to panther by a median difference of 1.066 by Mann-Whitney test of log-transformed copy numbers per cell ($U=122$, $p=0.0001$; Figure 6A). Despite lowered proviral load in Florida panther tissues, antigen production in tissues was trending toward greater production (median value 3.354 vs. 2.872) of FeLV capsid antigen p27, although this was not statistically significant by Mann-Whitney test ($U=438$, $p=0.1495$; Figure 6B).

DISCUSSION

FeLV shows a greater capacity to infect puma fibroblasts compared to domestic cat fibroblasts. FeLV has been documented to naturally infect free-ranging pumas (Brown et al., 2008; Chiu et al., 2019). Others

have speculated about the potential for enhanced virulence and adaptation of retroviruses, including FeLV, following introduction into novel hosts (Lee et al., 2017; Meli et al., 2009). Experimental infections *in vitro* were conducted to establish differences in FeLV infection between puma and domestic cat cells in the absence of immunological and physiological parameters encountered during natural infections. In fibroblast cultures, FeLV infection resulted in greater infection levels in puma cells than in domestic cat cells (Figure 2A). Additionally, enhanced viral replication occurred in naturally infected pumas as evidenced by FeLV antigen load (Figure 2B). Therefore, at a cellular level, puma cells appear to be a more than competent host for FeLV infection and replication.

enFeLV-LTR copy number is associated with resistance against FeLV infection and replication. Endogenous viruses are a sizable component of an animal's genome (Consortium, 2001) and in the majority of cases, solo LTRs vastly outnumber full pseudogenomes (Boeke and Stoye, 1997). In our limited sampling of domestic cat fibroblast enFeLV components, solo LTRs range from 32-74 copies per cell, while env ranges from 9-13 copies per cell (Figure 4). Previously reported measures of full length enFeLV range from 6-26 copies per cell (Chiu et al., 2018). LTR copy number variation, but not full enFeLV genomes, were associated with FeLV replication; enFeLV-env showed no correlation to either FeLV infection or replication (Figure 5B). In contrast, puma fibroblasts that lack enFeLV elements supported viral infection, and replication was greater than in domestic cat cells (Figure 2B).

Intrinsic cellular factors may modulate FeLV infection. Gammaretroviruses are believed to require the dissolution of the nucleus during mitosis in order to integrate into cells (Yamashita and Emerman, 2006). As such, rapidly dividing cells may be more susceptible to FeLV infection and replication. We measured cell count as a proxy for of cell cellular division and percent cell mortality as a measure of viability, and did not detect differences between puma and domestic cat cells (Figure 3). Puma cells had qualitatively smaller fibroblasts when compared to domestic cat fibroblasts (data not shown). Basic morphological differences between species appeared to be the main factor driving this difference as all cells achieved 100% confluency, with limited mortality. FeLV is not known to cause CPE in cell culture, and did not have any impact on cell mortality (Figure 3 C,D). The CrFK control cell line

displayed greater cell count at confluency and higher cell mortality (Figure 3). Immortalized cell lines have accumulated multiple changes that fundamentally alter their morphology and physiologic behaviors, including decreases in contact inhibition (Herbert et al., 1999). Experimental infections of CrFK were more consistent than individual infections of puma and domestic cat fibroblasts, which is characteristic of wild type derived primary tissue cultures. Taken together, we do not believe that differences in cellular replication contribute greatly to the differences between puma and domestic cat fibroblast infections.

Though FeLV is believed to be predominately lymphotrophic, domestic cat PBMCs do not support robust FeLV infection compared to fibroblast infections. Infection, measured by proviral load, was much lower in PBMC compared to domestic cat fibroblasts (Figure 2C). With regard to viral replication, PBMC infections only appeared positive at low level in two of six infections (Figure 2D). The PBMCs' reduced ability to become infected predicated the reduced viral replication. As such, any association that we attributed to enFeLV-LTR in fibroblast infections appears to be absent at a low level of infection.

FeLV *in vitro* infection kinetics are not entirely replicated in natural infections. Across all lymphoid tissues, naturally infected pumas with FeLV had lower proviral load than domestic cats, with the exception of two bone marrow samples, that achieved $2x10^7$ proviruses per $1x10^6$ cells (Figure 6A). Greater FeLV proviral loads hints at greater viral replication; however, the naturally infected Florida panthers appeared to be capable of generating the same amount of virus despite lower levels of infection measured by reduced proviral load (Figure 6B). While enFeLV-LTR may not play a large part in restricting against FeLV infection, it is possible that it would limit viral production. Furthermore, the samples we have acquired show a trend that is close to being statistically significant. Ultimately, we would like to be able to collect more samples from individuals that had died acutely, but are limited to natural infections that are few and far between, but potentially becoming more frequent.

The state and manner in which naturally FeLV-infected animal tissues were collected may have impacted our conclusions. Field collections were performed opportunistically on Florida panthers when animals were either found deceased or hit by vehicle, often hours to days after death occurred. In contrast,

FeLV positive shelter cats were euthanized when clinical signs were impeding quality of life, and tissues were collected rapidly following death. The timeliness of collection may have augmented the quality of the sample prior to DNA extraction. FeLV transmission may also impact subsequent infection kinetics. The initial spillover events of FeLV to pumas has been associated with predation, where as FeLV is believed to be transmitted through social interactions such as grooming in domestic cats. Grooming is often a sustained event and so domestic cats may be receiving a constant dose of FeLV. Unlike domestic cats, pumas are much more solitary and interactions between pumas outside of mother-offspring groups are believed to be antagonistic. Lots of factors at play in natural infections make it difficult to conclude the biological relevance of these data.

enFeLV-LTR-mediated restriction may occur through direct and/or indirect interference mechanisms. ERV-LTRs and Env protein have the potential to interact with their exogenous viral counterparts (Chapter 1). While they are transcriptionally active, ERV LTRs do not encode for proteins, but act as regulatory regions for viral propagation. The process of recombination and transposition, particularly early on in the endogenization process, can lead to the production and accumulation of solo-LTRs scattered in a random fashion across the host genome (Vitte and Panaud, 2003). LTRs may activate anti-viral or innate immune genes in close proximity to LTR insertion sites, result in protection against exogenous retroviruses. This indirect ERV modulation has been documented in other systems such as MLV, and can be mediated through *cis*-activation (promoter) or *trans*-activation (enhancer) of host genes (Thompson et al., 2016). Alternatively, it is possible that the enFeLV genetic elements may directly interfere with exFeLV infection by encoding for small interfering RNAs that activate host DICER complexes to specifically target FeLV transcripts, not yet described. Our results are more suggestive of direct interference mechanisms due to the linearity of the FeLV restriction afforded by enFeLV-LTRs.

Outside the realm of endogenous retroviruses, changes in infection capacity and virulence has been demonstrated in other viral systems following cross species transmission (Geoghegan and Holmes, 2018). In some cases, disease spillovers into novel species of the same family can result in dead-end hosts for the virus (Infectious hematopoietic necrosis virus; (Garver et al., 2006)). In other cases, disease

spillover may result in active infections that maintain persistent transmission (Mycoplasmosis; (Kellner et al., 2018)). Further still, some cases result in adaptation of the virus in novel hosts leading to increased morbidity and mortality (HIV; (Sharp and Hahn, 2011)). General trends have yet to be defined and appear to be dependent on the given specifics of various cases, despite our best efforts to locate and predict the next epidemic (PREDICT; (Consortium, 2014)). While it appears apparent that puma cells are more susceptible to FeLV infection *in vitro,* this may be due to intrinsic difference between cells physiology and presence of intrinsic restriction factors, and may or may not be indicative of viral outcomes *in vivo*.

In this report, we present information that demonstrates that enFeLV-LTR may confer protection against exFeLV replication. The exact mechanism by which these constituents act has yet to be determined, but may manifest as direct interference through RNA silencing mechanisms, or by indirect enFeLV-LTR-mediated promotion of host anti-viral genes. FeLV provides one of the few systems in which we can directly interrogate the mechanisms that govern related exogenous-endogenous retroviral interactions in an outbred and diverse population.

FeLV-61E – enFeLV-LTR – CCR5

AGAATTCTGACCAG***AGTTCGACCTTCCGCCTCAT***TTAAACTAACCAATCCCCATGCCTCTCG***CTTC***
TGTACGCGCGCTTTCTGCTATAAAACGAGCCATCAGCCCCCAACGGGCGCGCAAGTCTTTGCTGA
GACTTGACCGCCCCGGCAGGG***GTCTTATCCTAAGTCCACCGTTTA***GCTGCCAAACAGGATATCTG
TGGTCAGCCACCCGGCCCTAAGATAGCCACCTGGCCCTAAGATGGGAATGGAAAGTACTGACTCC
ACCC***GATAGACCCTAGAGATGAGCCTAG***TCAGCCACCCATCCGGCTTCGTGGGCAACCTGCTGGT
CGTCCTCATCCTCATCAACTGCAAAAAGCTGAGGGGCATGACTG***ACGTCTACCTGCTCAACCTGG***
CCATCTCCGACCTGCTCTTCCTTTTCACCCTCCCCTTCTGGGCCCACTATGCCGCCAACGGGTGGG
TCTTCGGG***GATGTGATGTGTAAGACGGTCACC***GGAATTCAA

FeLV-61E – enFeLV-env – CCR5

AGAATTCTGACCAG***AGTTCGACCTTCCGCCTCAT***TTAAACTAACCAATCCCCATGCCTCTCG***CTTC***
TGTACGCGCGCTTTCTGCTATAAAACGAGCCATCAGCCCCCAACGGGCGCGGG**TGTGGGCCCTG**
TTACGATAAAGCTGTTCACTCCTCGACAACGGGAGCTA**GTGAAGGGGGCCGGTG**CAGCTGGTC
GTCCTCATCCTCATCAACTGCAAAAAGCTGAGGGGCATGACTG***ACGTCTACCTGCTCAACCTGG***C
CATCTCCGACCTGCTCTTCCTTTTCACCCTCCCCTTCTGGGCCCACTATGCCGCCAACGGGTGGGT
CTTCGGG***GATGTGATGTGTAAGACGGTCACC***GGAATTCAA

Figure 4.1. Oligonucleotides were synthesized for qPCR quantification. Two gBlocks were synthesized through integrated DNA technologies (IDT, Coralville, IA). Both concatenated gBlocks contained sequences for FeLV-61E (blue) and CCR5 (yellow). One contained enFeLV-LTR sequence, where the other contained enFeLV-*env* sequence. Bold sequences represent forward and reverse primer binding sites.

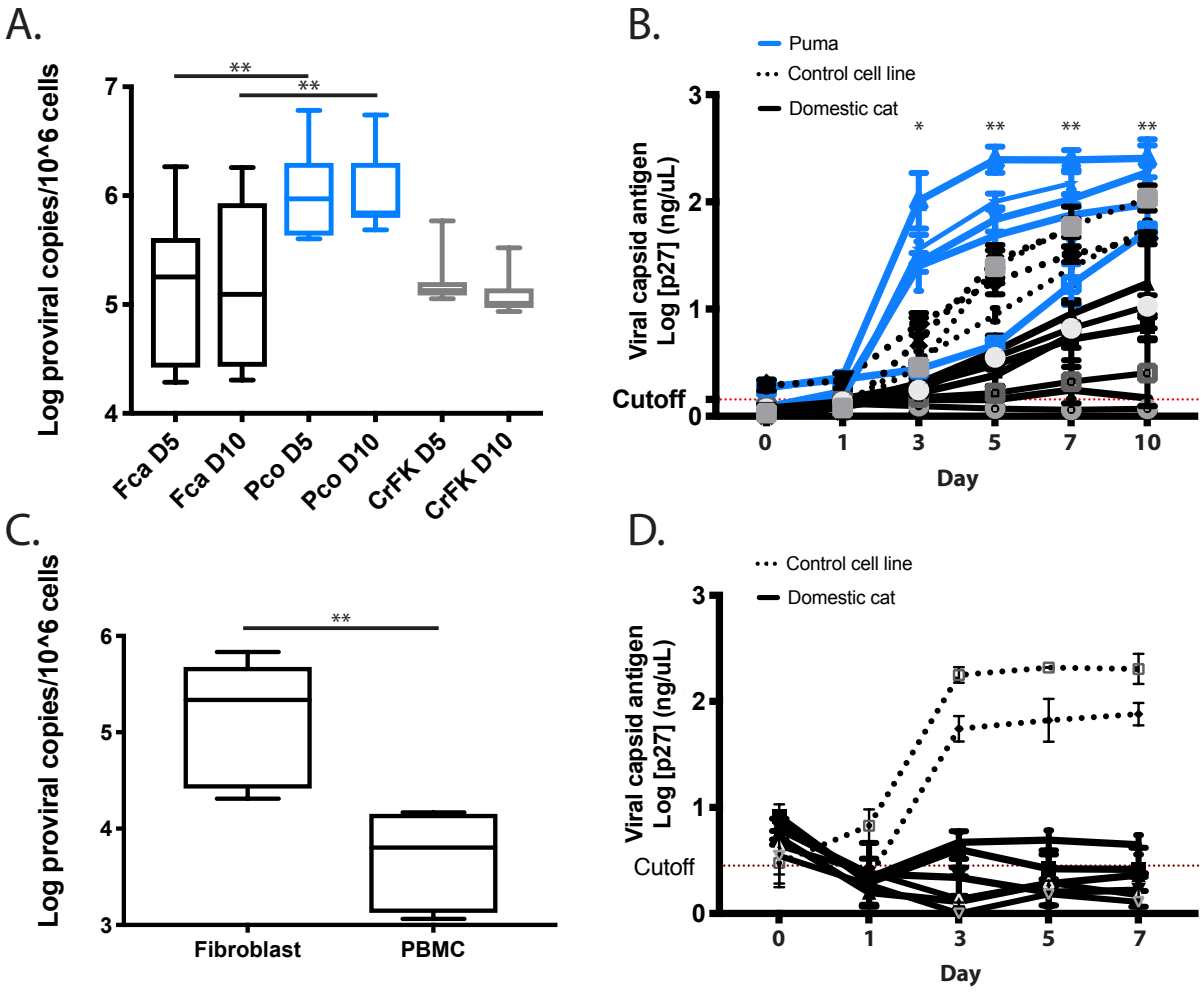

Figure 4.2: A) Puma fibroblasts have greater FeLV proviral load (copies per cell) compared to domestic cat fibroblasts. At both day 5 and day 10, puma cells (Pco) demonstrated significantly higher proviral copy numbers (Kruskal-Wallis; **=p<0.01). Variance between domestic cats (Fca) cells was greater than that of puma cells. CrFK cell infections displayed the least amount of variation. B) Puma fibroblast viral capsid antigen (p27) production is higher in than domestic cat cells. Beginning day 3, puma cells replicated more virus than domestic cat cells (repeated measure ANOVA; *=p<0.05, **=p<0.01). One puma, one domestic cat, and one control cat experiment had to be terminated at Day 7. Cutoff values were established by 3x standard error above average value for negative control wells (red line). C) Domestic cat PBMCs were more resistant to FeLV infection than fibroblasts. At day 5, median proviral load was 6,359 copies per 10^6 cells in PBMCS, compared to 119,155 copies per 10^6 cells in fibroblasts (Mann-Whitney U test; **=p<0.01). D) Domestic cat PBMCs supported low levels of virus replication. Only two PBMC cultures had transient infections that peaked above the negative cutoff value, established at 3x standard error above average value for negative control wells (red line).

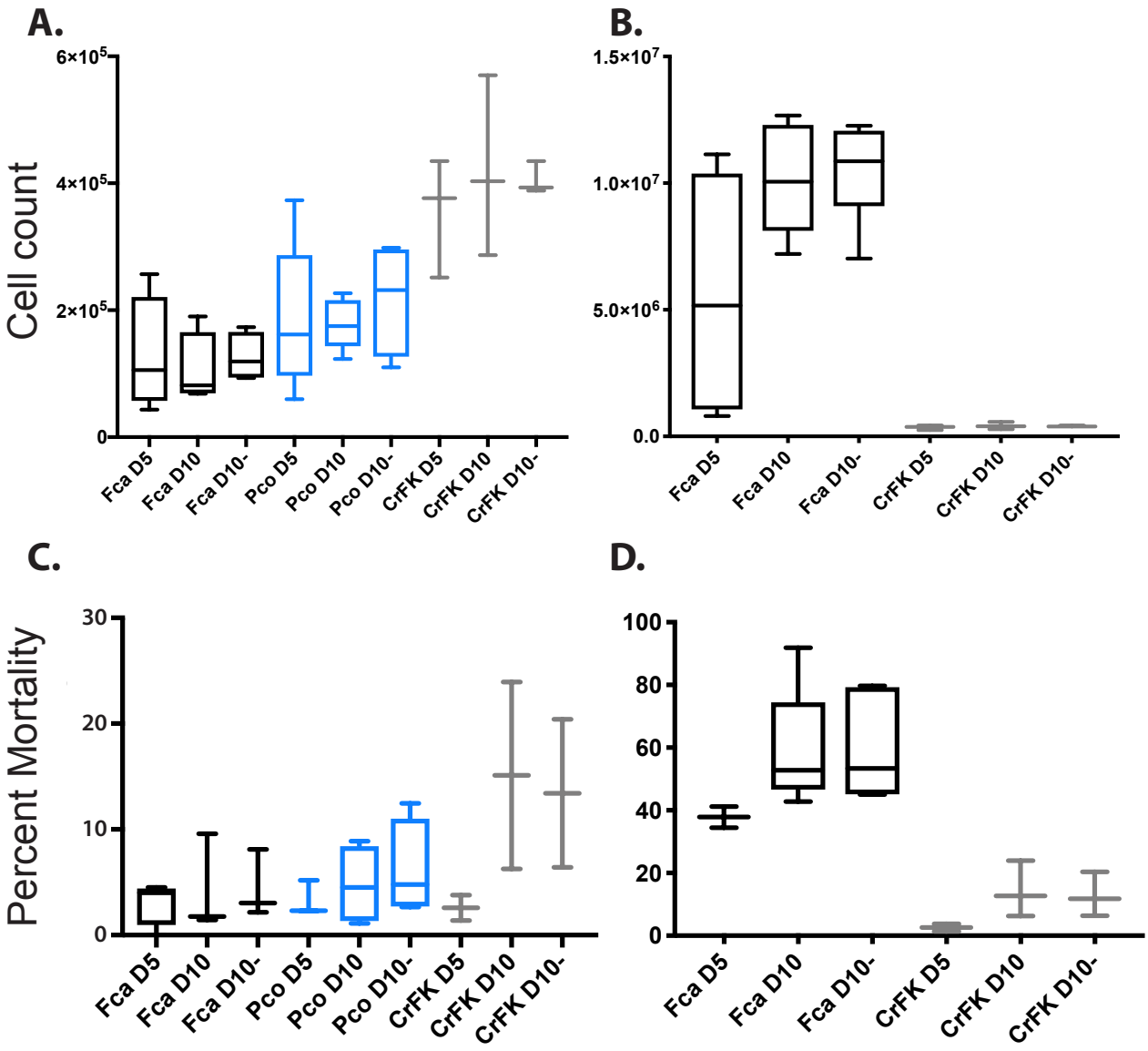

Figure 4.3: Viable cell numbers and cell mortality increase over time and are similar in puma and domestic cat cultures. A) Puma (blue) and domestic cat (black) fibroblast cell counts increased over time and reached 100% confluency by day 5. Initial seeding density was $5x10^4$ cells per $2cm^2$. Differences in domestic cat and puma fibroblast cell counts were not significant between day 5 and day 10. B) Domestic cat PBMCs continued to increase throughout the study. Initial seeding density was $1x10^6$ per mL. C) Puma (blue) and domestic cat (black) fibroblast mortality increased over time following 100% confluency at day 5. Differences in domestic cat and puma fibroblast cell counts were not significant between day 5 and day 10. D) Domestic cat PBMC mortality increased over time.

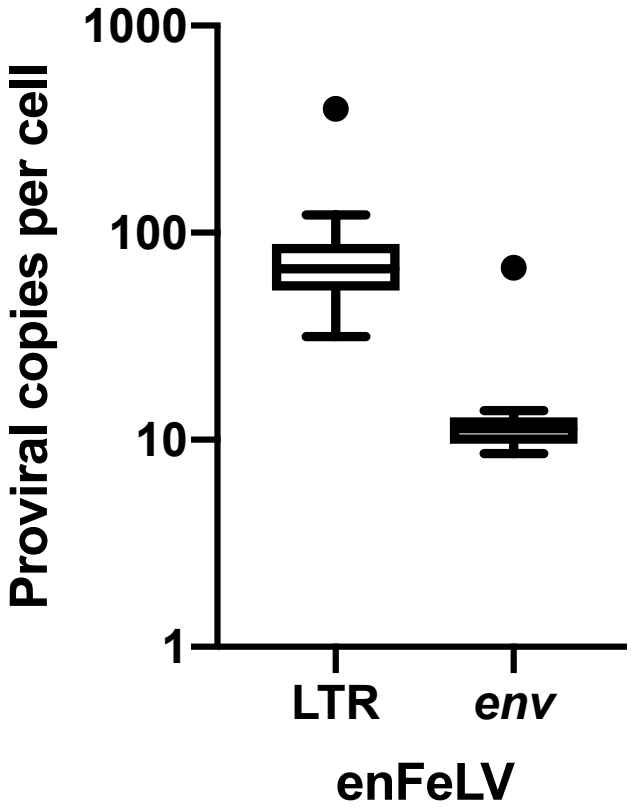

Figure 4.4: Domestic cat cells (n=13) display variation between enFeLV-LTRs and env. In individual cats, LTR copy number was greater than *env* copy number. With one exception, enFeLV-LTR ranged between 8-14 copies per cell. enFeLV-*env* was more variable ranging between 32-122 copies per cell. One domestic cat that provided PBMCs had 397 LTR and 68 *env* copies per cell.

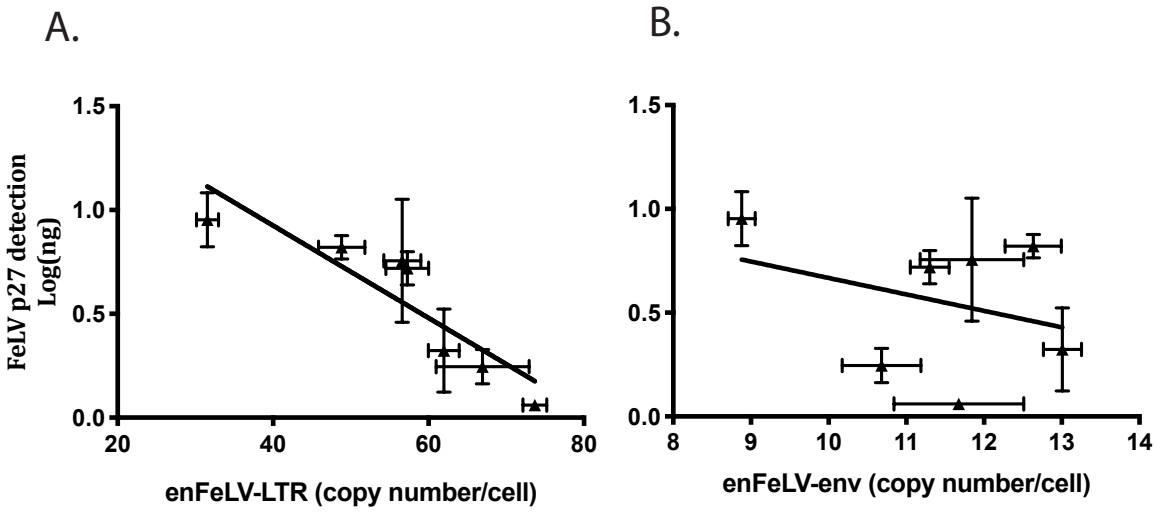

Figure 4.5: enFeLV genome elements correlate to viral replication. A) Domestic cat enFeLV-LTR in fibroblasts show a negative trend compared to FeLV antigen production (Pearson's coefficient= -0.8943; p= 0.0066). B) Domestic cat enFeLV-*env* loses negative trend between *env* copy number and FeLV antigen production (Pearson's coefficient= -0.1071; p=0.8397).

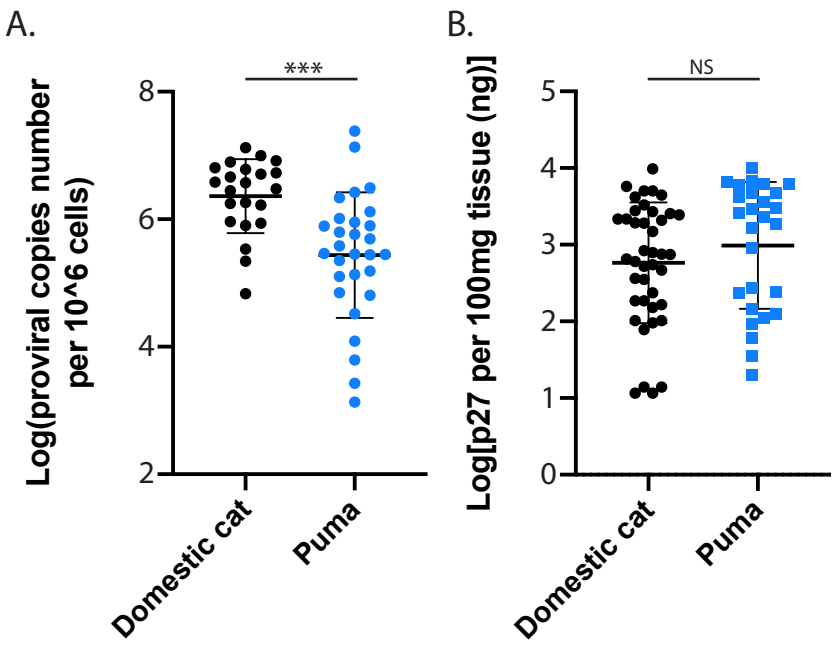

Figure 4.6: Lymphoid tissues from naturally infected FeLV-positive domestic cats and pumas have varying viral loads at end stage disease. A) Domestic cat lymphoid tissues displayed greater FeLV proviral load compared to puma tissues (Mann-Whitney test; ***$p<0.0001$). B) There is no statistical difference in antigen load between domestic cat lymphoid tissues and puma tissues; however puma tissues trended to greater amounts of viral antigen (Mann-Whitney test; $p=0.1495$).

Table 1. Primer sequences and cycling conditions used for qPCRs.

Region	Sequence	Cycling conditions	Source
FeLV-61E			[Tandon et al., 2008]
Forward	*5'-AGTTCGACCTTCCGCCTCAT*-3'		
Probe	*5'-TAAACTAACCAATCCCCATGCCTC*-3'		
Reverse	*5'-AGAAAGCGCGCGTACAGAAG*-3'		
enFeLV-LTR			[Powers et al., 2018]
Forward	*5'-GTCTTATCCTAAGTCCACCGTTTA*-3'		
Probe	*5'-CCTGGCCCTAAGATGGGAATGGAAA*-3'		
Reverse	*5'-CTAGGCTCATCTCTAGGGTCTATC*-3'	98˚C : 3 min;	
enFeLV-*env*		40 cycles of	
Forward	5'-*TGTGGGCCCTGTTACGATAAA*-3'	98˚C: 5 sec;	
Probe	5'-*CTGTTCACTCCTCGACAACGGGAGCT*-3'	60˚C: 15 sec;	
Reverse	5'-*CACCGGCCCCCTTCAC*-3'		
Forward	5'-*ACGTCTACCTGCTCAACCTGG*-3'		[Howard et al., 2010]
Probe	5'-*TCCGACCTGCTCTTCCTCTTCACCCTCC*-3'		
Reverse	5'-*ACCGTCTTACACATCCCATCCC*-3'		
CCR5 (puma)			Modified from [Howard et al., 2010]
Forward	5'-*ACGTCTACCTGCTCAACCTGG*-3'		
Probe	5'-*TCCGACCTGCTCTTCCTTTTCACCCTCC*-3'		
Reverse	5'-*GGTGACCGTCTTACACATCACATC*-3'		

Table 2. Tissues from FeLV-positive domestic cat and pumas available for qPCR and ELISA testing. Not all tissues were available from all individuals

Tissue	Domestic cat (n=6)	Puma (n=11)
Bone Marrow	6	6
Lymph Node	5	9
Spleen	6	10
Thymus	6	4

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CHAPTER FIVE

Endogenous Feline Leukemia Virus (enFeLV) siRNA May Drive Direct Interference with FeLV Infection

INTRODUCTION

Endogenous retroviruses (ERV) are scattered throughout the genome and impact normal biologic processes (Consortium, 2001; Griffiths, 2001). The degree to which ERVs impact an animal's biology is dependent on whether the ERV maintains the ability to produce proteins, or to enhance/promote transcription of other host genes. ERVs in their early stages of endogenization undergo changes by accumulating mutations that often render the newly endogenized virus defunct as host cells deal with foreign, potentially deleterious genetic material (Lober et al., 2018). In addition to accumulating mutations, ERVs can act as retro-transposable elements capable of inserting into novel locations in the genome and becoming removed from the genome via homologous recombination and other mechanisms that are not entirely understood, often times leaving small segments of their genome behind in the form of solo long terminal repeats (LTR) (Boeke and Stoye, 1997; Lober et al., 2018). While typically unable to produce infectious virions, many ERVs are still capable of undergoing transcription and may produce functional viral proteins (Knerr et al., 2004; Li and Karlsson, 2016). ERVs have been implicated in other biological processes such as placentation, oncogenesis, immune modulation, and infectious disease progression (Crittenden et al., 1987; Knerr et al., 2004; Umemura et al., 2000; Zeng et al., 2014).

ERVs have also been exapted to participate in anti-viral activities against exogenous homologues (Chapter 1). For example, endogenous mouse mammary tumor virus (MMTV)-encoded superantigen negatively selects against self-reacting T-cells, limiting the ability for certain exogenous MMTV strains to infect those T-cells (Holt et al., 2013). Endogenous jaagsiekte sheep retrovirus (JSRV) produces proteins that interfere with the regular trafficking mechanisms of exogenous JSRV, thereby reducing viral budding and maturation (Malfavon-Borja et al., 2015). A specific locus of endogenous murine leukemia virus (*Mtv*) LTR is situated 5 nt from exon2 of mouse APOBEC3, resulting in increased expression of the

potent antiretroviral host protein (Sanville et al., 2010). Here we report details about endogenous feline leukemia virus (FeLV) transcription in domestic cat cells as a model system to study other mechanisms by which ERVs and XRVs may interact in an outbred host, the domestic cat (Willett and Hosie, 2013).

In a study of a natural FeLV epizootic in a 65 hybrid domestic cat breeding colony, we showed a correlation between higher enFeLV-LTR copy number and cats that were able to contain exogenous FeLV infection (regressive/abortive infection), whereas cats with lower LTR copy number tended to develop progressive infection and accumulate virulent enFeLV-exogenous FeLV recombinants (Powers et al., 2018), We experimentally infected domestic cat fibroblasts with FeLV and likewise demonstrated that primary cells from cats with greater enFeLV-LTR copy number were more resistant to FeLV infection and viral replication (Chapter 4). This relationship was not observed when we examined FeLV infection and replication related to the enFeLV-*env* gene, which are found at considerably lower copy number than enFeLV-LTR (mean of 11 *env* copies/cell versus 67 LTR copies/cell; Chapter 4).

enFeLV-mediated restriction in our cell culture experiments appeared dose-dependent, leading to the hypothesis that enFeLV may directly interfere with FeLV XRV. This is in contrast to indirect interference mechanisms where enhancer and promoter functions of the LTR drive *cis*- or *trans*-activation of host transcription machinery to encode for anti-viral proteins, as is seen in MuLV (Sanville et al., 2010). Direct interference may involve transcription and production of small non-coding RNAs that regulate gene expression and viral reproduction by degrading target RNA. These include silencing RNA (siRNA, specific for one target) or microRNA (miRNA, less specific for many targets). Both siRNA and miRNAs result in a process known as RNA interference (RNAi), which leads to lead to activation of DICER, a ribonuclease that processes siRNA and miRNA and complexes them onto RNA-induced silencing complex (RISC) (Pratt and MacRae, 2009). Once incorporated into a RISC complex, ssRNA can find its full (siRNA) or partial (miRNA) complementary mRNA strand and signal it for translational repression, mRNA degradation, or mRNA cleavage. A comprehensive review of siRNA and miRNA can be found in (Lam et al., 2015).

Besides a copy-number and potentially dose-dependent resistance mechanism mediated by enFeLV-LTR, we have documented an inability of FeLV to productively infect primary peripheral blood mononuclear cells (PBMCs) in contrast to permissive infection of primary fibroblasts. This is despite the fact that FeLV is believed to be lymphotrophic in nature and primarily results in immunodepleting diseases (Khan et al., 1993; Shojima et al., 2006). To address these observations and to determine mechanisms underlying ERV-XRV interactions in the FeLV system, we approach the following questions: 1) Are enFeLV transcripts tissue dependent, and thus potentially directing FeLV infection tropism? and 2) Do enFeLV encode for miRNA which may suppress exogenous FeLV replication? We hypothesize that enFeLV transcription may be variable between tissues given the relative difficulty it is to infect and replicate FeLV in PBMCs. Furthermore, we believe that enFeLV may produce short non-coding RNAs that can participate in RNAi against FeLV. We approached this question by evaluating publicly available data from the NCBI Sequence Read Archive (SRA; www.ncbi.nlm.nih.gov/sra).

METHODS

Endogenous FeLV transcriptomic analysis

Domestic cat transcriptome and miRNAome data sets were acquired through the search function in the NCBI Sequence Reads Archive (SRA) using the search key words: “felis” and “rna-seq.” Datasets were included in the study if they were derived from healthy cats, identified the tissue of origin, and represented transcriptome (excluding miRNAome) datasets (Table 1). Two additional non-*Felis spp* felid transcriptome datasets (Accession numbers: SRX317246 and SRX3478130) were included as negative controls that would not be expected to harbor enFeLV transcripts (Table 1). Tissues analyzed included embryonic (fetus, embryo body, embryo head), neural (cerebellum, parietal lobe, occipital lobe, temporal lobe, hippocampus, spinal cord, retina), skin (skin, ear tip, ear cartilage), lymphoid (spleen, lymph node, bone marrow, thymus), and various organ (muscle, liver, uterus, kidney, testes, pancreas, heart, salivary gland) tissues. All data processing and analysis was completed using the Colorado State University College of Veterinary Medicine and Biomedical Science server (CVMRIT02). Transcriptome datasets were analyzed using a custom bioinformatics pipeline (Figure 1). Reads were trimmed for appropriate

adapters and by quality (q=20) using cutadapt (version 1.18). The first 600 bp and last 600 bp of the full-length enFeLV were discarded prior to creating the index due to the potential for transcripts to map to other host genomic elements that surrounded the enFeLV integration site. Indices were first generated for full-length domestic cat enFeLV (GenBank Accession Number: AY364319) and individual enFeLV gene regions, including LTR, *gag*, *pol*, and *env* separately using Bowtie2-build function (version 2.3.4.1). Transcriptome sequences were mapped to indices using "--sensitive" settings in local mode in Bowtie2 to allow for heterogeneity among different enFeLV genotypes. Alignments were visually inspected by importing mapped *.sam* files into Geneious 11.1.2. Exogenous FeLV was ruled out as the source of mapped reads by looking for exogenous FeLV-specific DNA segments. Transcriptome reads mapping to full-length FeLV were reported as reads per million reads (RPM). While the reads were mapped as paired-end reads, reported RPM was calculated as unpaired reads. Individual genome elements were reported as fragments per kilobase million (FPKM) to normalize for size of the respective gene region (full-length enFeLV- 8,448bp; LTR – 592bp; *gag* – 1,512bp; *pol* – 3,630bp; *env* – 2002 bp). Percent transcription for full-length FeLV was analyzed by ANOVA in Prism (version 7.0).

Feline miRNAome analysis

miRNAome datasets were accessed using the same criteria as the transcriptome datasets retrieved by querying "felis" and "rna-seq" that also identified miRNA-Seq in the library preparation strategy. Tissues analyzed included neural (cerebellum, cerebral cortex, brain stem), skin (skin, lip, tongue), lymphoid (spleen, lymph node), and various organ (pancreas, kidney, liver, lung, testis, ovary) tissues. Using the same full-length enFeLV index built using Bowtie2-build, miRNAome reads were mapped on local mode with a minimum threshold score set at 20 in Bowtie2 to account for miRNA's intrinsically short length. miRNA reads mapping to full-length enFeLV were reported as percent reads mapped to each genome element compared to total mapped reads to the full-length FeLV genome.

RESULTS

SRA accessed transcriptome data suggests tissue-specific enFeLV transcription

A total of 207 individual animal transcriptomic RNA-Seq datasets were retrieved from the SRA dataset inquiry. Fifty-six of these datasets were from healthy domestic cat tissues of various origins (e.g., embryonic, lymphoid, neural, etc.). Forty-two datasets were included following quality control analysis, representing two studies (99 Lives Cat Genome Sequencing Initiative, *unpublished*; (Fushan et al., 2015)). An RNA-seq datasets originating from a jaguar (*Panthera onca*) and one from a bobcat (*Lynx rufus*) were used as negative controls (Table 1).

enFeLV transcript levels were approximately 100 reads normalized per million reads (RPM) for most tissues. Outliers included: (1) embryonic tissues (*i.e.* head, body, whole), (2) lymphoid tissues, and (3) a single salivary gland sample. All of these tissues had consistently greater enFeLV transcription levels that were higher than neural, skin, reproductive, urinary, lung, digestive, circulatory, and liver tissues (Figure 2A). Strikingly, lymphoid (n=4) and salivary gland (n=1) tissues appeared to have the greatest enFeLV transcription, averaging approximately 10 fold greater transcription than other tissues.

Following normalization against the size of the gene, genetic segments were found to have differential expression profiles (Figure 2B). LTR, *gag*, *pol*, and *env* transcripts represented 0.4387, 0.0302, 0.0265, and 0.0453 FPKM of total transcripts, respectively. Relative expression of size-normalized gene segments as FPKM by tissue generally supports trends identified for full enFeLV; in that lymphoid tissues and the salivary gland account for the greatest level of transcription (Figure 3). Additionally, LTR transcription is approximately 10-fold greater than other enFeLV genes (Figure 3A).

Negative control datasets from bobcat and Siberian tiger also displayed hits to enFeLV in two focal regions of the enFeLV genome (Figure 4). One of these regions is a 29-bp poly-adenine stretch in the LTR. The other region is a 187-bp sequence with only 87-bp driving the alignment to enFeLV with 43 SNPs in the Siberian tiger dataset and a 179-bp sequence with 39 SNPs in the bobcat dataset. Both regions overlap with each other and the most conserved 87-bp region shares 100% identity to uncharacterized *Panthera pardus* (Leopard) LOC10927796 mRNA (Accession number XM_019467717.1). Further characterization of this mRNA using NCBI's BLAST tblastn function reveals a shared polymerase gene identity to feline endogenous retrovirus gamma4-A1 (Accession number:

LC176795). Nucleotide similarity between LC176795 and enFeLV is 60% over the region with 36 SNPs in 90 nucleotides. Pairwise identity between full length enFeLV (AY364319) and feline endogenous retrovirus LC176795 shares only 49% nucleotide identity.

SRA accessed miRNAome data suggests enFeLV-derived siRNA

From the 207 individual animal transcriptomic RNA-Seq datasets retrieved from the SRA, 27 datasets were prepared to characterize the feline miRNAome (Table 2). These consisted of small <30bp RNA fragments responsible for RNA silencing (Lagana et al., 2017). Mapped miRNA sequences accounted for 0.0163% of miRNA and sequences were concentrated primarily in approximately 75% of the sequences originated in the LTR (75.1% ± 21.0%) (Figure 5). miRNA that mapped to *gag*, *pol*, and *env* represented more than 10% of the mapped reads in 3-6 of the datasets. LTR miRNA was mapped to one region at nucleotide 557, 74 nucleotides downstream of the transcription start site (Figure 6). Sequence for LTR miRNA was (5'-TGGGGGCTCGTCCGGGAT-3'). miRNA alignments in the *env* were located focally at nucleotide 7,045 and is a 12-bp segment.

DISCUSSION

Feline miRNAome analysis suggests a possible direct inhibitory mechanism of exFeLV restriction, namely the production of siRNA, with very high coverage to the LTR region (Figure 5). The strongest signal of miRNA transcription mapped to the 3' end of the FeLV LTR, 3 nucleotides downstream from the 5'-LTR U3 region. All 27 miRNAome datasets contained miRNA transcripts that we believe would restrict exogenous FeLV replication given that endogenous and exogenous share approximately 86% nucleotide similarity (Chiu et al., 2018). This is in contrast to low number of alignments among other mapped miRNA sites in other genes (i.e., *gag*, *pol*, and *env*). In a limited number of tissue datasets, only a single peak was identified where miRNA mapped to *gag* and *env* genes (Figure 6). Only three tissue datasets showed miRNA reads that would map to the *pol* gene. This could be explained by the fact that the *pol* gene is generally conserved among related retroviruses, so we may be examining miRNA produced against many related virus *pol* genes. Furthermore, only 3 datasets contained more than 10% of total miRNA reads mapping to *pol*. The presence of miRNA indicates a

possible inhibitory sequence that would directly restrict against infection or replication through RNA degradation mechanisms. The specific mechanism is dependent on the percent identity the miRNA shares with the target sequence. The enFeLV-LTR miRNA identified through analysis of the miRNAome harbored one SNP between our reference enFeLV genome, which may signal a difference in genotype, rather than an incomplete match to enFeLV. Mapping the 20bp sequence to exogenous FeLVs demonstrates a complete match in some strains (61E; Accession number M18427), but not others (Rickard; Accession number AF052723). This would in theory, classify this non-coding RNA as a siRNA over miRNA. miRNAs are a broad-acting group of RNAs that do not specifically target sequences, but rather may be produced to interact with mRNA in a non-specific manner. siRNAs on the other hand are complementary matches to the mRNA they are destined to degrade by signaling the RISC complex to induce enzymatic digestion of RNA. Unlike enFeLV transcription, enFeLV miRNA was present only focal areas (Figure 6). This indicates that production of these miRNAs are not random and may have been selected for under evolutionary forces, particularly in the presence of a virulent virus such as FeLV. Hence, having greater loads of enFeLV-LTR may restrict against FeLV replication in a dose-dependent manner. Further studies may expand upon this by synthetically producing siRNA and measuring the direct impact of the 20bp siRNA on viral replication.

Analyzing the feline transcriptome, we show here enFeLV is transcribed in a tissue-specific manner and transcript level varies by gene segment (Figures 2B and 3). Transcription rates vary by cell type; in particular, lymphoid tissues and salivary gland have 10-fold greater transcription than other tissues (Figure 3). This is noteworthy as FeLV is presumed to be predominantly lymphotropic. The increased basal expression of enFeLV in lymphoid tissues may produce the conditions for a greater propensity for recombination to occur in these cells during FeLV infection as recombination is predicated by the co-packaging on endogenous and exogenous retrovirus transcripts (Stuhlmann and Berg, 1992). On the other hand, expression of particular ERV-derived proteins, namely Env, has been implicated in protection against exogenous retrovirus infections. With respect to FeLV, enFeLV Env can block FeLV-B infection through receptor interference (McDougall et al., 1994). Increased FeLV-*env* transcription is

suggestive of greater Env expression, which would provide protection for lymphocytes against a FeLV subgroup that has a change of tissue tropism towards lymphocytes following recombination. This has been documented before as increased ERV transcription in developing embryos has been attributed to having a protective role for resisting *in utero* infections (Crittenden and Fadly, 1985). Transcription of enFeLV may also be protective and could be increased in cells likely to be infected (i.e. lymphoid cells) or in cells responsible for replication and transmission of the virus.

LTRs from enFeLV are transcribed at much greater levels than *gag*, *pol*, and *env* (Figure 2B). The increased amount LTR transcription can likely be attributed to host genomes containing more copies of the LTR region than the viral coding genes (see Chapter 2). Curiously, not all of the reads that mapped to enFeLV may be enFeLV in origin, but instead may originate for another endogenous retrovirus. Alignment of non-*Felis* spp transcripts to enFeLV showed two sites where mRNA produced aligned to the enFeLV genome. One location in the LTR mapped to a 30bp poly-A stretch, where the remainder of the transcript did not match (Figure 4). *Felis catus* samples did not show transcripts mapping to this region. A second 90 bp region in the *pol* gene shares identity to both enFeLV and another endogenous gammaretroviral element in domestic cats, previously described in domestic cats as feline endogenous retrovirus gamma4-A1. The polymerase gene is necessary for all retroviruses and as such, it is considered one of the most conserved regions of retroviruses (Kamer and Argos, 1984). It is very likely we are seeing this 90bp *pol* transcript that maps to enFeLV due solely to the conservation that exists among different retroviral *pol* genes. Infection of this ERV may have pre-dated the divergence between the large and small felids. The remainder of the non-*Felis* spp. transcriptomes that we included in this study failed to map to enFeLV (Figure 4).

One limitation of this study is that the level of curation of submitted SRA datasets is variable. While many datasets are clearly labeled, lack of careful description severely limits the use of other datasets. While the SRA is rife with data that has yet to be explored and provides ample opportunities for interesting research questions, there are certainly fields of inquiry to a greater degree. For example, a simple inquiry of "HIV RNA-seq" yields 1,491 results as of the writing of this report, where as "FIV

RNA-seq" yields 8. As a result, we were limited in the number of available data sets for various tissues. Additional RNA-sequencing targeting enFeLV transcription in specific tissues may bolster our analyses, particularly with respect to the biological relevance of these findings we report.

Only, enFeLV Env has been hypothesized to play a protective role against infection with particular subgroups of FeLV, a mechanism that has been documented in other retroviral systems (McDougall et al., 1994; Robinson et al., 1981). enFeLV Gag and Pol proteins have yet to be examined with regard to interference with exFeLV infection. One rebuttal to the protective role of ERVs against a natural infection of a corresponding exogenous retrovirus is that Env, Gag, and Pol protein production may have consequences during immune system development. During embryonic development of the thymus, host genes (and by consequence, ERVs) are expressed and recognized by the autoimmune regulator (AIRE) so as not to elicit an autoimmune response against 'self' proteins (Crittenden et al., 1987). This may allow XRV to evade the adaptive immune response.

This analysis suggests evidence to support the hypotheses regarding the direct restriction enFeLV may confer against exogenous FeLV infection and replication. enFeLV is transcribed at much higher rates in tissues that are relevant in FeLV infection. Furthermore, the feline miRNAome contains siRNA that may elicit FeLV degradation via the RISC degradation pathway. While we believe that these mechanisms support the dose dependent effect of enFeLV-mediated protection against FeLV we have previously described (Chapter 4), this study only provides circumstantial evidence for these interactions. We intend to follow up and test siRNA-mediated restriction against exogenous FeLV replication mechanistically through siRNA knockdown experiments using siRNA identified through the miRNAome analysis. The findings of this study do not preclude additional indirect impacts enFeLV-LTRs may have on FeLV infection. The LTR is not a protein-encoding region, rather the LTR harbors both promoter and enhancer regions that acts by inducing transcription and read-through fusion transcripts that may be processed into functional proteins or other units (Berry et al., 1988). This phenomenon has been documented for specific host genes that propagate and inhibit disease processes depending on integration site, including anti-viral proteins such as APOBEC3C (Löwer, 1999; Sanville et al., 2010). If enFeLV LTRs integrate near anti-

viral restriction factors, it could ostensibly prime cells to be more resistant to viral infection. Increased numbers of solo-LTRs, a result of retro-transposition of ERVs, could lead to more random integrations with fixation occurring in advantageous sites through positive selective forces. Examination of LTR integration sites may therefore prove useful in determining what host genes may be impacted by increased transcription or expression.

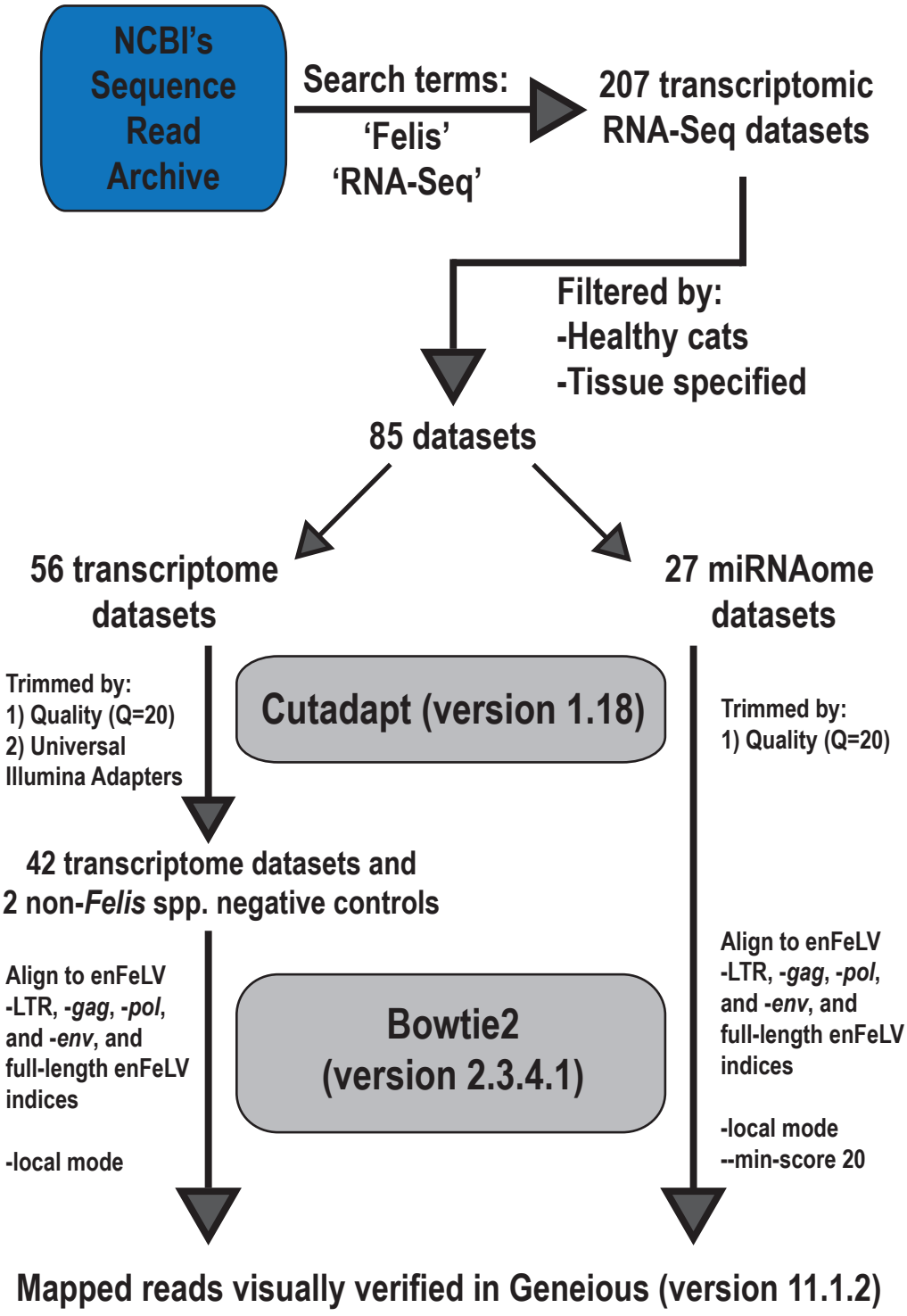


Figure 5.1: Bioinformatics pipeline. We originally identified 207 RNA-seq datasets, but after filtering for healthy cats with tissue of origin defined, we ended with 56 datasets for our transcriptome analysis and 27 datasets for our miRNAome analysis. Out of our 56 transcriptome datasets, only 33 satisfied quality controls in our bioinformatics pipeline. Two non-*Felis* spp. transcriptome datasets were included as negative controls.

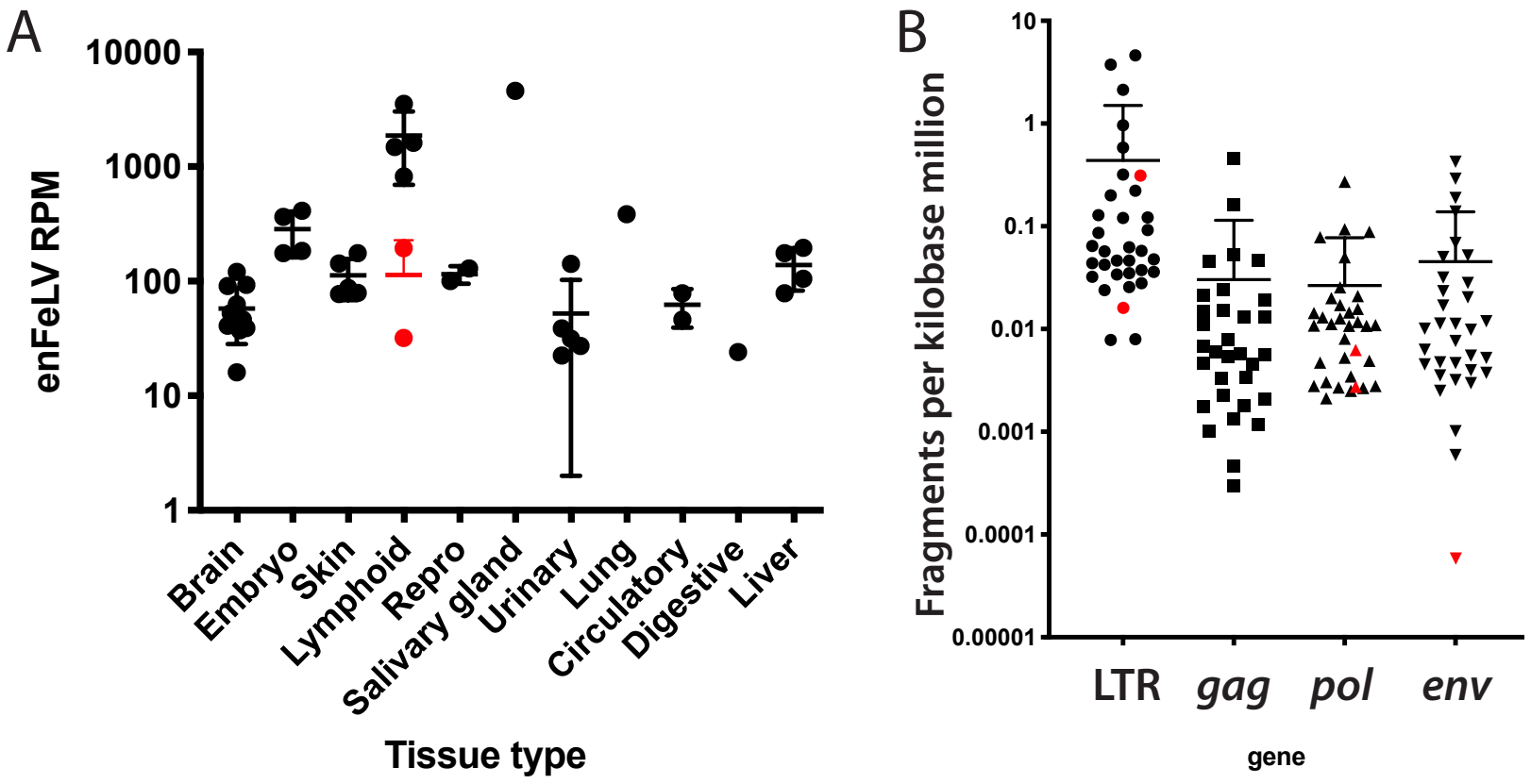


Figure 5.2: enFeLV transcription is tissues and gene specific. A) enFeLV reads appear to be transcribed at greatest levels in lymphoid and salivary gland tissues. Statistics could not be performed due to the limited sample sizes available in the SRA. RPM equals 'reads per million' and is a measure of comparison to all other available transcripts in the transcriptome dataset. Data shown here represent accession numbers SRX211594-211596; SRX211644-211646; SRX211688-211690; SRX1610301-1610326; SRX1625943-1625949. Negative control datasets (SRX317246 and SRX3478130) are shown in red. B) enFeLV-LTR is transcribed at greater levels than other enFeLV genes. Multiple comparisons following ANOVA demonstrated an average 10-fold increase in enFeLV-LTR compared to *gag* (p=0.0079), *pol* (p=0.0073), and *env* (p=0.0111). FPKM = fragments per kilobase million. Data shown here represent accession numbers SRX211594-211596; SRX211644-211646; SRX211688-211690; SRX1610301-1610326; SRX1625943-1625949. Negative control datasets (SRX317246 and SRX3478130) are shown in red and did not always have reads map to the enFeLV reference genome in all genes.

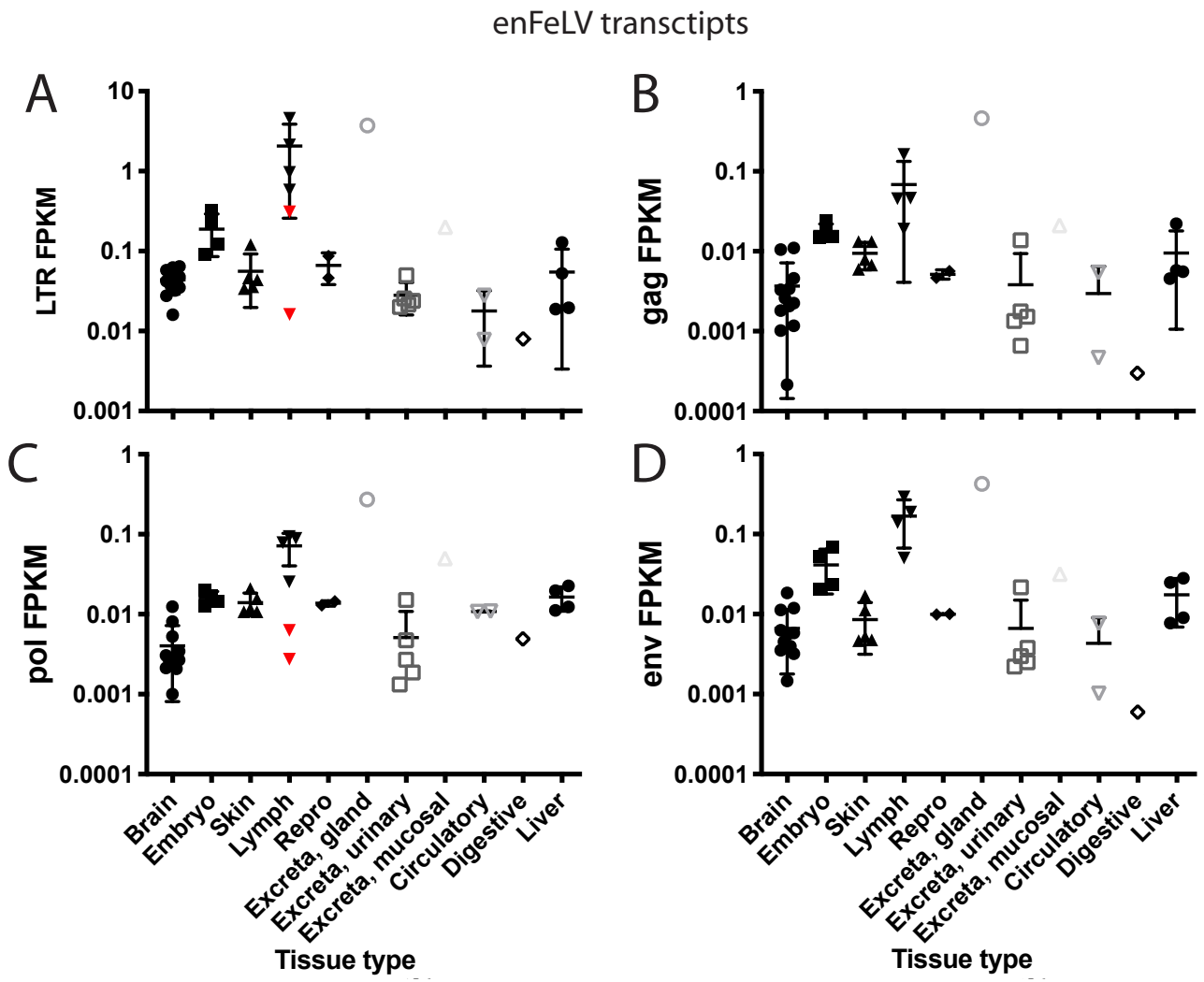
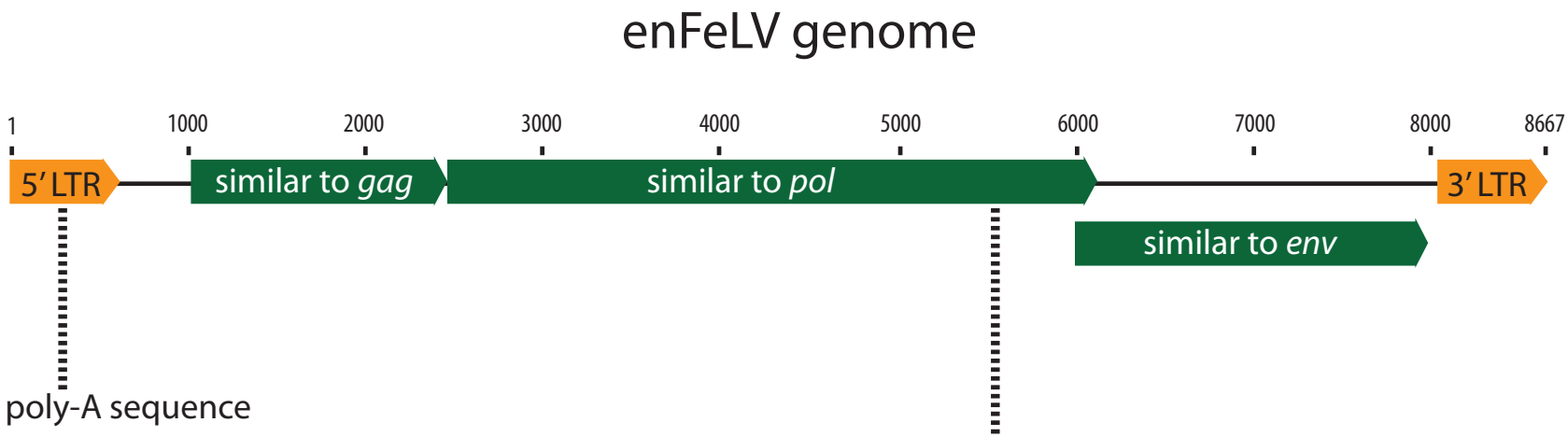


Figure 5.3: enFeLV genome elements are transcribed variably between all tissue types. A) enFeLV-LTR is transcribed 10 times greater than *gag* (B), *pol* (C), and *env* (D). Lymphoid tissues and the salivary gland experience the greatest amount of transcription across all genome elements. Negative control datasets (SRX317246 and SRX3478130) are shown in red and did not always have reads map to the enFeLV reference genome in all genes.



Accession number	Species	Genome position	Sequence
SRX317246	*Panthera tigris altaica*	5421-5608	ATGATAGGGTCAGACAATGGACCTGCATTCGTCTCTAAGGTAAGTCAGGAACTGGCTTCCATAC TTGGGGCTGATTGGAAATTACATTGTGCATACCGACCCCAAAGCTCAGGACAGGTAGAGAGA ATGAACAGAACATTAAAGGAGACCCTAACCAAATTGACCATGGAGACTGGCGTTAATTGG
SRR6384483	*Lynx rufus*	5427-5608	GGGTCAGACAATGGACCTGCATTCGTCTCTAAGGTAAGTCAGGAACTGGCTGCCATACTTGGG GCTGATTGGAAATTACATTGTGCATACCGACCCAAAGCTCAGGACAGGCAGAGAGAATGAACA GAGCATTAAAGGAGACCCTAACCAAATTGACCATGGAGACTGGCGCTAATTG

Figure 5.4: enFeLV genome elements are found in bobcat and Siberian tiger transcriptomes. RNA mapped to a poly-A region of the LTR (nt 275-304) and a variable region in *pol* (nt 5,421-5,608). The poly-A region only skewed negative control datasets and did not have an impact on *Felis catus* transcriptome analysis following visual verification. The *pol* mapped reads represented a conserved region in *pol* that appears to map to an uncharacterized feline endogenous retrovirus that may be distantly related to enFeLV and is found in both the bobcat and Siberian tiger. The sequences highlighted in green were responsible for driving alignment to enFeLV *pol*. Nucleotides highlighted in red represent SNPs.

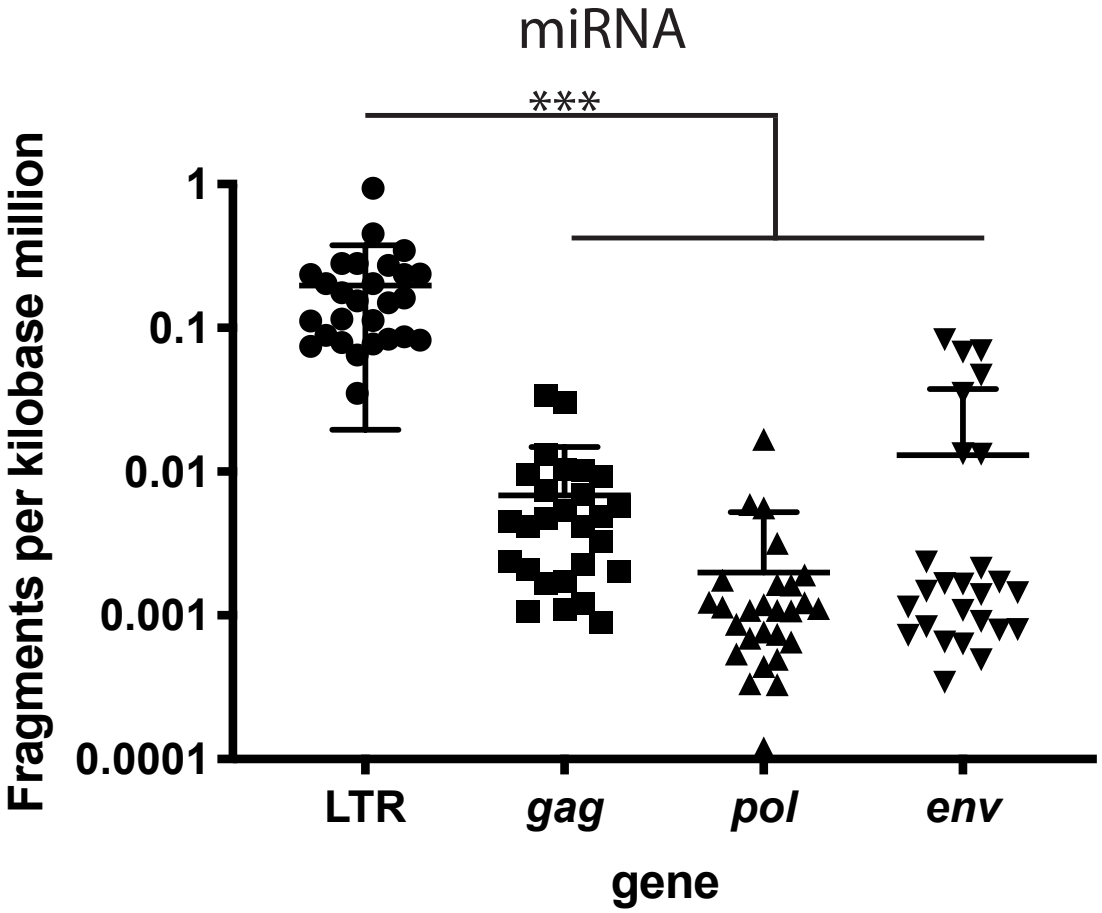


Figure 5.5: miRNA could be detected for all gene regions in enFeLV but mapped most frequently to enFeLV-LTR (ANOVA; $p<0.001$). The contribution of miRNA attributed to *gag*, *pol,* or *env* rarely exceeded 0.01 fragments per kilobase million (FPKM).

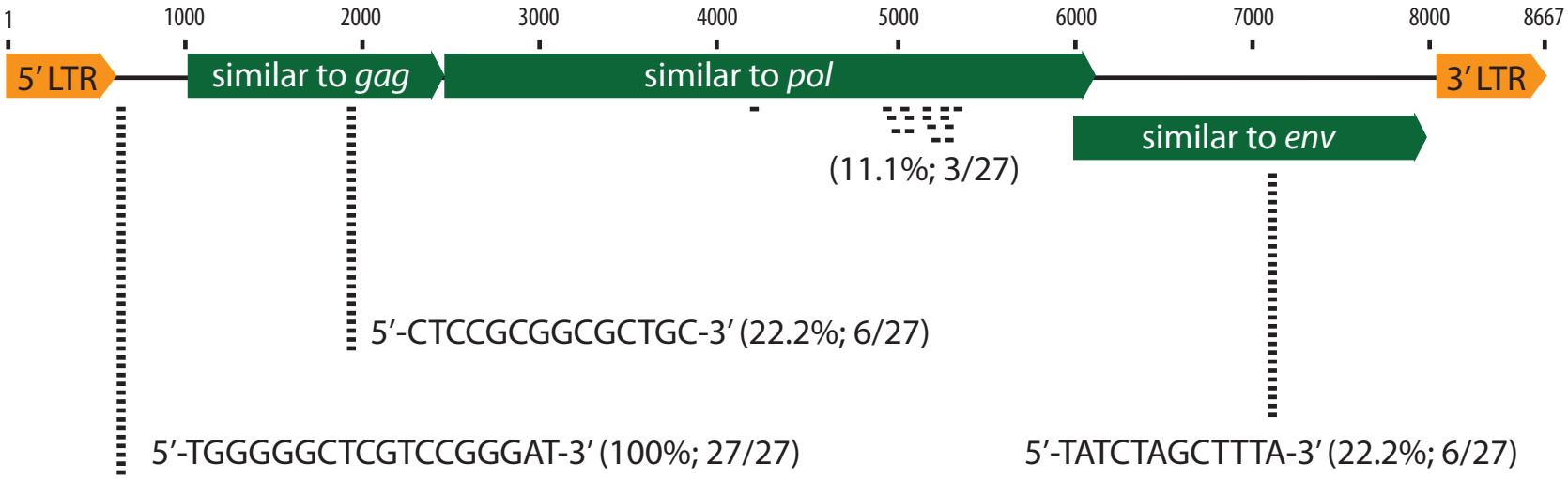


Figure 5.6: enFeLV miRNA maps only to two specific regions of the enFeLV genome. Of the 27 miRNA datasets, reads mapping to LTR, *gag*, *pol*, and *env* genome segments greater than 10% occurred in 27, 6, 3, and 6 datasets, respectively. Reads mapping to *pol* were diffuse and did not center on a particular locus, perhaps indicating contributions from various closely related endogenous gammaretroviruses.

Table 5.1: RNA-seq datasets used in this study

Accession number	Tissue type	Tissue class	Sequencer	Study
SRX211594	Liver	Organ	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211595	Liver	Organ	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211596	Liver	Organ	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211644	Kidney	Organ	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211645	Kidney	Organ	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211646	Kidney	Organ	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211688	Brain	Brain	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211689	Brain	Brain	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX211690	Brain	Brain	Illumina Genome Analyzer Ilx	Mammalian transcriptome study
SRX1610301	fetus	Embryo	Illumina HiSeq 2500	99 Lives project
SRX1610302	lung	Lymph	Illumina HiSeq 2500	99 Lives project
SRX1610303	fetus	Embryo	Illumina HiSeq 2500	99 Lives project
SRX1610304	muscle	Organ	Illumina HiSeq 2500	99 Lives project
SRX1610305	hippocampus	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610306	liver	Organ	Illumina HiSeq 2500	99 Lives project
SRX1610307	ear tip	Skin	Illumina HiSeq 2500	99 Lives project
SRX1610308	ear cartilage	Skin	Illumina HiSeq 2500	99 Lives project
SRX1610309	cerebellum	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610310	retina	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610311	uterus	Organ	Illumina HiSeq 2500	99 Lives project
SRX1610312	bone marrow	Lymph	Illumina HiSeq 2500	99 Lives project
SRX1610313	kidney	Organ	Illumina HiSeq 2500	99 Lives project
SRX1610314	temporal lobe	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610315	testes	Organ	Illumina HiSeq 2500	99 Lives project
SRX1610316	occipital lobe	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610317	embryo head	Embryo	Illumina HiSeq 2500	99 Lives project
SRX1610318	retina	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610319	spinal cord	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610320	skin	Skin	Illumina HiSeq 2500	99 Lives project
SRX1610321	embryo body	Embryo	Illumina HiSeq 2500	99 Lives project
SRX1610322	pancreas	Organ	Illumina HiSeq 2500	99 Lives project
SRX1610323	skin	Skin	Illumina HiSeq 2500	99 Lives project
SRX1610324	heart	Organ	Illumina HiSeq 2500	99 Lives project
SRX1610325	parietal lobe	Brain	Illumina HiSeq 2500	99 Lives project
SRX1610326	kidney	Organ	Illumina HiSeq 2500	99 Lives project
SRX1625943	skin	Skin	Illumina HiSeq 2500	99 Lives project
SRX1625944	spleen	Lymph	Illumina HiSeq 2500	99 Lives project
SRX1625945	thymus	Lymph	Illumina HiSeq 2500	99 Lives project
SRX1625946	spleen	Lymph	Illumina HiSeq 2500	99 Lives project
SRX1625948	salivary gland	Organ	Illumina HiSeq 2500	99 Lives project
SRX1625949	cerebellum	Brain	Illumina HiSeq 2500	99 Lives project
SRX317246	Blood	Negative	Illumina HiSeq 2000	Siberian Tiger Negative Control
SRR6384483	Blood	Negative	Illumina HiSeq RNA	Lynx rufus Negative Control

Table 5.2: miRNAome datasets included in this study.

Accession number	Tissue type	Tissue class	Sequencer	Notes
SRX2164134	brain stem	Brain	ABI Solid 5500	healthy cats
SRX2164135	cerebellum	Brain	ABI Solid 5500	healthy cats
SRX2164136	pancreas	Organ	ABI Solid 5500	healthy cats
SRX2164137	tongue	Skin	ABI Solid 5500	healthy cats
SRX2164138	kidney	Organ	ABI Solid 5500	healthy cats
SRX2164139	liver	Organ	ABI Solid 5500	healthy cats
SRX2164140	lung	Organ	ABI Solid 5500	healthy cats
SRX2164141	lymph node	Lymph	ABI Solid 5500	healthy cats
SRX2164142	skin	Skin	ABI Solid 5500	healthy cats
SRX2164143	spleen	Lymph	ABI Solid 5500	healthy cats
SRX2164144	kidney	Organ	ABI Solid 5500	healthy cats
SRX2164145	liver	Organ	ABI Solid 5500	healthy cats
SRX2164146	cerebral cortex	Brain	ABI Solid 5500	healthy cats
SRX2164147	lung	Lymph	ABI Solid 5500	healthy cats
SRX2164148	skin	Skin	ABI Solid 5500	healthy cats
SRX2164149	spleen	Lymph	ABI Solid 5500	healthy cats
SRX2164150	testis	Repro	ABI Solid 5500	healthy cats
SRX2164151	testis	Repro	ABI Solid 5500	healthy cats
SRX2164152	ovary	Repro	ABI Solid 5500	healthy cats
SRX2164153	ovary	Repro	ABI Solid 5500	healthy cats
SRX2164154	pancreas	Organ	ABI Solid 5500	healthy cats
SRX2164155	tongue	Skin	ABI Solid 5500	healthy cats
SRX2164156	lip	Skin	ABI Solid 5500	healthy cats
SRX2164157	cerebellum	Brain	ABI Solid 5500	healthy cats
SRX2164158	cerebral cortex	Brain	ABI Solid 5500	healthy cats
SRX2164159	kidney	Organ	ABI Solid 5500	healthy cats
SRX2164160	liver	Organ	ABI Solid 5500	healthy cats

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CHAPTER SIX

Characterization of Endogenous Feline Leukemia Virus (enFeLV) Long Terminal Repeat (LTR) Integration Site Diversity

INTRODUCTION

Arising from ancient viral infections, endogenous retroviruses (ERV) stably integrated into the genome have lost the ability to produce infectious virions but continue to interact with related exogenous retroviruses as outlined in Introduction to this dissertation (Boeke and Stoye, 1997). Through the course of ERV-host evolution, these viral remnants have assumed a multitude of cellular processes, including immune modulation, placentation, and oncogenesis (Bannert et al., 2018; Denner, 2016; Frank and Feschotte, 2017; Grandi and Tramontano, 2018). Common interactions between ERVs and their exogenous retrovirus (XRV) correlates are recombination, where the genotypes of two related viruses are combined due to template switching during reverse transcription (Luo and Taylor, 1990), and receptor interference, where envelope proteins (Env) that are responsible for cellular entry bind and inactivate receptors intra-cellularly before they can be transported to the cell surface (Rasmussen, 1997). Less commonly, clonal deletion of T-cells (Holt et al., 2013) and dis-regulation of virion trafficking (Murcia et al., 2007) can result from specific proteins that are similar between ERV-XRV couplets.

ERV-XRV interactions can also arise from unique features of specific retroviral genomic elements. Animal genomes contain over 40% endogenous retroviral elements, with approximately 8% of that genomic material consisting of full-length ERV (Consortium, 2001). All retroviral genomes contain three genes – *gag*, *pol*, and *env* – flanked on both ends by long terminal repeats (LTRs) oriented in the same direction (Coffin et al., 1992). LTRs contain three regions, the U5, R, and U3 regions, containing enhancer, promotor, and regulatory functions (Coffin et al., 1992). The number of LTRs in a genome is not directly associated with the copy number of full-length ERVs (see Chapter 2). This is possible due to fact that ERVs undergo retrotransposition, the process in which retrotransposable elements are excised

from the host genome and get inserted into other loci. As a consequence of retrotransposition, LTRs become disarticulated from their ERV progenitors (Vitte and Panaud, 2003). In some cases, LTRs maintain their enhancer and promoter functions, which serve important biological purposes, including the regulation of normal gene functions (Thompson et al., 2016). While not all solo-LTRs will modulate transcription of host genes, those that do likely have selective advantages that results in retention of LTR in the host genome. For example, in the case of murine leukemia virus (MuLV), the expression of the anti-viral APOBEC gene is promoted by a specific MuLV-derived LTR locus (Sanville et al., 2010). In this way, ERV LTRs contribute indirectly to viral restriction.

ERV-derived solo-LTRs may augment host gene expression in two main ways: *cis*-activation of host genes via the promoter, and *trans*-activation of host genes via the enhancer (Thompson et al., 2016). Evidence of both these interactions has been previously reported, although enhancer functions are more difficult to detect and attribute to specific LTR loci (Thompson et al., 2016). This is due to the fact that the enhancer can function and increase transcription of host genes up to 1Mbp away in both downstream and upstream orientations (Chisholm et al., 2019; Maston et al., 2006; Pennacchio et al., 2013). Thus, the restriction properties of enFeLV-LTRs associated with FeLV infections could be a result of up regulation of antiviral host genes (Chiu, specific aim 2 and 3).

The enFeLV-LTR is believed to have maintained both enhancer and promoter functions (Berry et al., 1988). As noted previously, we have documented that full-length enFeLV proviral copy numbers vary by approximately 8-12 copies per cell (in line with previous literature reports describing 8-24 copies per cell), while LTR copy number may exceed 80 copies per cell. In this chapter, we evaluate whether endogenous feline leukemia virus (enFeLV) LTRs are associated with host genes that may indirectly result in exFeLV disease modulation (described in Chapter 4), acting as a restriction factor or activator of immune responses that diminish exFeLV infection.

METHODS

Sample collection, and DNA extraction

Full thickness skin biopsies were collected during cat necropsies performed at the Colorado State Veterinary Diagnostic Laboratory. Primary fibroblasts were isolated as previously described (Vangipuram et al., 2013) and cultured and expanded in 20% FBS-supplemented DMEM high glucose media and 1x antibiotic-antimycotic solution (Gibco, penicillin/streptomycin/fungizone). Blood was drawn from specific pathogen free (SPF; free of FeLV, FIV, FFV) domestic cats maintained as a closed colony for at least two decades with one set of new genetic donors in 1993. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by ficoll-gradient centrifugation as previously described (Riedhammer et al., 2014). PBMCs were cultured in 20% FBS-supplemented RPMI media supplemented with 100 ng/mL interleukin-2 (Sigma, USA) and 50 ng/mL concanavalin A (Sigma, USA). Blood from domestic cat/Asian leopard cat (*Prionailurus bengalensis*) hybrids (Bengal cats) maintained in a semi-closed colony for a decade were collected, processed, and viably frozen using previously reported methods (Powers et al., 2018). DNA was extracted from 7 domestic cat fibroblast cultures, 7 SPF domestic cat PBMC cultures, and 6 Bengal cat hybrid PBMCs using a DNeasy blood and tissue DNA extraction kit (Qiagen, US). All procedures were approved by the CSU IACUC and followed appropriate animal care protocols.

DNA Linker-mediated PCR and sequencing

In order to determine FeLV-LTR integration sites, we employed a strategy to directly enrich sequencing results for LTR integrations, adapted from a method used to determine retroviral integration sites (Figure 1A; (Maldarelli et al., 2014)). DNA was sheared by ultrasonication using a Covaris M220, with specifications to target DNA fragments with an average length of 400 base pairs (Peak incident power, 50; 200 cycles per burst; duty factor, 10%; 70 seconds). Sheared DNA was further size selected with AMPure beads (Agilent) at a concentration of 0.8x beads per DNA. Enzyme-facilitated end repair and 3'-dA-tailing was completed under manufacturer's directions (New England Biolabs, Ipswich, MA). Duplexed linker sequences 5'-ACTATAGGGCTCCGCTTAAGGGACT-3' and 5'-GTCCCTTAAGCGGAG-3' were synthesized with one 3'-T overhang. Linkers (500nM) were ligated to size-selected, sheared DNA with T4 DNA Ligase (New England BioLabs). Flanking 3'-dT's on the linker

and 3'-dA's on the DNA selected against the formation of DNA-DNA and linker-linker complexes. Unligated linkers were removed with an AMPure bead cleanup at a concentration of 0.8x beads per DNA. Library amplification was performed using a nested PCR, with Illumina sequencing adapters being incorporated with primers in the first round, and second round primers containing unique barcodes to allow for identification of individual samples. Each round was 15 cycles and completed using, 9µl of template, 10µl of KAPA high fidelity polymerase, and 1µl of primer. Libraries underwent a final cleanup and size selection with AMPure beads at a concentration of 0.6x beads per DNA volume in order to filter out small complexes. Quality control of the library was performed after each step of library preparation using a High Sensitivity D1000 DNA tape on an Agilent 2200 tape station (Agilent, US). The 20 unique dual-indexed fibroblast and PBMC libraries were combined and sequenced on an Illumina MiSeq using a MiSeq Reagent 2x250bp Nano kit v2 (Illumina, San Diego, CA) at Colorado State University's Next Generation Sequencing Core.

Bioinformatics Analysis: Integration sites

Domestic cat LTR integration sites were determined using a custom bioinformatics pipeline (Figure 1B). Reads were filtered with cutadapt (version 2.7) first with quality scores (Q>20) and then by removing sequences associated with the linker sequence in the first read (i7 adapter) of the paired end reads. Any sequence in which the linker sequence was absent was filtered out and paired reads were reconciled. Then, the 5' LTR sequence was removed from read 2 (i5 adapter). 3'-LTR and linkers were removed from both reads. Reads were aligned with Bowtie2 (version 2.3.4.3) to the *Felis catus* whole genome 9.0 (NC_018723-41) with the addition of the domestic cat Y-chromosome (KP081775) as the whole genome was sequenced from an Abyssinian female. Alignments were performed with minimum local alignment score of 50 to allow for short sequences and genomic differences from outbred domestic cats. Bowtie2 .sam output files were converted to .bam files with samtools (version 1.9) and then to .bed files with bedtools (version 2.27.1). Bedtools genomecov –bg function was used to calculate genome coverage in both exonic and intronic regions with collapsed viewing format. LTR integration sites were determined manually if they satisfied both a length >100bp and a total coverage >50x. A cut-off value of

>100bp was established as shorter reads did not provide enough material to confidently align to the cat genome. We used a greater than >50x coverage by calculating that theoretical level of coverage per sample.

LTR integration sites were first identified and then confirmed visually in Geneious (version 11.1.2), which required both linker and LTR sequences in softclips generated by cutadapt in addition to a Poisson distribution of genome coverage in .sam files aligned to the domestic cat genome. Polarity of the LTR integration was determined based on the directionality of the Poisson distribution as the 5'-LTR start site will appear as a blunt end to the Poisson distribution. Integration sites were removed if the adjacent genomic regions mapped to enFeLV-*env* genes as these regions are these regions are set in the reference genome and thus may not represent the true integration site in the specific cats sequenced. Chromosomal integration sites were visually identified by scanning the annotated feline genome for genes 1Mbp upstream and downstream of the integration sites using chromosomes imported into Geneious.

Statistics

The difference between number of total integrations and number of unique integration sites between the three cat cohorts was measured for statistical significance using a One-way ANOVA, following a Kolmogorov-Smirnov test for normality in Prism v8.

CRFK cell culture and fluorescent in situ hybridization (FISH)

To ensure loci detected by NGS analysis represented a normal distribution of enFeLV integrations, we conducted FISH on the feline-derived cell line Crandall Reese Feline Kidney (CRFK), which were determined to have 7 copies of enFeLV proviral copies and 63 enFeLV-LTRs (Chapter 4). CRFK cells were cultured in 1X antibiotic/antimycotic supplemented DMEM media in a T-75 flask with 5% CO_2 at 37˚C. Puma (*Puma concolor*) fibroblasts, negative for FeLV, were used as negative controls. Puma full skin biopsies were collected by Colorado Parks and Wildlife and primary fibroblasts were isolated using methods previously reported in Chapter 4. Cells were passaged once they reached 80-90% confluence. Forty nucleotide FeLV probes were designed against the entire 8.5kb enFeLV proviral genome (Genbank Accession number: AY364318; KromaTid, Fort Collins CO). Actively dividing CRFK

and puma cells were treated with 0.1ug/mL colcemid and incubated for 4 hours. Cells were washed with sterile PBS, typsinized with 0.05% trypsin (Hycyclone), collected and centrifuged. Cells were then fixed with 3:1 methanol:acetic acid and dropped on cold slides and fixed under heat. Slides were checked visibly for metaphase spreads before being rehydrated in PN buffer for 10 minutes before being dehydrated in 75%, 85%, and 100% ethanol for 2 minutes each. After air-drying the slides, they were incubated in a 75% formamide/2xSSC solution at 75˚C for 8 minutes and immediately dehydrated again in 75%, 85%, and 100% ethanol for 2 minutes each before being allowed to air dry again. During incubation, a 30uL FISH probe hybridization mix made of 2 uL FeLV probe and 28 uL FISH hybridization buffer was incubated at 75˚C for 5 minutes and then chilled on ice. The hybridization mix was added to each slide and a coverslip was rubber cemented in place to reduce evaporation. Slides were incubated overnight in a humid chamber at 37˚C. After incubation, slides were immediately washed 5 times for 15 minutes in 42˚C 2x SSC buffer. Finally, slides were washed in PBS before Dapi was added. Telomere-FISH was used as a positive control for the FISH protocol following previously reported methods (telomere probes generously provided by Dr. Susan Bailey; (McKenna et al., 2017)). Metaphase spread images were acquired using an Olympus microscope outfitted with a Coolsnap ES^2 camera and running MetaVue Imaging System software (Molecular Devices, San Jose, CA).

RESULTS

Integration site identification

Among the 20 unique domestic cats sequenced (7 outbred domestic cat, 7 SPF domestic cat from a colony closed, and 6 cats from a partially-closed colony with hybrid Bengal cat ancestry), we identified a total of 1,112 total integration sites that achieved at least 50x read depth and were at least 100 nucleotides long (Figure 1B). The MiSeq Reagent Nano Kit v2 (Illumina, San Diego, CA) ideally yields 1 million reads, producing 50,000 reads per sample. Given previously reported enFeLV-LTR copy numbers, each sample should have on average 50 integration sites, thereby generating approximately 1,000 reads per site. The sonication method is random, and as such may be more efficient in generating cuts in some parts of the genome, compared to others. Any integration site that breached the 50x cutoff

achieved 5% efficiency from the 'ideal' coverage, accounting for library pooling, sonication, and PCR biases. After filtering out reads mapping to enFeLV-*env*, 588 integration sites remained that were identified by a Poisson distribution achieved following library preparation protocol and contained both synthesized linker and 5' LTR sequences that were functionally removed in our bioinformatics pipeline, but are still visible in Geneious (Figure 1). The 588 individual integration sites identified in among all 20 cats represent a total of146 integration sites shared between all 20 cats, with 54 sites exclusive to individual cats (Figure 3, Table 1). The number of total integration sites between the three cohorts was not statistically different (Figure 2A). Outbred cats had a significantly greater number of unique integration sites (i.e., not shared by other cats) per cat compared to SPF colony cats (4 vs. 2; ANOVA $t=2.928$, $p<0.05$; Figure 2B). Cats from the Bengal hybrid colony harbored an intermediary number of unique integration sites.

Integration sites among all cats were scattered across all chromosomes; however we were unable to locate integration sites in the Y-chromosome (Figure 4). Two integrations sites were identified in all 20 cats, and 19 integration sites were in at least 10 individuals (Figure 4, Table 2). Visual confirmation in Geneious revealed that between cats, the starting nucleotide position in 11 integration sites were offset and required reconciliation by shifting the start site by a maximum of 6 nt. The shift was necessary to adjust for insertions and deletions introduced upstream to the start site by other LTR integration sites, that artificially adjusted the integration position when compared to the reference genome.

Integration site locations

In total, all 146 integration sites were within 1MB of 2,463 protein-coding genes. The 19 LTR integration sites shared by at least 10 cats were within 1Mbp of 255 individual genes (Table 3). Two LTR integration sties (chrA2:7199456 and chrA3:3320238) were not in close proximity to any identified coding regions. Four LTR integration sites (chrA3:97445, chrB2:32150838, chrD1:117070340, and chrF1:44637013) were in proximity of 168 host genetic loci. The remaining integration sites were associated in an average of 12 coding regions (Table 3). Only 7 genes were associated with the two sites found in all 20 cats (Figure 5). While the majority of gene classes with proximity to LTR integration sites

were not represented more than one time, four zinc finger genes were in proximity to at least three integration sites, including one integration represented in all 20 cats (chrC2:160924756) (Figure 5). Two zinc finger loci (ZNF445 and ZNF35) were in exceptionally close proximity to the chrC2:160924756 LTR integration site. ZNF35 is 176,186bp away from the 3' end of the integration site and ZNF445 is 6,240bp upstream of the LTR integration site. One LTR integration site in two cats was found to be associated with APOBEC1, a potent anti-retroviral protein (chrB4: 42584509).

FeLV-FISH

enFeLV-LTR and full-length enFeLV integration distributions were validated by FISH. Hybridization for FeLV in CrFK cells displayed distribution reminiscent of FeLV-LTR integrations (Figure 6B). Sites appear to be located in centromeric and telomeric sites and everywhere in between. Telomeric sites are not represented in every chromosome, unlike what is shown following telomere-FISH (Figure 6A). FeLV was not hybridized in tiger cells (Figure 6C).

DISCUSSION

One hundred forty six enFeLV-LTR integration sites were identified in a 20 cats from three populations. The presence of integration sites on all chromosomes was expected as insertion of the provirus is considered a random process in both retrotransposition and novel exogenous retroviral infections. There are a few retroviral integration sites in other viral systems that may be more commonly targeted, but the idea of site fidelity is believed to be retrovirus specific (Li et al., 1984). LTR integration sites that we have identified are the culmination of every integration and retrotransposition event in the cat's pedigree. The two sites found in all cats may be the result of an older evolutionary history or represent a selective advantage. If the integration site were one of the earliest proviral integrations they would have been maintained for generations and may have become fixed among domestic cats. In addition, if the enFeLV-LTR integration sites are beneficial for the host, likely by promoting a nearby host gene, then the presence of this integration would provide an evolutionary advantage and selection could have resulted in the loci became fixed in domestic cat populations.

There are more unique domestic cat enFeLV-LTR integration sites in outbred cats compared to cats bred in a closed colony. Despite this anticipated finding, the average number of total LTR integration sites was not different between the three groups of cats (SPF colony, hybrid colony, outbred cats). Population level changes in LTR-integration number are difficult to appreciate because of high variation enFeLV-LTR numbers (see Chapter 2). Although the specific location of integration sites vary, the sites are passed along through Mendelian inheritance so they should be similar between closely related individuals barring any chromosomal rearrangement (e.g., inversions, duplications, translocations, etc.). For this reason, one would expect outbred cats to harbor more unique integration sites when compared to a subset of cats from closed colonies. Likewise, if we were able to sample from closely related outbred cats, we would expect to find fewer unique integration sites. Another mechanism that may be at play is the size of the colony. With more cats in the hybrid cat colony, there are more potential 'family groups' from which to select animals and as such, an intermediate level of diversity between the SPF cat colony and outbred cats. Despite all of this, the majority of integration sites are represented in all three groups.

Integration sites that are shared by a majority of cats (10+) are in the vicinity of 255 protein-coding genes. Association was defined by integration site proximity to genes fewer than 1Mbp away as this distance is generally accepted as the maximal effective distance for LTR enhancer function (Maston et al., 2006; Pennacchio et al., 2013), although enhancers are also believed to be able to be trans-activators for genes on different chromosomes. Curiously, some integration sites are not close to any domestic cat genes. Taking into account all 146 integration sites, there are 2,463 associated protein-coding genes (Table 3). Many gene classes were associated with enFeLV-LTR integration sites. The majority of cases in which associated genes belonged to the same class occurred in association with a single integration site. One exception and a notable example of a class of proteins that is associated with enFeLV-LTR integrations is zinc finger proteins accounting for 49 genes across 22 individual integrations sites, described in more detail below.

One integration site shared by all cats is found within 200 kb of two two zinc finger genes (ZNF35 and ZNF445), with the closest approximately 6kb upstream of the LTR integration. Zinc finger

proteins represent one of the most abundant classes of regulatory and transcription factor proteins that have a variety of functions, but the basic mechanisms center on DNA recognition, DNA binding, and transcription down/up-regulation. The physiological roles of ZNFs include differentiation of cells from pluripotent progenitors (e.g., skin, muscle, intestine, stem cells, etc), regulation of proto-oncogene and tumor suppressor genes, among other functions (Cassandri et al., 2017). In addition to tissue homeostasis, zinc fingers have also been documented to have anti-viral activities, often through the action of zinc finger anti-viral proteins (ZAP) (Zheng et al., 2017); however, the ZNF35 and ZNF445 genes found here have not been noted to have ZAP activity. ZNF445 has recently been described as a potent regulator of genomic imprinting, the mechanism by which epigenetic markers are transmitted parentally (Takahashi et al., 2018). There are approximately 150 known imprinted genes in mice and the majority of imprinted genes are responsible for embryonic development and placentation formation (Peters, 2014). ZNF35 is not as well characterized, but the protein-binding site has been identified (Pengue et al., 1993) and it has been associated with spermatogenesis in mice and humans (Przyborski et al., 1998; Zhou et al., 2010). The degree to which enFeLV-LTRs may influence biological processes is difficult to discern particularly with our findings of LTR integration sites associated to genes that function as regulators of cellular processes. This study however, lays the groundwork for future interrogation of enFeLV-LTR integration by identifying sites to target for genome editing impacts to domestic cat gene expression.

We were unable to characterize LTR integration sites in the Y-chromosome nor the integration sites that were associated with full-length enFeLV. The methods used in this report have identified host genome integration sites that are upstream, relative to the orientation of the LTR. While full-length enFeLVs have two LTRs, both LTRs are positioned in the same direction. As such, we can only find the integration 5' of the 5'-LTR. The region 5' to the 3'-LTR only yields the enFeLV-*env* gene. There are many copies of enFeLV-*env* in the domestic cat genome and these have not been well annotated. Curiously, we were not able to map locations to the Y-chromosome, which was constructed independently from the rest of the feline whole genome-sequencing project (Li et al., 2013). Furthermore, the Y-chromosome is made up of a large percentage of mobile elements that do not undergo

recombination and so the Y-chromosome available may not be representative of all Y-chromosomes. We were, however, able to map enFeLV-*env* in two loci on the Y-chromosome, indicating we had properly indexed the Y-chromosome for our bioinformatic approach. Earlier reports documented males having more enFeLV-LTR copy numbers compared to females (Powers et al., 2018) and our inability to map integration sites to the Y-chromosome make it difficult to confirm this through our methods.

Fifty enFeLV-LTR integration sites were at flanking regions of each chromosome (i.e., within the first and last 10% of the chromosome). enFeLV integration may be a random process, but the consequences of integration are far-reaching long after the enFeLV has undergone retrotransposition. While we cannot speculate as to why enFeLV has a propensity to integrate into the terminal regions, their presence in terminal regions may increase their enhancer functions. As regulatory elements, enFeLV-LTRs present in sub-telomeric ends may benefit from telomere position effect over long distances (TPE-OLD;(Robin et al., 2014)). Telomeres are themselves regulatory elements and the process of TPE-OLD causes chromosome looping that may exert regulatory functions by 'relocating' the telomeres to genes far beyond the 1Mb generally understood to be the normal range of action of regulatory sequences.

enFeLV-LTRs maintain functional enhancer and promoter functions and we have documented potential targets for increased transcription, some of which have additional regulatory functions. While we were unable to document significant integration sites (i.e., sites were found in a majority of cats) that were associated specifically with anti-viral activities, there were individual cats that showed LTR integration to these sites; the LTR integration site of two cats was found within 1MB of APOBEC1, an anti-viral cytidine deaminase that disrupts normal retroviral replication. We cannot be sure that the integration site actually had an impact on gene expression in this site or in any site although future examination of the transcriptome of samples where the integration sites have been identified will provide light on that matter. We may also explore how the transcriptome changes in light of FeLV infection to further interrogate the system. Given our data, we believe enFeLV-LTRs has the potential to have an impact on host gene transcription, and establishing the degree LTRs functionally augment gene expression is an area for future inquiry.

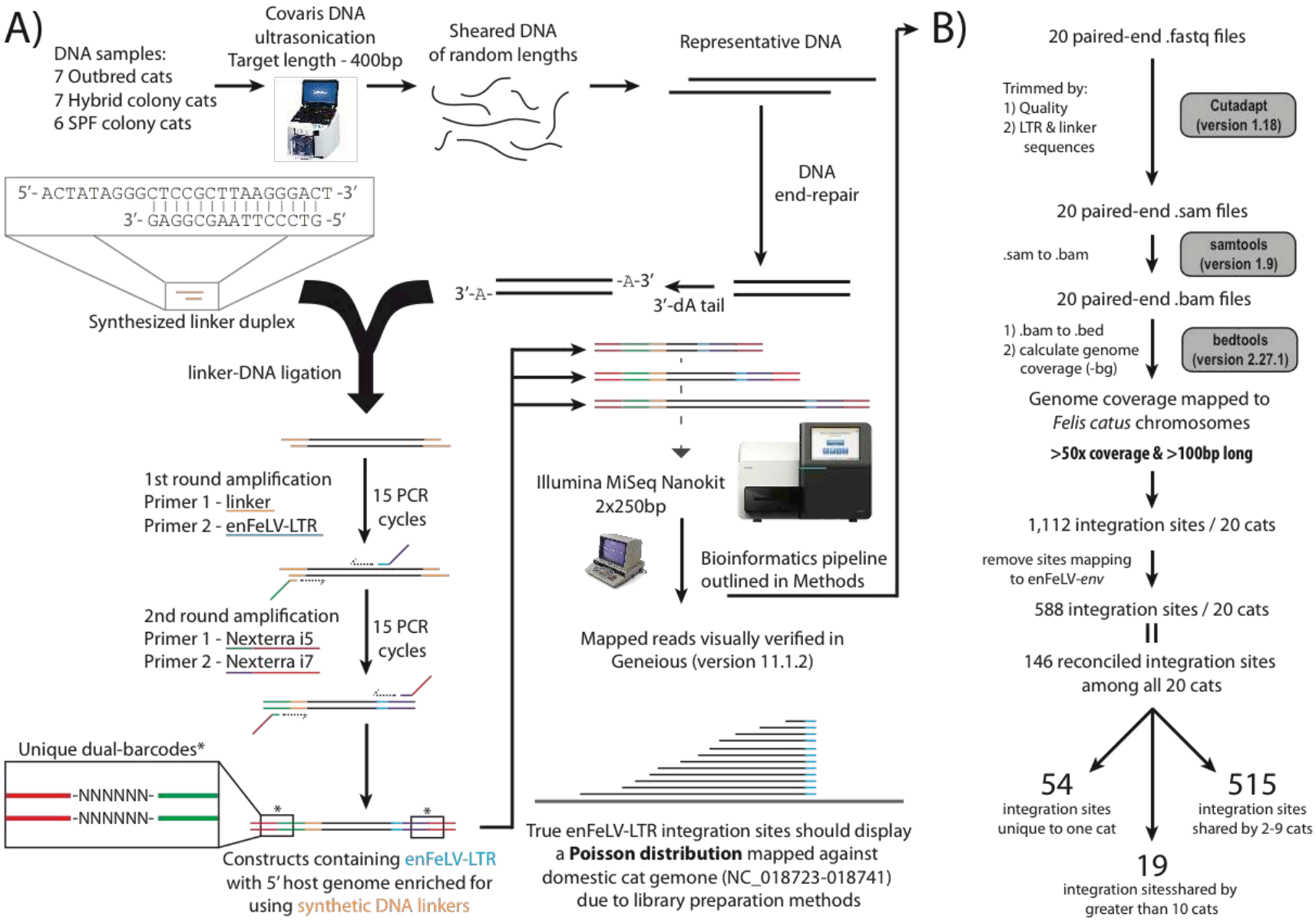
A)
DNA samples:
7 Outbred cats
7 Hybrid colony cats
6 SPF colony cats
Covaris DNA ultrasonication
Target length - 400bp
Sheared DNA of random lengths
Representative DNA
DNA end-repair
3'-dA tail
5'- ACTATAGGGCTCCGCTTAAGGGACT -3'
3'- GAGGCGAATTCCCTG -5'
Synthesized linker duplex
linker-DNA ligation
1st round amplification
Primer 1 - linker
Primer 2 - enFeLV-LTR
15 PCR cycles
2nd round amplification
Primer 1 - Nexterra i5
Primer 2 - Nexterra i7
15 PCR cycles
Unique dual-barcodes*
-NNNNNN-
-NNNNNN-
Constructs containing enFeLV-LTR with 5' host genome enriched for using synthetic DNA linkers
Illumina MiSeq Nanokit 2x250bp
Bioinformatics pipeline outlined in Methods
Mapped reads visually verified in Geneious (version 11.1.2)
True enFeLV-LTR integration sites should display a Poisson distribution mapped against domestic cat gemone (NC_018723-018741) due to library preparation methods
B)
20 paired-end .fastq files
Trimmed by:
1) Quality
2) LTR & linker sequences
Cutadapt (version 1.18)
20 paired-end .sam files
.sam to .bam
samtools (version 1.9)
20 paired-end .bam files
1) .bam to .bed
2) calculate genome coverage (-bg)
bedtools (version 2.27.1)
Genome coverage mapped to Felis catus chromosomes
>50x coverage & >100bp long
1,112 integration sites / 20 cats
remove sites mapping to enFeLV-env
588 integration sites / 20 cats
146 reconciled integration sites among all 20 cats
54
integration sites unique to one cat
515
integration sites shared by 2-9 cats
19
integration sitesshared by greater than 10 cats

Figure 6.1: Stylized library preparation and bioinformatics pipeline. A) DNA from outbred, hybrid colony, and SPF colony domestic cats was sheared and ligated to synthesized linkers to selectively amplify enFeLV-LTR integration sites. Due to library preparation methods, integration sites displayed a Poisson distribution. B) Bioinfomatics pipeline used to generate mapped LTR integration identified unique and common integration sites shared between all cats included in this study.

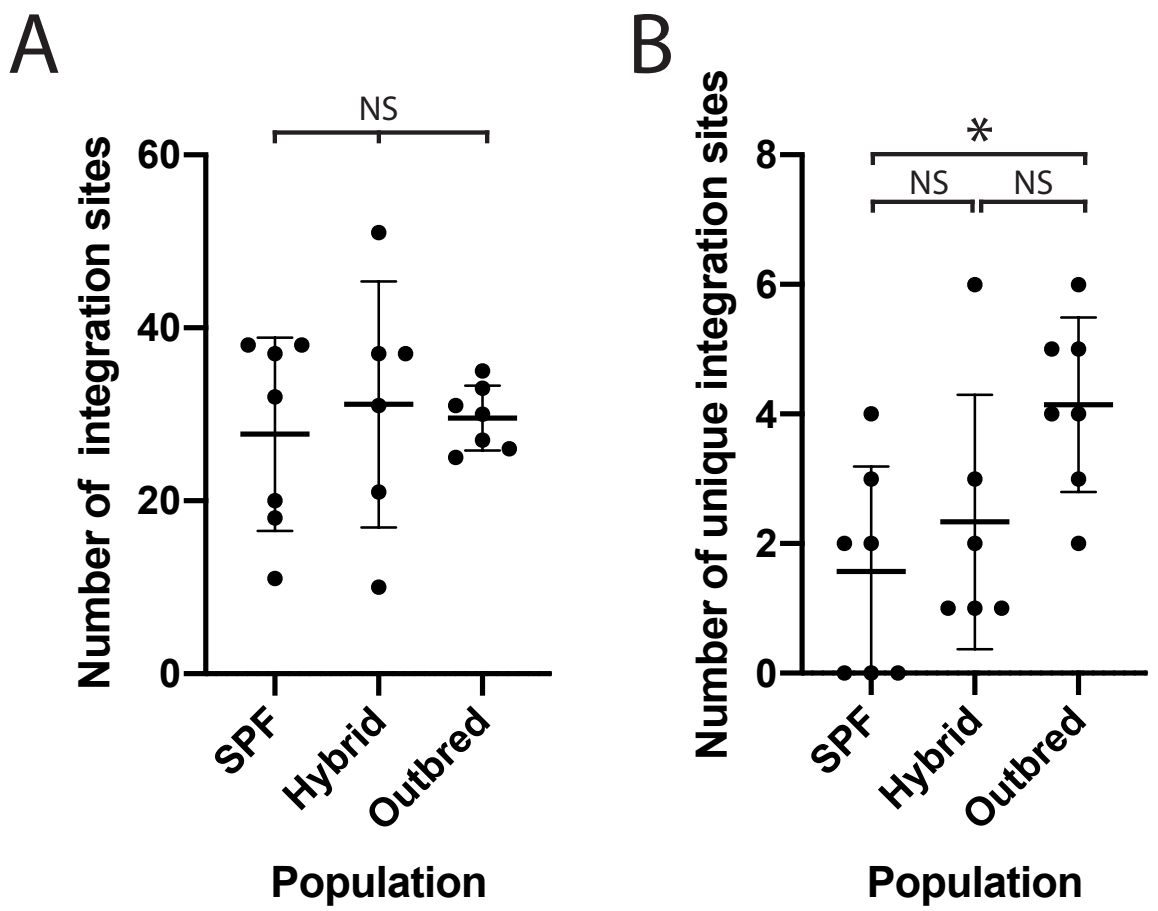

Figure 6.2: The source of tested cats had an impact on the location, but not the number of integration sites. A) Cats, regardless of their source, had on average the same number of integration sites (ANOVA; NS= $p>0.05$). B). Cats from an outbred group had on average, greater number of unique integration sites compared to SPF cats (n=26) (ANOVA; *= $p<0.05$). Cats from a hybrid cat colony (n=65) have an intermediary number of unique integration sites.

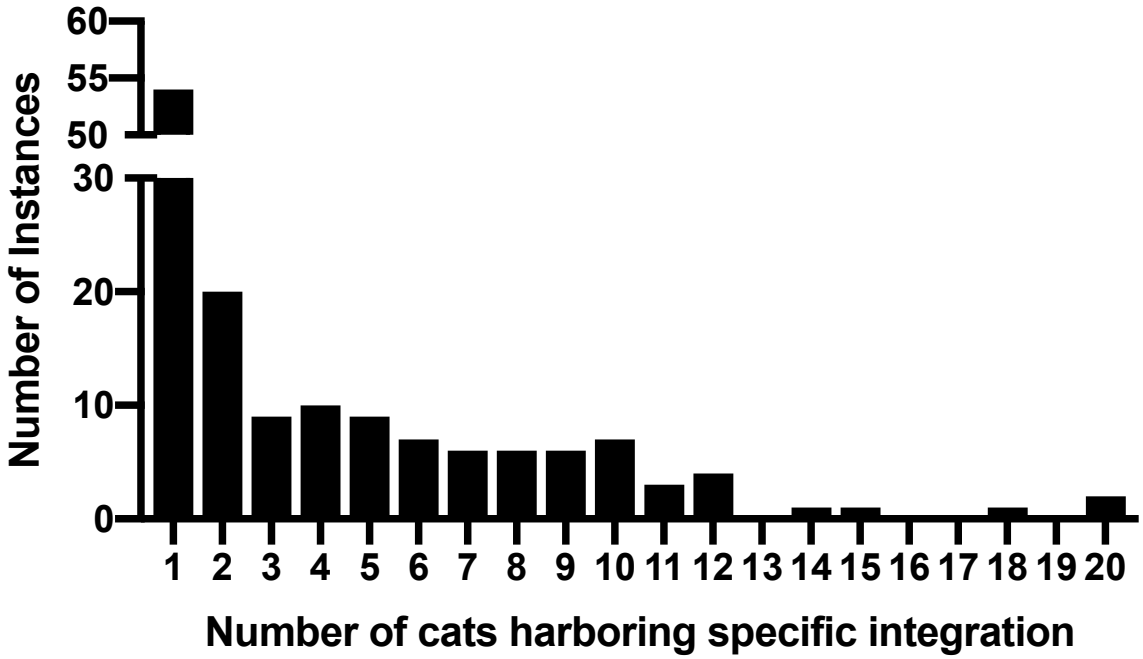

Figure 6.3: Integration sites documented in domestic cats appear more often than not to be unique within a population of cats. Only two sites were shared by all twenty cats. Among all 20 cats, there were 54 unique sites not identified in other cats.

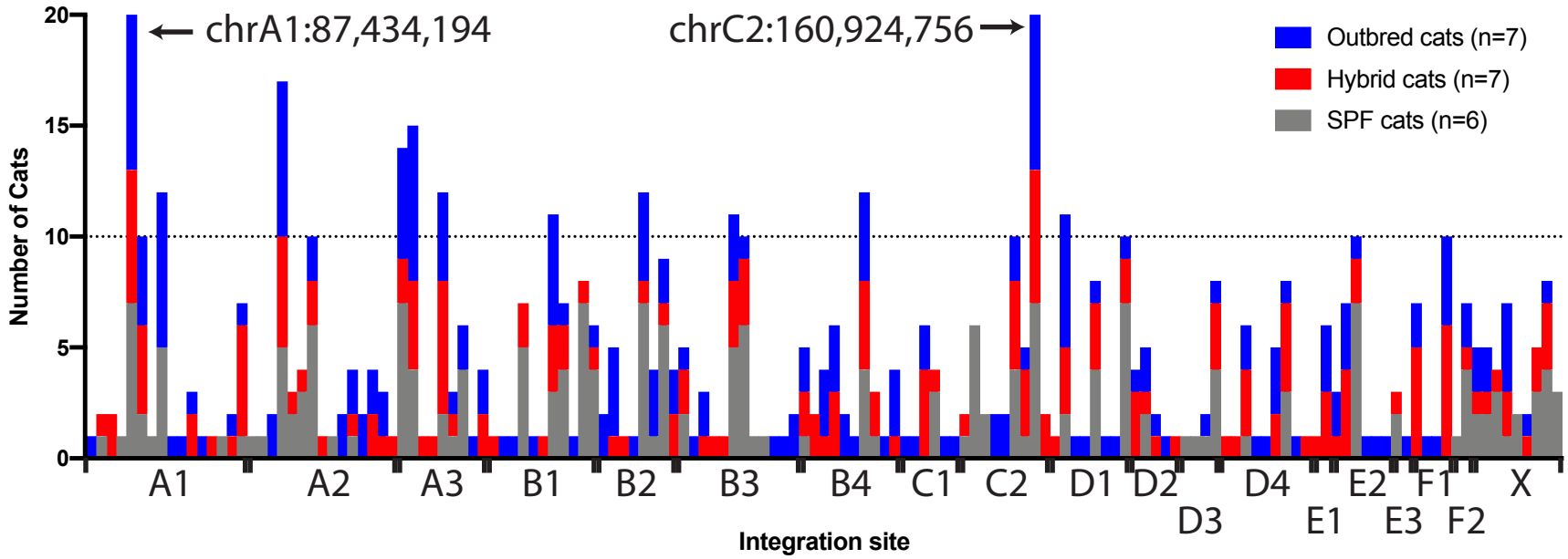

Figure 6.4: One hundred forty six enFeLV-LTR integration sites are distributed across all chromosomes of the domestic cat genome. Only two sites found in chromosomes A1 and C2 are shared by all 20 cats. Very few non-unique integration sites are made up of cats solely from one cohort.

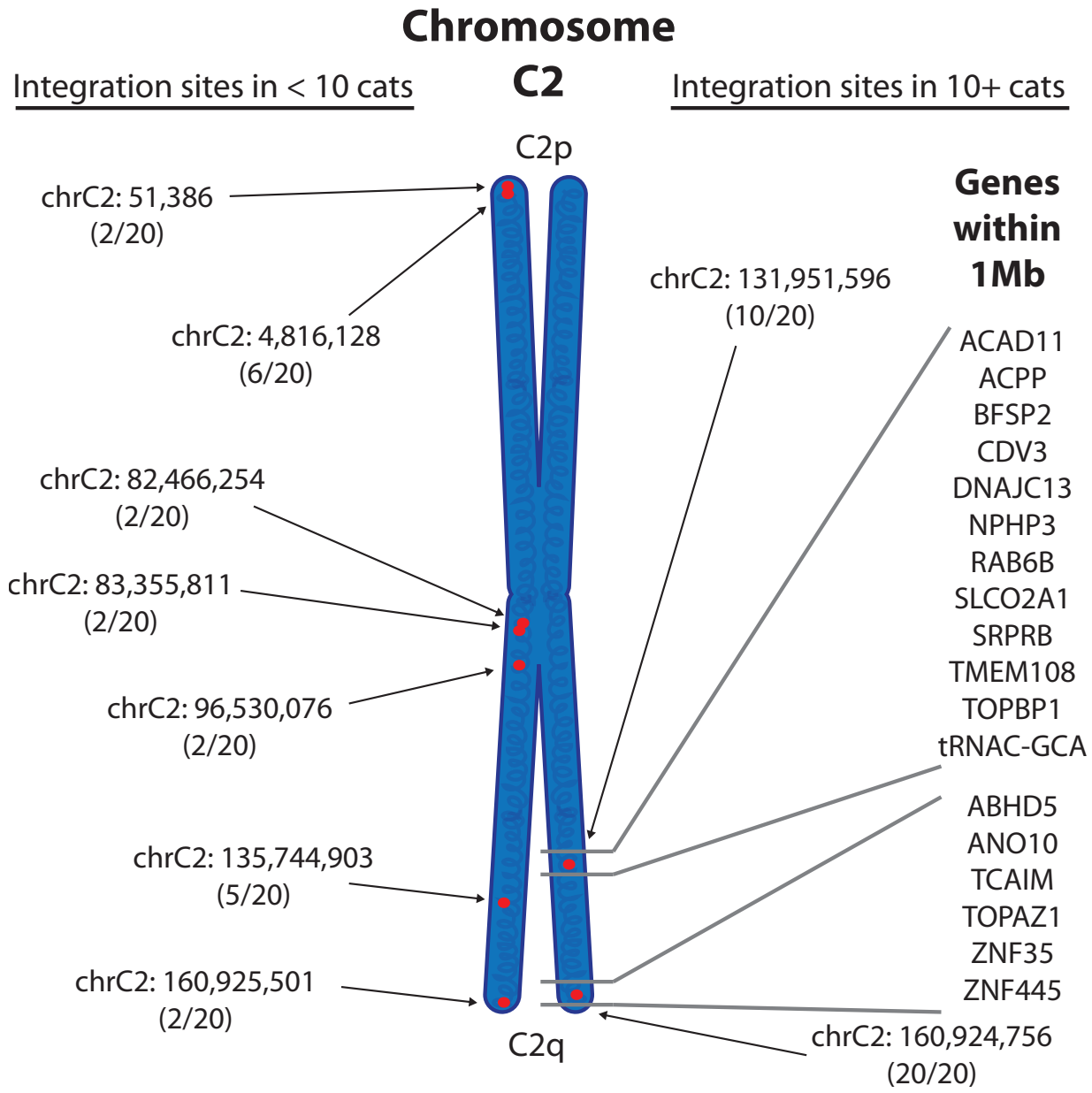

Figure 6.5: Nine LTR integrations can be found on chromosome C2. Seven of the 9 integration sites are found in fewer than 10 cats. Only 2 integrations are found in at least 10 cats. In these 2 integration sites, 18 genes are found within 1Mb.

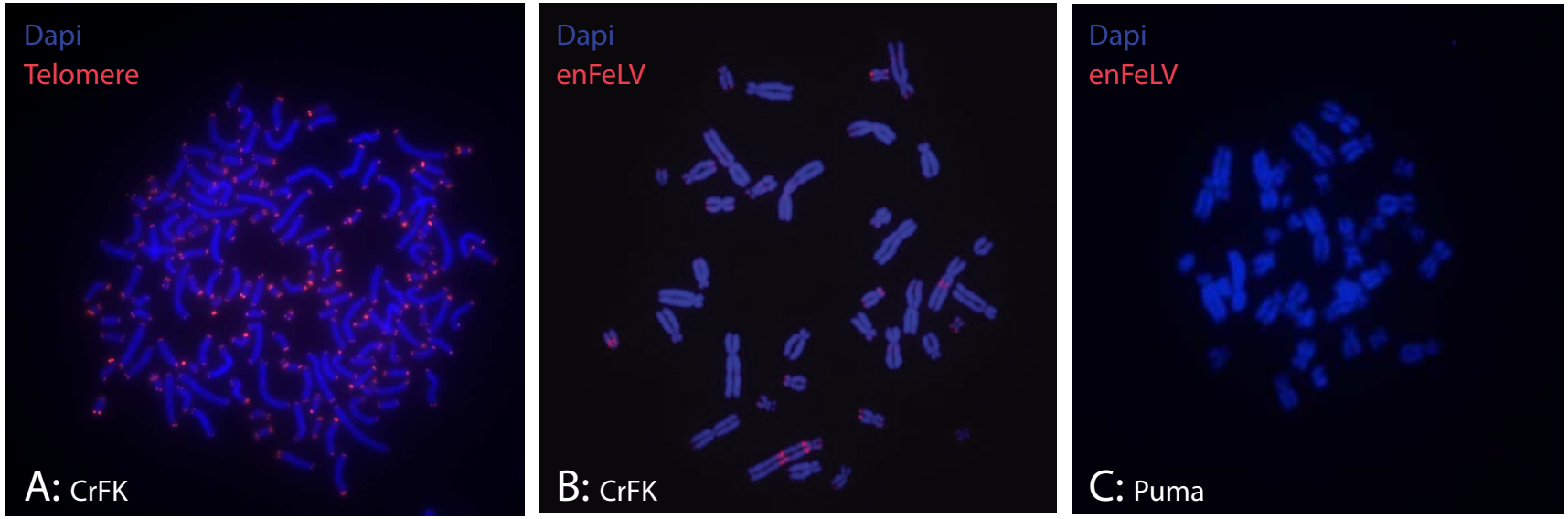

Figure 6. Fluorescent *in situ* hybridization allows for the visualization of specific genomic elements in Crandall Reese Feline Kidney metaphase spreads. A) Probes specific for telomeres can be found at both ends of each chromosome. B) Probes specific for enFeLV appear to hybridize along the majority of chromosomes. The majority of signals are punctate, possibly indicating solo-LTRs, while some larger signals may indicate full-length enFeLV. C) Puma cells lack enFeLV and as such do not show signal to enFeLV probes.

Table 1. Integration site summaries by individual cat identifying sex and number of total and unique integrations. ND = not determined.

Cat ID	Number of total integrations	Number of unique integrations	enFeLV-LTR Copy Number by qPCR	Sex
156	51	3	26	Male
178	37	1	47	Male
260	31	6	25	Female
282	10	1	60	Male
369	37	1	29	Female
377	21	2	42	Male
4438	37	2		Female
4460	38	4		Female
4474	38	3		Female
4501	20	2	36	Male
4504	11	0	121	Male
4510	32	0	89	Male
4520	18	0		Male
DC1	33	6	67	unknown
DC2	30	3	57	unknown
DC4	35	5	32	unknown
DC6	31	5	57	unknown
x2654	25	2	74	unknown
x2656	27	4	62	unknown
x2657	26	4	49	unknown

Table 2. Curated and compiled list of all integration sites. Unique integration sites are indicated by colored blocks.

Accession No.	Chromosome	Integration site	Coverage	Polarity	Cat
NC_018723	A1	25175154	351	-	DC6
NC_018723	A1	37559850	143	-	260
NC_018723	A1	37559850	123	-	4510
NC_018723	A1	75673842	50	+	282
NC_018723	A1	75673842	618	+	369
NC_018723	A1	84436185	82	+	4501
NC_018723	A1	87434194	1774	+	156
NC_018723	A1	87434194	499	+	178
NC_018723	A1	87434194	1879	+	260
NC_018723	A1	87434194	87	+	282
NC_018723	A1	87434194	530	+	369
NC_018723	A1	87434194	120	+	377
NC_018723	A1	87434194	1859	+	4438
NC_018723	A1	87434194	1994	+	4460
NC_018723	A1	87434194	2178	+	4474
NC_018723	A1	87434194	122	+	4501
NC_018723	A1	87434194	108	+	4504
NC_018723	A1	87434194	1456	+	4510
NC_018723	A1	87434194	212	+	4520
NC_018723	A1	87434194	541	+	DC1
NC_018723	A1	87434194	52	+	DC2
NC_018723	A1	87434194	752	+	DC4
NC_018723	A1	87434194	84	+	DC6
NC_018723	A1	87434194	58	+	x2654
NC_018723	A1	87434194	115	+	x2656
NC_018723	A1	87434194	782	+	x2657
NC_018723	A1	87661908	787	+	156
NC_018723	A1	87661908	497	+	178
NC_018723	A1	87661908	1604	+	260
NC_018723	A1	87661908	83	+	282
NC_018723	A1	87661908	968	+	4438
NC_018723	A1	87661908	877	+	4460
NC_018723	A1	87661908	356	+	DC1
NC_018723	A1	87661908	551	+	DC4
NC_018723	A1	87661908	64	+	DC6
NC_018723	A1	87661908	133	+	x2656
NC_018723	A1	91818785	161	+	4460
NC_018723	A1	91986082	979	-	156

NC_018723	A1	91986082	511	-	178
NC_018723	A1	91986082	904	-	260
NC_018723	A1	91986082	62	-	282
NC_018723	A1	91986082	124	-	377
NC_018723	A1	91986082	447	-	DC1
NC_018723	A1	91986082	654	-	DC4
NC_018723	A1	91986082	322	-	DC6
NC_018723	A1	91986082	203	-	x2654
NC_018723	A1	91986082	78	-	x2656
NC_018723	A1	91986082	376	-	x2657
NC_018723	A1	114054753	82	+	x2656
NC_018723	A1	125566171	635	+	DC2
NC_018723	A1	125666539	983	-	260
NC_018723	A1	125666539	72	-	377
NC_018723	A1	125666539	625	-	DC1
NC_018723	A1	128356700	76	+	x2656
NC_018723	A1	143377630	488	+	178
NC_018723	A1	144879177	156	-	4460
NC_018723	A1	208330102	74	+	377
NC_018723	A1	208330102	326	+	x2657
NC_018723	A1	213612022	1191	+	156
NC_018723	A1	213612022	100	+	377
NC_018723	A1	241024242	825	-	156
NC_018723	A1	241024242	506	-	178
NC_018723	A1	241024242	476	-	369
NC_018723	A1	241024242	89	-	4501
NC_018723	A1	241024242	73	-	x2656
NC_018724	A2	3504617	494	+	4460
NC_018724	A2	7197372	393	+	DC1
NC_018724	A2	7197372	116	+	x2654
NC_018724	A2	7199456	867	+	156
NC_018724	A2	7199456	894	+	178
NC_018724	A2	7199456	899	+	260
NC_018724	A2	7199456	1020	+	369
NC_018724	A2	7199456	176	+	377
NC_018724	A2	7199456	892	+	4438
NC_018724	A2	7199456	51	+	4474
NC_018724	A2	7199456	123	+	4501
NC_018724	A2	7199456	497	+	4510
NC_018724	A2	7199456	103	+	4520
NC_018724	A2	7199456	420	+	DC1

NC_018724	A2	7199456	608	+	DC2
NC_018724	A2	7199456	600	+	DC4
NC_018724	A2	7199456	601	+	DC6
NC_018724	A2	7199456	411	+	x2654
NC_018724	A2	7199456	85	+	x2656
NC_018724	A2	7199456	282	+	x2657
NC_018724	A2	48890477	58	+	156
NC_018724	A2	48890477	72	+	4474
NC_018724	A2	48890477	58	+	4510
NC_018724	A2	56416655	523	+	178
NC_018724	A2	56416655	766	+	4438
NC_018724	A2	56416655	884	+	4474
NC_018724	A2	56416655	76	+	4501
NC_018724	A2	70794561	58	-	156
NC_018724	A2	70794561	541	-	178
NC_018724	A2	70794561	1194	-	4438
NC_018724	A2	70794561	1183	-	4460
NC_018724	A2	70794561	1245	-	4474
NC_018724	A2	70794561	177	-	4504
NC_018724	A2	70794561	859	-	4510
NC_018724	A2	70794561	123	-	4520
NC_018724	A2	70794561	205	-	x2654
NC_018724	A2	70794561	124	-	x2656
NC_018724	A2	72785472	81	-	377
NC_018724	A2	84283614	52	-	4474
NC_018724	A2	123648711	523	+	DC1
NC_018724	A2	123648711	85	+	x2656
NC_018724	A2	123657108	376	-	156
NC_018724	A2	123657108	503	-	4438
NC_018724	A2	123657108	238	-	DC1
NC_018724	A2	123657108	332	-	DC2
NC_018724	A2	123764091	343	-	x2654
NC_018724	A2	123764091	128	-	x2656
NC_018724	A2	143119473	724	+	156
NC_018724	A2	143119473	451	+	178
NC_018724	A2	143119473	488	+	DC1
NC_018724	A2	143119473	112	+	x2654
NC_018724	A2	158856806	584	-	178
NC_018724	A2	158856806	574	-	DC4
NC_018724	A2	158856806	414	-	DC6
NC_018724	A2	7199456	82	+	282

NC_018725	A3	97445	324	-	178
NC_018725	A3	97445	78	-	377
NC_018725	A3	97445	1291	-	4438
NC_018725	A3	97445	565	-	4460
NC_018725	A3	97445	1401	-	4474
NC_018725	A3	97445	146	-	4501
NC_018725	A3	97445	75	-	4504
NC_018725	A3	97445	864	-	4510
NC_018725	A3	97445	122	-	4520
NC_018725	A3	97445	270	-	DC2
NC_018725	A3	97445	300	-	DC6
NC_018725	A3	97445	153	-	x2654
NC_018725	A3	97445	64	-	x2656
NC_018725	A3	97445	202	-	x2657
NC_018725	A3	3320238	1521	+	178
NC_018725	A3	3320238	68	+	260
NC_018725	A3	3320238	201	+	282
NC_018725	A3	3320238	211	+	377
NC_018725	A3	3320238	304	+	4501
NC_018725	A3	3320238	146	+	4504
NC_018725	A3	3320238	1698	+	4510
NC_018725	A3	3320238	238	+	4520
NC_018725	A3	3320238	1116	+	DC1
NC_018725	A3	3320238	995	+	DC2
NC_018725	A3	3320238	52	+	DC4
NC_018725	A3	3320238	863	+	DC6
NC_018725	A3	3320238	868	+	x2654
NC_018725	A3	3320238	329	+	x2656
NC_018725	A3	3320238	998	+	x2657
NC_018725	A3	53432908	1034	-	260
NC_018725	A3	54762799	52	-	4474
NC_018725	A3	77917350	843	-	156
NC_018725	A3	77917350	393	-	178
NC_018725	A3	77917350	752	-	260
NC_018725	A3	77917350	67	-	282
NC_018725	A3	77917350	491	-	369
NC_018725	A3	77917350	61	-	377
NC_018725	A3	77917350	1009	-	4474
NC_018725	A3	77917350	60	-	4504
NC_018725	A3	77917350	570	-	DC4
NC_018725	A3	77917350	392	-	DC6

NC_018725	A3	77917350	59	-	x2654
NC_018725	A3	77917350	299	-	x2657
NC_018725	A3	81636796	392	-	156
NC_018725	A3	81636796	474	-	4474
NC_018725	A3	81636796	61	-	x2654
NC_018725	A3	102855515	866	+	4460
NC_018725	A3	102855515	194	+	4501
NC_018725	A3	102855515	667	+	4510
NC_018725	A3	102855515	62	+	4520
NC_018725	A3	102855515	441	+	DC1
NC_018725	A3	102855515	88	+	x2656
NC_018725	A3	108613548	282	+	DC6
NC_018725	A3	142200006	598	-	156
NC_018725	A3	142200006	408	-	178
NC_018725	A3	142200006	289	-	DC6
NC_018725	A3	142200006	319	-	x2657
NC_018726	B1	7050694	747	+	156
NC_018726	B1	17135834	56	+	x2656
NC_018726	B1	38181490	290	+	x2657
NC_018726	B1	67270589	57	+	156
NC_018726	B1	67270589	654	+	369
NC_018726	B1	67270589	1244	+	4438
NC_018726	B1	67270589	1196	+	4460
NC_018726	B1	67270589	1061	+	4474
NC_018726	B1	67270589	120	+	4501
NC_018726	B1	67270589	815	+	4510
NC_018726	B1	95538792	295	+	DC6
NC_018726	B1	107463005	874	-	260
NC_018726	B1	136552326	180	-	156
NC_018726	B1	136552326	124	-	178
NC_018726	B1	136552326	105	-	369
NC_018726	B1	136552326	97	-	4438
NC_018726	B1	136552326	103	-	4460
NC_018726	B1	136552326	62	-	4510
NC_018726	B1	136552326	114	-	DC1
NC_018726	B1	136552326	104	-	DC2
NC_018726	B1	136552326	115	-	DC4
NC_018726	B1	136552326	80	-	DC6
NC_018726	B1	136552326	80	-	x2657
NC_018726	B1	156352163	166	+	156
NC_018726	B1	156352163	76	+	260

NC_018726	B1	156352163	146	+	4438
NC_018726	B1	156352163	58	+	4460
NC_018726	B1	156352163	153	+	4474
NC_018726	B1	156352163	74	+	4510
NC_018726	B1	156352163	76	+	DC4
NC_018726	B1	200852188	612	-	DC6
NC_018726	B1	201827018	91	+	156
NC_018726	B1	201827018	1800	+	4438
NC_018726	B1	201827018	1531	+	4460
NC_018726	B1	201827018	1699	+	4474
NC_018726	B1	201827018	99	+	4501
NC_018726	B1	201827018	68	+	4504
NC_018726	B1	201827018	1074	+	4510
NC_018726	B1	201827018	119	+	4520
NC_018726	B1	207201969	112	+	178
NC_018726	B1	207201969	109	+	4438
NC_018726	B1	207201969	124	+	4460
NC_018726	B1	207201969	144	+	4474
NC_018726	B1	207201969	127	+	4510
NC_018726	B1	207201969	135	+	DC2
NC_018727	B2	2325283	528	+	DC1
NC_018727	B2	2325283	638	+	DC4
NC_018727	B2	3487959	135	-	369
NC_018727	B2	3487959	92	-	DC1
NC_018727	B2	3487959	118	-	DC2
NC_018727	B2	3487959	240	-	DC4
NC_018727	B2	3487959	158	-	x2657
NC_018727	B2	14821672	791	+	156
NC_018727	B2	24664832	68	+	DC1
NC_018727	B2	32150838	67	+	156
NC_018727	B2	32150838	814	+	4438
NC_018727	B2	32150838	906	+	4460
NC_018727	B2	32150838	1888	+	4474
NC_018727	B2	32150838	94	+	4501
NC_018727	B2	32150838	109	+	4504
NC_018727	B2	32150838	588	+	4510
NC_018727	B2	32150838	190	+	4520
NC_018727	B2	32150838	389	+	DC6
NC_018727	B2	32150838	260	+	x2654
NC_018727	B2	32150838	160	+	x2656
NC_018727	B2	32150838	309	+	x2657

NC_018727	B2	140538177	104	-	4520
NC_018727	B2	140538177	721	-	DC4
NC_018727	B2	140538177	91	-	x2656
NC_018727	B2	140538177	709	-	x2657
NC_018727	B2	146684233	489	+	178
NC_018727	B2	146684233	809	+	4438
NC_018727	B2	146684233	1628	+	4460
NC_018727	B2	146684233	856	+	4474
NC_018727	B2	146684233	191	+	4501
NC_018727	B2	146684233	583	+	4510
NC_018727	B2	146684233	67	+	4520
NC_018727	B2	146684233	424	+	DC2
NC_018727	B2	146684233	546	+	DC4
NC_018727	B2	150861037	917	-	156
NC_018727	B2	150861037	479	-	178
NC_018727	B2	150861037	501	-	DC1
NC_018727	B2	150861037	536	-	DC2
NC_018728	B3	80634	140	+	156
NC_018728	B3	80634	56	+	369
NC_018728	B3	80634	174	+	4438
NC_018728	B3	80634	164	+	4474
NC_018728	B3	80634	78	+	DC4
NC_018728	B3	5040633	217	+	x2654
NC_018728	B3	25546595	875	+	260
NC_018728	B3	25546595	523	+	DC1
NC_018728	B3	25546595	366	+	DC2
NC_018728	B3	42564061	108	+	260
NC_018728	B3	52016984	497	-	369
NC_018728	B3	74694913	440	-	178
NC_018728	B3	74694913	392	-	369
NC_018728	B3	74694913	97	-	377
NC_018728	B3	74694913	657	-	4438
NC_018728	B3	74694913	819	-	4460
NC_018728	B3	74694913	83	-	4501
NC_018728	B3	74694913	500	-	4510
NC_018728	B3	74694913	88	-	4520
NC_018728	B3	74694913	519	-	DC4
NC_018728	B3	74694913	260	-	DC6
NC_018728	B3	74694913	157	-	x2656
NC_018728	B3	79470596	949	+	156
NC_018728	B3	79470596	1089	+	178

NC_018728	B3	79470596	981	+	260
NC_018728	B3	79470596	1164	+	4438
NC_018728	B3	79470596	1014	+	4460
NC_018728	B3	79470596	1092	+	4474
NC_018728	B3	79470596	131	+	4504
NC_018728	B3	79470596	700	+	4510
NC_018728	B3	79470596	194	+	4520
NC_018728	B3	79470596	57	+	DC6
NC_018728	B3	129403506	56	+	4474
NC_018728	B3	136800478	58	+	4438
NC_018728	B3	140527233	1068	-	DC4
NC_018728	B3	141210920	113	-	DC2
NC_018728	B3	141212382	348	-	DC2
NC_018728	B3	141212382	323	-	x2657
NC_018729	B4	1508837	682	-	156
NC_018729	B4	1508837	501	-	369
NC_018729	B4	1508837	716	-	4460
NC_018729	B4	1508837	464	-	DC1
NC_018729	B4	1508837	349	-	DC6
NC_018729	B4	12275158	844	+	260
NC_018729	B4	12275158	131	+	377
NC_018729	B4	24617796	765	+	156
NC_018729	B4	24617796	565	+	DC6
NC_018729	B4	24617796	159	+	x2654
NC_018729	B4	24617796	87	+	x2656
NC_018729	B4	29973213	781	+	156
NC_018729	B4	29973213	1536	+	260
NC_018729	B4	29973213	451	+	369
NC_018729	B4	29973213	66	+	x2654
NC_018729	B4	29973213	66	+	x2656
NC_018729	B4	29973213	381	+	x2657
NC_018729	B4	42584509	655	-	DC2
NC_018729	B4	42584509	289	-	DC6
NC_018729	B4	59253465	526	-	DC1
NC_018729	B4	64351846	259	-	156
NC_018729	B4	64351846	150	-	178
NC_018729	B4	64351846	234	-	260
NC_018729	B4	64351846	124	-	369
NC_018729	B4	64351846	194	-	4438
NC_018729	B4	64351846	274	-	4460
NC_018729	B4	64351846	291	-	4474

NC_018729	B4	64351846	202	-	4510
NC_018729	B4	64351846	126	-	DC1
NC_018729	B4	64351846	72	-	DC2
NC_018729	B4	64351846	156	-	DC4
NC_018729	B4	64351846	56	-	x2657
NC_018729	B4	64792601	630	+	156
NC_018729	B4	64792601	602	+	260
NC_018729	B4	64792601	81	+	4520
NC_018729	B4	82998470	507	-	DC4
NC_018729	B4	93341701	598	-	369
NC_018729	B4	93341701	436	-	DC1
NC_018729	B4	93341701	449	-	DC2
NC_018729	B4	93341701	483	-	x2657
NC_018730	C1	3060916	658	+	DC1
NC_018730	C1	6358251	425	+	DC6
NC_018730	C1	58764761	313	+	178
NC_018730	C1	58764761	743	+	260
NC_018730	C1	58764761	465	+	369
NC_018730	C1	58764761	104	+	377
NC_018730	C1	58764761	385	+	DC2
NC_018730	C1	58764761	136	+	x2654
NC_018730	C1	68452792	151	-	156
NC_018730	C1	68452792	94	-	4438
NC_018730	C1	68452792	124	?	4474
NC_018730	C1	68452792	110	-	4510
NC_018730	C1	188394950	490	+	DC1
NC_018730	C1	221278200	617	-	x2657
NC_018731	C2	51386	740	-	260
NC_018731	C2	51386	928	-	4438
NC_018731	C2	4816128	783	-	4438
NC_018731	C2	4816128	805	-	4460
NC_018731	C2	4816128	869	-	4474
NC_018731	C2	4816128	72	-	4501
NC_018731	C2	4816128	1051	-	4510
NC_018731	C2	4816128	131	-	4520
NC_018731	C2	82466254	928	-	4438
NC_018731	C2	82466254	992	-	4474
NC_018731	C2	83355811	273	-	DC2
NC_018731	C2	83355811	560	-	DC4
NC_018731	C2	96530076	458	-	DC1
NC_018731	C2	96530076	398	-	DC2

NC_018731	C2	131951596	58	-	156
NC_018731	C2	131951596	455	-	178
NC_018731	C2	131951596	58	-	282
NC_018731	C2	131951596	1064	-	369
NC_018731	C2	131951596	1026	-	4438
NC_018731	C2	131951596	1724	-	4460
NC_018731	C2	131951596	1012	-	4474
NC_018731	C2	131951596	93	-	4520
NC_018731	C2	131951596	618	-	DC4
NC_018731	C2	131951596	51	-	DC6
NC_018731	C2	135744903	88	-	156
NC_018731	C2	135744903	53	-	178
NC_018731	C2	135744903	52	-	369
NC_018731	C2	135744903	61	-	4438
NC_018731	C2	135744903	66	-	DC1
NC_018731	C2	160924756	2039	-	156
NC_018731	C2	160924756	673	-	178
NC_018731	C2	160924756	1957	-	260
NC_018731	C2	160924756	76	?	282
NC_018731	C2	160924756	624	-	369
NC_018731	C2	160924756	207	-	377
NC_018731	C2	160924756	2089	-	4438
NC_018731	C2	160924756	2169	-	4460
NC_018731	C2	160924756	2066	-	4474
NC_018731	C2	160924756	263	-	4501
NC_018731	C2	160924756	136	-	4504
NC_018731	C2	160924756	1493	-	4510
NC_018731	C2	160924756	224	-	4520
NC_018731	C2	160924756	1075	-	DC1
NC_018731	C2	160924756	888	-	DC2
NC_018731	C2	160924756	1363	-	DC4
NC_018731	C2	160924756	959	-	DC6
NC_018731	C2	160924756	229	-	x2654
NC_018731	C2	160924756	230	-	x2656
NC_018731	C2	160924756	633	-	x2657
NC_018731	C2	160925501	549	+	178
NC_018731	C2	160925501	619	+	369
NC_018732	D1	7411697	1010	+	260
NC_018732	D1	34510046	450	+	156
NC_018732	D1	34510046	228	+	178
NC_018732	D1	34510046	294	+	369

NC_018732	D1	34510046	189	+	4438
NC_018732	D1	34510046	239	+	4460
NC_018732	D1	34510046	319	+	DC1
NC_018732	D1	34510046	180	+	DC2
NC_018732	D1	34510046	351	+	DC4
NC_018732	D1	34510046	181	+	DC6
NC_018732	D1	34510046	106	+	x2654
NC_018732	D1	34510046	219	+	x2657
NC_018732	D1	42346635	335	-	DC2
NC_018732	D1	70433473	382	+	DC2
NC_018732	D1	94144838	90	+	156
NC_018732	D1	94144838	91	+	260
NC_018732	D1	94144838	61	-	369
NC_018732	D1	94144838	61	+	4438
NC_018732	D1	94144838	103	+	4460
NC_018732	D1	94144838	108	+	4474
NC_018732	D1	94144838	176	+	4510
NC_018732	D1	94144838	86	+	x2657
NC_018732	D1	108715902	87	-	x2656
NC_018732	D1	108968976	663	-	DC1
NC_018732	D1	117070340	70	-	156
NC_018732	D1	117070340	53	-	178
NC_018732	D1	117070340	926	-	4438
NC_018732	D1	117070340	1668	-	4460
NC_018732	D1	117070340	1916	-	4474
NC_018732	D1	117070340	198	-	4501
NC_018732	D1	117070340	102	-	4504
NC_018732	D1	117070340	1251	-	4510
NC_018732	D1	117070340	199	-	4520
NC_018732	D1	117070340	411	-	DC6
NC_018733	D2	1922130	1244	+	156
NC_018733	D2	1922130	821	+	178
NC_018733	D2	1922130	865	+	369
NC_018733	D2	1922130	228	+	x2656
NC_018733	D2	12595596	704	-	369
NC_018733	D2	12595596	1299	-	4460
NC_018733	D2	12595596	1267	-	4474
NC_018733	D2	12595596	69	-	x2656
NC_018733	D2	12595596	574	+	x2657
NC_018733	D2	32724795	644	-	369
NC_018733	D2	32724795	347	-	x2657

NC_018733	D2	40076847	516	-	DC4
NC_018733	D2	64430451	61	+	156
NC_018734	D3	18283759	92	+	4501
NC_018734	D3	26724054	289	-	4460
NC_018734	D3	71626706	164	-	4438
NC_018734	D3	71626706	56	-	DC6
NC_018734	D3	84470684	330	-	156
NC_018734	D3	84470684	176	+	260
NC_018734	D3	84470684	86	+	369
NC_018734	D3	84470684	844	+	4438
NC_018734	D3	84470684	156	+	4460
NC_018734	D3	84470684	427	+	4474
NC_018734	D3	84470684	254	+	4510
NC_018734	D3	84470684	168	+	DC4
NC_018735	D4	2891083	840	-	260
NC_018735	D4	10921105	251	+	178
NC_018735	D4	14464243	1426	+	260
NC_018735	D4	14464243	446	+	369
NC_018735	D4	14464243	152	+	377
NC_018735	D4	14464243	97	+	4501
NC_018735	D4	14464243	622	+	DC4
NC_018735	D4	14464243	160	+	x2654
NC_018735	D4	30619296	750	-	DC4
NC_018735	D4	75206367	405	+	DC6
NC_018735	D4	75720082	720	-	156
NC_018735	D4	75720082	477	-	369
NC_018735	D4	75720082	287	-	DC1
NC_018735	D4	75720082	415	-	DC4
NC_018735	D4	75720082	69	-	x2654
NC_018735	D4	76548648	848	-	156
NC_018735	D4	76548648	400	-	178
NC_018735	D4	76548648	563	-	369
NC_018735	D4	76548648	95	-	377
NC_018735	D4	76548648	835	-	4438
NC_018735	D4	76548648	1027	-	4474
NC_018735	D4	76548648	545	-	4510
NC_018735	D4	76548648	410	-	DC6
NC_018735	D4	90423962	137	-	DC1
NC_018735	D4	94404358	68	-	377
NC_018736	E1	7054245	581	+	369
NC_018736	E1	45368200	412	-	178

NC_018736	E1	45368200	401	-	369
NC_018736	E1	45368200	61	-	377
NC_018736	E1	45368200	227	-	DC6
NC_018736	E1	45368200	74	-	x2654
NC_018736	E1	45368200	151	-	x2656
NC_018737	E2	5650623	708	-	156
NC_018737	E2	5650623	436	-	x2654
NC_018737	E2	5650623	86	-	x2656
NC_018737	E2	23620399	898	+	156
NC_018737	E2	23620399	571	+	178
NC_018737	E2	23620399	547	+	369
NC_018737	E2	23620399	123	+	377
NC_018737	E2	23620399	453	+	DC2
NC_018737	E2	23620399	115	+	x2654
NC_018737	E2	23620399	791	+	x2657
NC_018737	E2	29101585	53	+	156
NC_018737	E2	29101585	472	+	369
NC_018737	E2	29101585	876	+	4438
NC_018737	E2	29101585	1888	+	4460
NC_018737	E2	29101585	948	+	4474
NC_018737	E2	29101585	182	+	4501
NC_018737	E2	29101585	100	+	4504
NC_018737	E2	29101585	611	+	4510
NC_018737	E2	29101585	79	+	4520
NC_018737	E2	29101585	191	+	x2654
NC_018737	E2	31833385	680	-	DC4
NC_018737	E2	51149544	720	+	x2657
NC_018737	E2	51190684	705	+	x2657
NC_018738	E3	144548	61	+	156
NC_018738	E3	144548	51	+	4460
NC_018738	E3	144548	57	+	4474
NC_018738	E3	20648884	480	-	DC4
NC_018739	F1	18740066	827	+	156
NC_018739	F1	18740066	1070	+	178
NC_018739	F1	18740066	2017	+	260
NC_018739	F1	18740066	520	+	369
NC_018739	F1	18740066	139	+	377
NC_018739	F1	18740066	575	+	DC4
NC_018739	F1	18740066	237	+	x2654
NC_018739	F1	25497226	280	+	DC2
NC_018739	F1	34758843	501	+	DC1

NC_018739	F1	44637013	771	-	156
NC_018739	F1	44637013	543	-	178
NC_018739	F1	44637013	891	-	260
NC_018739	F1	44637013	69	-	282
NC_018739	F1	44637013	1005	-	369
NC_018739	F1	44637013	175	-	377
NC_018739	F1	44637013	362	-	DC2
NC_018739	F1	44637013	623	-	DC4
NC_018739	F1	44637013	132	-	x2656
NC_018739	F1	44637013	335	-	x2657
NC_018740	F2	30387404	82	+	4438
NC_018740	F2	85487944	70	-	156
NC_018740	F2	85487944	98	-	4438
NC_018740	F2	85487944	61	-	4460
NC_018740	F2	85487944	110	-	4474
NC_018740	F2	85487944	68	-	4510
NC_018740	F2	85487944	64	-	DC1
NC_018740	F2	85487944	76	-	DC6
NC_018741	X	75504	318	+	369
NC_018741	X	75504	295	+	4460
NC_018741	X	75504	172	+	4510
NC_018741	X	75504	115	+	DC2
NC_018741	X	75504	222	+	DC4
NC_018741	X	1254459	193	+	369
NC_018741	X	1254459	117	+	4460
NC_018741	X	1254459	57	+	4510
NC_018741	X	1254459	54	+	DC2
NC_018741	X	1254459	106	+	DC4
NC_018741	X	8831199	81	+	156
NC_018741	X	8831199	69	+	4460
NC_018741	X	8831199	54	+	4474
NC_018741	X	8831199	95	+	4510
NC_018741	X	45785700	116	-	156
NC_018741	X	45785700	56	-	178
NC_018741	X	45785700	72	-	4460
NC_018741	X	45785700	78	-	DC1
NC_018741	X	45785700	60	-	DC2
NC_018741	X	45785700	148	-	DC4
NC_018741	X	45785700	59	-	DC6
NC_018741	X	52608326	50		4460
NC_018741	X	52608326	52	?	4474

NC_018741	X	95727165	634	+	260
NC_018741	X	95727165	58	+	x2656
NC_018741	X	98027273	94	+	156
NC_018741	X	98027273	62	+	260
NC_018741	X	98027273	114	+	4438
NC_018741	X	98027273	94	+	4474
NC_018741	X	98027273	56	+	4510
NC_018741	X	122403865	186	-	156
NC_018741	X	122403865	130		178
NC_018741	X	122403865	228	-	260
NC_018741	X	122403865	168	-	4438
NC_018741	X	122403865	176	-	4460
NC_018741	X	122403865	211	-	4474
NC_018741	X	122403865	162	-	4510
NC_018741	X	122403865	64	-	DC4
NC_018741	X	126845602	1849	-	4460
NC_018741	X	126845602	957	-	4474
NC_018741	X	126845602	91	-	4501

Table 3. 2,463 protein-coding genes are associated with all 146 integration sites. Integration sites by

Accession No.	Chromosome	Integration site	Number of cats	Genes within 1MB
NC_018723	A1	25175154	1	COG3
NC_018723	A1	25175154	1	CPB2
NC_018723	A1	25175154	1	ERICH6B
NC_018723	A1	25175154	1	ESD
NC_018723	A1	25175154	1	GPALPP1
NC_018723	A1	25175154	1	GTF2F2
NC_018723	A1	25175154	1	HTR2A
NC_018723	A1	25175154	1	KCTD4
NC_018723	A1	25175154	1	LCP1
NC_018723	A1	25175154	1	LRCH1
NC_018723	A1	25175154	1	LRRC63
NC_018723	A1	25175154	1	NUFIP1
NC_018723	A1	25175154	1	RUBCNL
NC_018723	A1	25175154	1	SIAH3
NC_018723	A1	25175154	1	SLC25A30
NC_018723	A1	25175154	1	SPERT
NC_018723	A1	25175154	1	TPT1
NC_018723	A1	25175154	1	TRNAE-UUC
NC_018723	A1	25175154	1	ZC3H13
NC_018723	A1	37559850	2	TDRD3
NC_018723	A1	75673842	2	NONE
NC_018723	A1	84436185	1	FELCATV1R-PS78
NC_018723	A1	84436185	1	FELCATV1RPS61
NC_018723	A1	84436185	1	LYPD8
NC_018723	A1	84436185	1	PGBD2
NC_018723	A1	84436185	1	SH3BP5L
NC_018723	A1	84436185	1	TRNAE-CUC
NC_018723	A1	84436185	1	TRNAL-CAA
NC_018723	A1	84436185	1	ZNF672
NC_018723	A1	87434194	20	MTUS2
NC_018723	A1	87661908	10	MTUS2
NC_018723	A1	91818785	1	BTNL9
NC_018723	A1	91818785	1	CANX
NC_018723	A1	91818785	1	CBY3
NC_018723	A1	91818785	1	CNOT6
NC_018723	A1	91818785	1	FELCATV1R-PS12
NC_018723	A1	91818785	1	FELCATV1R2
NC_018723	A1	91818785	1	FELVCATV1R-PS58
NC_018723	A1	91818785	1	FLT4

NC_018723	A1	91818785	1	GFPT2
NC_018723	A1	91818785	1	LTC4S
NC_018723	A1	91818785	1	MAML1
NC_018723	A1	91818785	1	MAPK9
NC_018723	A1	91818785	1	MGAT1
NC_018723	A1	91818785	1	MGAT4B
NC_018723	A1	91818785	1	MRNIP
NC_018723	A1	91818785	1	RACK1
NC_018723	A1	91818785	1	RASGEF1C
NC_018723	A1	91818785	1	RNF130
NC_018723	A1	91818785	1	SCGB3A1
NC_018723	A1	91818785	1	SQSTM1
NC_018723	A1	91818785	1	TBC1D9B
NC_018723	A1	91818785	1	TRANAV-CAC
NC_018723	A1	91818785	1	TRIM41
NC_018723	A1	91818785	1	TRIM52
NC_018723	A1	91818785	1	TRIM7
NC_018723	A1	91818785	1	TRNAA-UGC
NC_018723	A1	91818785	1	TRNAK-CUU
NC_018723	A1	91818785	1	TRNAK-CUU
NC_018723	A1	91818785	1	TRNAP-UGG
NC_018723	A1	91818785	1	TRNAT-UGU
NC_018723	A1	91818785	1	TRNAV-AAC
NC_018723	A1	91818785	1	ZFP62
NC_018723	A1	91986082	12	ubiquitin like 3
NC_018723	A1	114054753	1	CA1H5orf15
NC_018723	A1	114054753	1	CA1H5orf24
NC_018723	A1	114054753	1	CAMLG
NC_018723	A1	114054753	1	CATSPER3
NC_018723	A1	114054753	1	CDKL3
NC_018723	A1	114054753	1	CDKN2AIPNL
NC_018723	A1	114054753	1	CXCL14
NC_018723	A1	114054753	1	DDX46
NC_018723	A1	114054753	1	FBXL21
NC_018723	A1	114054753	1	IL9
NC_018723	A1	114054753	1	JADE2
NC_018723	A1	114054753	1	LECT2
NC_018723	A1	114054753	1	NEUROG1
NC_018723	A1	114054753	1	PCBD2
NC_018723	A1	114054753	1	PITX1
NC_018723	A1	114054753	1	PPP2CA

NC_018723	A1	114054753	1	SAR1B
NC_018723	A1	114054753	1	SEC24A
NC_018723	A1	114054753	1	SKP1
NC_018723	A1	114054753	1	SLC25A48
NC_018723	A1	114054753	1	SMAD5
NC_018723	A1	114054753	1	TCF7
NC_018723	A1	114054753	1	TGFBI
NC_018723	A1	114054753	1	TIFAB
NC_018723	A1	114054753	1	TXNDC15
NC_018723	A1	114054753	1	UBE2B
NC_018723	A1	114054753	1	VDAC1
NC_018723	A1	125566171	1	CA1H5orf46
NC_018723	A1	125566171	1	CCNO
NC_018723	A1	125566171	1	CDC20B
NC_018723	A1	125566171	1	DDX4
NC_018723	A1	125566171	1	DHX29
NC_018723	A1	125566171	1	DPYSL3
NC_018723	A1	125566171	1	GPX8
NC_018723	A1	125566171	1	GZMA
NC_018723	A1	125566171	1	IL31RA
NC_018723	A1	125566171	1	JAKMIP2
NC_018723	A1	125566171	1	MARCOL
NC_018723	A1	125566171	1	MCIDAS
NC_018723	A1	125566171	1	MTREX
NC_018723	A1	125566171	1	PLPP1
NC_018723	A1	125566171	1	PPP2R2B
NC_018723	A1	125566171	1	SCGB3A2
NC_018723	A1	125566171	1	SLC38A9
NC_018723	A1	125566171	1	SPINK1
NC_018723	A1	125566171	1	SPINK14
NC_018723	A1	125566171	1	SPINK5
NC_018723	A1	125566171	1	SPINK6
NC_018723	A1	125566171	1	STK32A
NC_018723	A1	125666539	3	CA1H5orf46
NC_018723	A1	125666539	3	CCNO
NC_018723	A1	125666539	3	CDC20B
NC_018723	A1	125666539	3	DDX4
NC_018723	A1	125666539	3	DHX29
NC_018723	A1	125666539	3	DPYSL3
NC_018723	A1	125666539	3	GPX8
NC_018723	A1	125666539	3	GZMA

NC_018723	A1	125666539	3	IL31RA
NC_018723	A1	125666539	3	IL6ST
NC_018723	A1	125666539	3	JAKMIP2
NC_018723	A1	125666539	3	MARCOL
NC_018723	A1	125666539	3	MCIDAS
NC_018723	A1	125666539	3	MTREX
NC_018723	A1	125666539	3	PLPP1
NC_018723	A1	125666539	3	PPP2R2B
NC_018723	A1	125666539	3	SCGB3A2
NC_018723	A1	125666539	3	SLC38A9
NC_018723	A1	125666539	3	SPINK1
NC_018723	A1	125666539	3	SPINK14
NC_018723	A1	125666539	3	SPINK5
NC_018723	A1	125666539	3	SPINK6
NC_018723	A1	125666539	3	STK32A
NC_018723	A1	128356700	1	ACTBL2
NC_018723	A1	128356700	1	GAPT
NC_018723	A1	128356700	1	GPBP1
NC_018723	A1	128356700	1	MIER3
NC_018723	A1	128356700	1	PLK2
NC_018723	A1	128356700	1	RAB3C
NC_018723	A1	128356700	1	SETD9
NC_018723	A1	143377630	1	AGGF1
NC_018723	A1	143377630	1	CRHBP
NC_018723	A1	143377630	1	F2R
NC_018723	A1	143377630	1	F2RL1
NC_018723	A1	143377630	1	F2RL2
NC_018723	A1	143377630	1	IQGAP2
NC_018723	A1	143377630	1	OTP
NC_018723	A1	143377630	1	PDE8B
NC_018723	A1	143377630	1	POC5
NC_018723	A1	143377630	1	S100Z
NC_018723	A1	143377630	1	SV2C
NC_018723	A1	143377630	1	TBCA
NC_018723	A1	143377630	1	WDR41
NC_018723	A1	143377630	1	ZBED3
NC_018723	A1	144879177	1	AP3B1
NC_018723	A1	144879177	1	ARSB
NC_018723	A1	144879177	1	BHMT2
NC_018723	A1	144879177	1	BHMTJMY
NC_018723	A1	144879177	1	DMGDH

NC_018723	A1	144879177	1	HOMER1
NC_018723	A1	144879177	1	LHFPL2
NC_018723	A1	144879177	1	OTP
NC_018723	A1	144879177	1	SCAMP1
NC_018723	A1	144879177	1	TBCA
NC_018723	A1	144879177	1	WDR41
NC_018723	A1	208330102	2	C6
NC_018723	A1	208330102	2	C7
NC_018723	A1	208330102	2	CA1H5orf51
NC_018723	A1	208330102	2	CARD6
NC_018723	A1	208330102	2	CCDC152
NC_018723	A1	208330102	2	FBXO4
NC_018723	A1	208330102	2	GHR
NC_018723	A1	208330102	2	MROH2B
NC_018723	A1	208330102	2	OXCT1
NC_018723	A1	208330102	2	PLCXD3
NC_018723	A1	208330102	2	PRKAA1
NC_018723	A1	208330102	2	RPL37
NC_018723	A1	208330102	2	TTC33
NC_018723	A1	213612022	2	CAPSL
NC_018723	A1	213612022	2	IL7R
NC_018723	A1	213612022	2	LMBRD2
NC_018723	A1	213612022	2	NADK2
NC_018723	A1	213612022	2	SKP2
NC_018723	A1	213612022	2	SPEF2
NC_018723	A1	241024242	5	AHRR
NC_018723	A1	241024242	5	BRD9
NC_018723	A1	241024242	5	CA1H5orf38
NC_018723	A1	241024242	5	CCDC127
NC_018723	A1	241024242	5	CEP72
NC_018723	A1	241024242	5	EXOC3
NC_018723	A1	241024242	5	IRX2
NC_018723	A1	241024242	5	IRX4
NC_018723	A1	241024242	5	LPCAT1
NC_018723	A1	241024242	5	LRRC14B
NC_018723	A1	241024242	5	MRPL36
NC_018723	A1	241024242	5	NDUFS6
NC_018723	A1	241024242	5	NKD2
NC_018723	A1	241024242	5	PDCD6
NC_018723	A1	241024242	5	SDHA
NC_018723	A1	241024242	5	SLC12A7

NC_018723	A1	241024242	5	SLC6A3
NC_018723	A1	241024242	5	SLC9A3
NC_018723	A1	241024242	5	TERT
NC_018723	A1	241024242	5	TPPP
NC_018723	A1	241024242	5	TRIP13
NC_018724	A2	3504617	1	AARDC5
NC_018724	A2	3504617	1	ANKRD24
NC_018724	A2	3504617	1	APBA3
NC_018724	A2	3504617	1	ATCAY
NC_018724	A2	3504617	1	CA2H19orf71
NC_018724	A2	3504617	1	CACTIN
NC_018724	A2	3504617	1	CCDC94
NC_018724	A2	3504617	1	CELF5
NC_018724	A2	3504617	1	CHAF1A
NC_018724	A2	3504617	1	CREB3L3
NC_018724	A2	3504617	1	DAPK3
NC_018724	A2	3504617	1	DOHH
NC_018724	A2	3504617	1	DPP9
NC_018724	A2	3504617	1	EBI3
NC_018724	A2	3504617	1	EEF2
NC_018724	A2	3504617	1	FEM1A
NC_018724	A2	3504617	1	FSD1
NC_018724	A2	3504617	1	FZR1
NC_018724	A2	3504617	1	GIPC3
NC_018724	A2	3504617	1	HDGFL2
NC_018724	A2	3504617	1	HMG20B
NC_018724	A2	3504617	1	LRG1
NC_018724	A2	3504617	1	MAP2K2
NC_018724	A2	3504617	1	MATK
NC_018724	A2	3504617	1	MFSD12
NC_018724	A2	3504617	1	MPND
NC_018724	A2	3504617	1	MRPL54
NC_018724	A2	3504617	1	MYDGF
NC_018724	A2	3504617	1	NCLN
NC_018724	A2	3504617	1	NFIC
NC_018724	A2	3504617	1	PIAS4
NC_018724	A2	3504617	1	PIN5
NC_018724	A2	3504617	1	PIP5K1C
NC_018724	A2	3504617	1	PLIN3
NC_018724	A2	3504617	1	PLIN4
NC_018724	A2	3504617	1	PTPRS

NC_018724	A2	3504617	1	RAX2
NC_018724	A2	3504617	1	S1PR4
NC_018724	A2	3504617	1	SEMA6B
NC_018724	A2	3504617	1	SH3GL1
NC_018724	A2	3504617	1	SHD
NC_018724	A2	3504617	1	SIRT6
NC_018724	A2	3504617	1	STAP2
NC_018724	A2	3504617	1	TBXA2R
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CONCLUSION

FeLV can no longer be classified as just a domestic cat pathogen, as evidenced by infections and outbreaks in a variety of wild felid species. The horizontal transmission of a recombinant oncogenic variant FeLV-B during the contemporary outbreak described in Chapter Three demonstrates Florida panthers are susceptible to the more pathogenic form of FeLV, which is considered not to be horizontally transmissible. Chapter Four demonstrated that puma cells infected with FeLV produce the virus at greater levels than domestic cat cells further suggesting greater vulnerability of wild pumas than domestic cats to FeLV infections on a cellular level. This could, in part, be a result of the lack of enFeLV in pumas as our data show that enFeLV reduces exFeLV infection in domestic cats. This is evidenced by the direct negative correlation between enFeLV-LTR copy number and FeLV infection and replication, indicating restriction against FeLV infection mitigated by enFeLV-LTR. Chapter Five revealed that domestic cat PBMCs, which were less susceptible to in vitro infection than fibroblasts, transcribe enFeLV more than other tissue types. Furthermore, we identified specific sequences produced by enFeLV-LTR that may act as silencing RNAs that could down-regulate FeLV replication. Finally, Chapter Six identified regions of enFeLV-LTR integration that could be interrogated in future studies to determine the capacity that LTRs modulate host gene expression. The increased susceptibility of pumas and potentially other endangered felids that lack these endogenous elements to FeLV is problematic in a time when increasing urbanization is increasing contact between wild and domesticated felids. Conservation action plans for wild and large felids should consider the possibility of disease transmission from domestic cats. Importantly, beyond FeLV, we have added important knowledge about the function and distribution of enFeLV in domestic cats, with important inferences about ways that endogenous and exogenous retroviruses interact. This work provides several avenues for additional investigation in ERV-XRV interactions.