Exploring the role of Human Endogenous Retroviral Gag in the formation and content of Extracellular Vesicles

Danielle McCulloch

Thesis submitted to the University of Ottawa in partial Fulfillment of the requirements for the M.Sc. program in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

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Abstract

Human Endogenous Retroviruses (HERV) are derived from exogenous retroviruses that infected inheritable germline tissues millions of years ago and account for 8% of the human genome. Like other retroviruses HERVs encode Gag, Pol and sometimes Env proteins. During a retroviral infection, retroviral Gag recruits the hosts Endosomal Sorting Complex Required for Transport (ESCRT) and associated proteins (ALIX and TSG101) to produce precisely sized viruses from endosomes or the plasma membrane. The ESCRT machinery is also involved in cytokinesis and control growth factor receptor signalling. HERV-K is the most recent HERV family to insert into the genome and is still able to produce mostly intact transcripts, including Gag. When expressed, Gag causes cells to release Virus-Like Particles (VLP) that lack HERV genomes. These retroviral VLP are remarkably similar to a sub-category of extracellular vesicles (EVs) called exosomes. Exosomes require ALIX, TSG101 and the ESCRT machinery for their production. It is possible that HERV-K Gag is required for exosome production or that HERV VLPs are a major contaminant of exosome preparations that account for many of the functions attributed to exosomes. Our data shows that HERV-K Gag over-expression or knockdown did not change the number of EVs released per cell in two cell lines. As well there was no difference in the amount of ALIX and TSG101 in the EVs in these conditions. The most intriguing observation made was the increase of cell number with expression of HERV-K Gag and decrease when HERV-K Gag was knocked down in HEK293T. We are currently unable to conclude the role of HERV-K Gag on EV production and content. We speculate that HERV-K Gag might affect cells through controlling cell proliferation or death, for example by competing with ESCRT machinery to impact signalling through growth factor receptors. This study begins to outline the potential effects HERV-K Gag might have on EV release and cell proliferation.
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List of Abbreviations

ALIX: aka. Programmed Cell Death 6 Interacting Protein (PCD6IP)
ALS: Amyotrophic Lateral Sclerosis
Arc: Activity Regulated Cytoskeleton Associated Protein
ATCC: American Type Culture Collection
bp: base pairs
CA: Capsid
cDNA: complementary DNA
CL: Cell Lysate
CMV: Cytomegalovirus promoter
Ctrl: Control
DMEM: Dulbecco’s Modified Eagle Medium
DNA: Deoxyribonucleic acid
dsDNA: double stranded DNA
EGFR: Epidermal Growth Factor Receptor
Env: Envelope
ERK: Extracellular signal-regulated kinases
ERV: Endogenous Retrovirus
ESCRT: Endosomal Sorting Complex Required for Transport
EtBr: Ethidium bromide
EV: Extracellular Vesicles
FBS: Fetal bovine serum
Fwd: Forward
Gag: Group-specific antigen
GFP: Green fluorescent protein
GRB2: Growth factor receptor-bound protein 2
GRCh38: Genome Research Consortium human build 38
HDR: Homology directed repair
HEK293T: Human embryonic kidney cells T antigen of SV40
HeLa: Henrietta Lacks
HERV: Human Endogenous Retrovirus
HIV-1: Human immunodeficiency virus 1
HML: Human MMTV-like
ILV: Intraluminal vesicles
IN: Integrase
Kb: kilobase
kDa: Kilodalton
LD: Ladder
L-domains: Late binding domains
LTR: Long terminal repeats
MA: Matrix
MCF-7: Michigan Cancer Foundation-7
MEF: Mouse embryonic fibroblast
MEK: Mitogen-activated protein kinase kinase
MHC-II: major histocompatibility complex class II
MLV: Murine leukemia virus
MMTV: Mouse mammary tumor virus
mRNA: Messenger RNA
MSC GMP: Mesenchymal stem cells collected under Good Manufacturing Production
MSC TERT: Mesenchymal stem cells transformed with Telomerase reverse transcriptase
MVB: Multivesicular bodies
N2a: Neuro2a
NC: nucleocapsid
Nedd4: Neuronal precursor cell Expressed Developmentally Downregulated 4
NHEJ: Non-homologous end joining
NTA: Nanoparticle tracking analysis
ORF: Open-reading frame
PAM: Protospacer Adjacent Motif
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
Pol: Polymerase
PRM: Proline rich motifs
Raf: Rapidly Accelerated Fibrosarcoma
Rev: Reverse
RNA: Ribonucleic acid
RNA-seq: RNA sequencing
RT: Reverse transcriptase
sgRNA: Single guide RNA
shRNA: Short hairpin RNA
siRNA: Small interfering RNA
SOS: Son of sevenless
tRNA: transfer RNA
TSG101: Tumour susceptibility gene 101
VLP: Viral-like particles
WT: Wild-type
Acknowledgments

First, I want to thank Dr. Derrick Gibbings for taking me on in his lab and giving me a chance to tackle a new project idea. It has been a challenging process to say the least, but extremely rewarding to see our ideas come to fruition. As well, I would like to thank my co-supervisor Dr. William Stanford and thesis advisory committee Dr. Marc-André Langlois and Dr. Tommy Alain for their guidance and help along the way. I would especially like to thank Dr. Langlois for providing the MLV and HIV plasmids used in this thesis.

Next, I would like to thank everyone in the Gibbings lab for being such an amazing and supportive group that always makes time to help with trouble shooting. Everyone in the lab has helped me in some way or another during this project. As well, you have all been great for insightful conversations and lunchtime debates. Thank you to Tanya Foley and members of the Jasmin and Russell lab for your friendship and fresh perspectives on ideas and life.

I would like to especially like to thank Maneka Chitiprolu, Alyssa Pastic, and Olanta Negeri. You girls were with me from the beginning and I have learned so much from each of you about science and about life. You are amazing friends and the memories of laughter we have shared together will never be forgotten. You have kept me sane these last two years and I will be forever grateful for our friendship.

I would like to thank my mom Eleonora and my father Danny. You have both been supportive of me and my education from the beginning. You have always seen the potential in me, even if I could not. Thank you for never letting me give up on the difficult things in life and helping me achieve my dreams. Lastly, I would like to thank my love Geoffrey. You have endless patience and understanding for my crazy work hours. You have been supportive throughout every aspect of my research and your willingness to listen means the world. Without you my life would be a little less bright and fun, so thank you.
Introduction

Endogenous Retroviruses (ERVs) Integration into the Genome

Transposable elements (TEs) are mobile genetic elements capable of moving within genomes (Friedli & Trono, 2015). Two classes of TE are DNA transposons and retrotransposons (Pace & Feschotte, 2007). DNA transposons use a cut-and-paste method for moving from one genomic location to another, but they have not been mobile for millions of years (Pace & Feschotte, 2007). Retrotransposons replicate via an RNA intermediate and insert into a new location in the genome (Craig et al. 2002). Retrotransposons can be broken down further by the presence or absence of long terminal repeats (LTRs) (Cordaux & Batzer, 2009; Bannert & Kurth, 2006). Non-LTR retrotransposons, include elements such as LINE-1 (long interspersed element 1), Alu, and SVA elements all of which are currently active (Hancks & Kazazian, 2016; Deininger & Batzer, 1999). These non-LTR retrotransposons have been shown to cause genetic disorders by inserting into new genomic locations and disrupting gene expression (Hancks & Kazazian, 2016; Deininger & Batzer, 1999). LTR retrotransposons are another class of retrotransposons with a subclass being endogenous retrovirus (ERVs) (Cordaux & Batzer, 2009; Bannert & Kurth, 2006; Friedli & Trono, 2015).

Endogenous retroviruses (ERVs) are remnants of ancient infections from exogenous retroviruses that infected the inheritable germline tissue (Young et al., 2013). The retrovirus will infect the host cell and reverse transcribe its viral single-stranded RNA (ssRNA) to a double-stranded DNA (dsDNA). This dsDNA will integrate into the host genome as a provirus and if this occurs in germline cells, the retroviral DNA can be transmitted vertically from parent to offspring (Agoni et al., 2013 & Barbulescu et al., 1999; Young et al., 2012; Stoye, 2012). After the first integration, it is suggested that some ERV elements were able to amplify, thus
increasing their copy number (Dewannieux & Heidmann, 2013; Gifford & Tristem, 2003). One way this could be done was through newly integrated ERVs producing viral particles that could infect other germline cells (Dewannieux & Heidmann, 2013). In the host cells, there are tremendous negative selection on ERVs (Gifford & Tristem, 2003). This causes amplification to decline due to methylation and mutations of ERV inserted during host replication (Gifford & Tristem, 2003). As ERVs acquire mutations another way they could amplify is by intracellular retrotransposition where viral RNA is made and then reverse transcribed and inserted back into the genome at a different location (Dewannieux & Heidmann, 2013). The frequency of an ERV in a population will depend on the host selection pressures (Gifford & Tristem, 2003). ERVs will either be lost in the population or become fixed depending on a variety of factors including; position of ERV insertion and changes in host population size (Gifford & Tristem, 2003). Eventually over time ERVs will no longer be able to amplify becoming inactivated and fixed in the genome, where they will decay over millions of years to junk DNA (Dewannieux & Heidmann, 2013; Gifford & Tristem, 2003). Most ERVs are highly mutated beyond the point of being functional through inactivation, mutation and truncation (Johnson, 2015). In most cases if an ERV is active it is a small proportion of the total ERVs present in the genome but this can vary between species and cell type (Johnson, 2015; Bhardwaj et al., 2015).

**Human Endogenous Retrovirus Family K (HERV-K) is the Earliest HERV Integration**

ERVs were initially discovered in the late 1960s by combining immunology and virology methods with Mendelian genetics (Weiss, 2006). At this time three ERVs were discovered almost simultaneously; avian leucosis virus (ALV), murine leukemia virus (MLV) and mouse mammary tumor virus (MMTV). Since then, ERVs have been discovered in a variety of
vertebrate animals including; pigs, sheep and humans (Yang et al., 2015; Dewannieux & Heidmann, 2013; Garcia-Etxebarria et al., 2014; Weiss, 2006; Tristem, 2000). In humans, these HERVs (Human ERVs) account for ~8% of the total genome (Shin et al., 2013; Belshaw et al., 2003; Barbulescu et al., 1999).

HERVs are derived from a relatively small number of endogenization events that occurred in the germline at different time points in human evolution (Bannert & Kurth, 2006; Gifford & Tristem, 2003). A family of HERVs, HERV-K, is thought to have its first endogenization event dating back 55 million years ago with ERV-K (HML-5), while the most recent known integration of this family, the HERV-K (HML-2) element, occurred approximately 750-250 thousand years ago (Figure 1; Hanke et al., 2016; Dewannieux & Heidmann, 2013). From the beginning, there were different methods to categorize HERVs (Escalera-Zamudio & Greenwood, 2016). Some researchers based categorization on the tRNA used at the primer binding site, which is a region on the viral RNA where host tRNA would bind to prime reverse transcription (i.e. HERV-K uses lysine tRNA while HERV-W uses tryptophan at the primer binding site) (Ono et al., 1986; Shin et al., 2013; Hanke et al., 2013; Tristem, 2000). Others categorized HERVs based on sequences and their similarities to exogenous retroviruses (Escalera-Zamudio & Greenwood, 2016). For example, HERV-K (HML-2) is categorized as a betaretrovirus-like retrovirus that is related to exogenous mouse mammary tumor virus (MMTV), hence these HERVs were called HML (‘Human MMTV-like’) (Ono et al., 1986; Monde et al., 2012; Subramnian et al., 2011; Broecker et al., 2016). Until there is a set nomenclature for HERVs an overlap will remain in identification methods. For this thesis, the family studied was HERV-K because it inserted more recently into the human genome and is likely the most active (Hanke et al., 2016).
HERVs are Heavily Regulated by Host Machinery

Uncontrolled replication and insertion of HERVs could be detrimental to the host genome (Hanke et al., 2016). There have been full-length HERVs documented, but none that remain infectious (Boller et al., 2008; Hanke et al., 2016; Subramnian et al., 2011). The majority of HERVs are functionally inactivated by regulation of their expression (Bhardwaj et al., 2015). This includes switching off promoters by methylation of DNA or histones or other modifications to the chromatin structure (Lavie et al. 2004; Rowe & Trono, 2011). Molecules such are Trim28 (also known as Kap1) have been shown to decrease the transcription of HERVs in human neural progenitor cells as well as ERVs in embryonic stem cells (Grow et al., 2015; Brattas et al., 2017). Regulation also occurs during host DNA replication by introducing mutations, insertions or deletions in coding regions; regulating transcription and protein expression (Zhao et al., 2011;
Young et al., 2012; Subramnian et al., 2011). An extreme form of control used by the host to inactivate ERVs is by internal homologous recombination deleting the entire proviral insertion except for the LTRs (Bannert & Kurth, 2006; Stoye, 2012).

**HERV-K Transcribes Group-specific antigen (Gag)**

When HERVs initially integrate into the genome as a provirus they contain genes for; group-specific antigen (*gag*), protease (*pro* or *prt*), polymerase (*pol*), and envelope (*env*) that play an essential role in the maturation and release of a fully functional retrovirus (George et al., 2011; Lavie et al., 2004; Kraus et al., 2011). The provirus is flanked by two long-terminal repeats (LTRs) which contain promoter and enhancer sequences for transcription (Contreras-Galindo et al., 2008; Slokar & Hasler, 2016). Besides the essential genes to produce functional retroviral proteins, HERV proviruses also contain accessory proteins such as Rec and Np9. Rec acts as a nuclear export factor, but many properties and functions of these accessory proteins are still not well understood (Schmitt et al., 2015; Buscher et al., 2005). There are three main open reading frames (ORFs) for full-length HERV proviruses and because of this the main genes overlap at a “slippery sequence”, a common betaretrovirus-like feature of genome organization (Dewannieux & Heidmann, 2013; Grandi et al., 2017; Broecker et al., 2016; Contreras-Galindo et al., 2008; Ono et al., 1986; George et al., 2011). These “slippery sequences” cause the ribosomal machinery to skip the stop codon of one gene and to continue reading and transcribing the next gene in a process known as ribosomal frameshifting (George et al., 2011; Hohn et al., 2013). This allows HERVs to produce *gag-pro* and *gag-pro-pol* transcripts that can be cleaved by viral Protease in order to form the main structural proteins (George et al., 2011; Hohn et al., 2013; Henzy et al., 2013; Aktar et al., 2014).
Group-specific antigen (Gag), encoded by *gag*, forms the structural core proteins of the virus and will recruit host proteins to aid in the budding and release of the viral particle. Protease (Pro), encoded by *pro*, is involved in the cleavage of Gag and maturation of the virion after release from the host cell (Kassiotis & Stoye, 2016; Freed, 2015; Contreras-Galindo et al., 2015). The gene *pol* encodes Reverse Transcriptase (RT) and Integrase (IN). Reverse Transcriptase and Integrase work together at the beginning of the retroviral life cycle. RT will convert the retroviral RNA into cDNA and IN will aid in the insertion of the retroviral cDNA into the host genome as a provirus. (Subramnian et al., 2011; Contreras-Galindo et al., 2015). Env, encoded by *env*, forms the precursor glycoproteins that will be located on the outside of the virion and will be involved with the recognition and attachment of virion to the host cell and its fusion and delivery of viral genome into the host cell (Scarlata & Carter, 2003; Bannert & Kurth, 2006). Most of what is known about HERV proteins comes from studying well-known retroviruses like HIV-1 and MMTV (Lee & Bieniasz, 2007; Ono, 2010; Boller et al., 2008). A typical retrovirus replication is summarized in Figure 2.

Currently most HERVs in the genome are not full-length functional proviruses, with many containing non-functional and mutated Env and RT (Hanke et al., 2016). Some families of HERVs that have integrated more recently, such as HERV-K (HML-2), still contain open reading frames (ORFs) for certain proteins. For example, there have been reports of different HERV-K insertions that are still able to produce Gag transcripts (Bhardwaj et al., 2015; Mueller-Lantzsch et al., 1993).
Figure 2. Typical retrovirus life cycle (HIV-1 shown). Initially a mature virion will bind to a receptor through the Env protein. From here the virion is internalized and the main capsid is disassembled revealing the ssRNA (purple). These will undergo reverse transcription by Reverse Transcriptase (orange diamond) to produce a cDNA that will be inserted into the genome by Integrase (dark blue circle). The now inserted provirus will be transcribed by host machinery to produce the mRNA required for retroviral replication. The cDNA can be reverse transcribed again, inserting into a new genome location (retrotransposition) or it can be used to translate Gag or GagPol polyproteins in the cytosol. GagPol is produced from the frameshift event that happens during translation due to the “slippery sequence”. Env glycoprotein formed on the rough endoplasmic reticulum will be transported to the Golgi and then in a vesicle to the plasma membrane (not shown). Gag attaches to the plasma membrane by the matrix domain and recruits’ viral RNA by the nucleocapsid zinc-fingers domains. As the virion starts to form and bud from the plasma membrane, TSG101 (a subunit on ESCRT-I) directly bind to the PTAP motif on p6 domain. The p6 domain also contains a YPX\textsubscript{a}L motif that direct binds ALIX, which is the bridge protein between ESCRT-I and ESCRT-III that will also be recruited to the budding site. This will drive the membrane scission and release of the immature virion. Viral protease will cleave Gag and GagPol polyproteins leading to maturation of the virion (not shown).
**HERV-K Gag can form viral-like particles (VLPs)**

Gag, which is the protein explored in this thesis, is required for the formation and release of the virion from the host cell and multiple HERV-K insertions have been shown to produce Gag transcripts (Bhardwaj et al., 2015; Mueller-Lantzsch et al., 1993; Monde et al., 2017). Gag alone can oligomerize creating viral-like particles (VLPs) that resemble an immature virus but lack the viral genome (Accola et al., 2000; Ako-Adjei et al., 2005; Kraus et al., 2011). VLPs derived from HERV-K Gag have been shown to form even with a dead protease or non-functional envelope protein, reinforcing the idea that HERV-K Gag can be expressed alone and form VLPs (George et al., 2011; Lee & Bieniasz, 2007; Boller et al., 2008). HERV-K Gag was first shown to produce viral-like particles in teratocarcinoma cell lines (Boller et al., 1983; Gotzinger et al., 1996). Since then, HERV-K Gag-derived VLPs have been reported in autoimmune disorders, prostate cancer, and even human blastocysts (Nelson et al., 2004; Reis et al., 2013; Grow et al., 2015).

**HERV-K Gag Shares Similar Motifs to HIV-1 Gag**

There are four main domains in a full-length HERV-K Gag; MA (matrix), CA (capsid), NC (nucleocapsid), p15 as well as short peptide sequences, SP1, QP1, and QP2 (George et al., 2011; Kraus et al., 2011; Monde et al., 2012). The functions of HERV-K Gag’s domains are not understood as well as HIV-1 Gag, which has been thoroughly studied, but the two do share similar functional motifs and both are reported to undergo virion assembly at the plasma membrane (Lee & Bieniasz, 2007; Ono, 2010; Boller et al., 2008; Freed, 2015; Scarlata & Carter, 2003; Wiegers et al., 1998; Garnier et al., 1998). For these reasons, it is possible that the domains of HERV-K Gag are similar to HIV-1 Gag (George et al., 2011; Monde et al., 2012; Kraus et al.,
The principal domains of HIV-1 Gag are MA, CA, NC, and p6 (Lee & Bieniasz, 2007; Ono, 2010; Boller et al., 2008). The MA domain promotes the binding of Gag to the plasma membrane while CA is involved in Gag-Gag interactions which forms the capsid core of the virus (Ako-Adjei et al., 2005). The NC domain contains two zinc finger motifs that will bind the viral RNA. The p6 domain contains late-binding motifs (L-domains) which will recruit host proteins to aid in the release of the virus (Adamson & Freed, 2007; Scarlata & Carter, 2003).

For HERV-K Gag the NC domain also has two conserved zinc finger motifs that can bind RNA (Kraus et al., 2011; George et al., 2011). The N-terminal of the MA domain has been shown to be essential for HERV Gag to bind to the plasma membrane (Monde et al., 2012). There are conserved homology regions in the CA domain that will be involved in the formation of the virion core (George et al., 2011). Between MA and CA is a p15 domain which differs from HIV-1 Gag. This p15 domain contains L-domain motifs PT/SAP and YPX_{n}L and acts in a very similar way to HIV-1 Gag p6 domain by binding TSG101 and ALIX respectively (Chudak et al., 2013). When these L-domain motifs are modified, it hinders the release of HERV-K viral-like particles (Figure 3; Chudak et al., 2013; Monde et al., 2012).

**L-domains Connect Retroviral Gag to the ESCRT Pathway**

There are three classes of late-binding motifs also known as L-domains; PT/SAP, YPX_{n}L and PPXY (Chudak et al., 2013). Different viruses will utilize different L-domains or a combination of the three (Freed, 2002; Chudak et al., 2013). These L-domains are the direct connection between retroviral Gag and the host’s endosomal sorting complex required for transport (ESCRT) machinery which will aid the budding and release of the virus (Monde et al.,
The ESCRT pathway is an intracellular network composed of more than thirty proteins that come together to form the main ESCRT complexes (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and Vps4 complexes) (Henne et al., 2015). These complexes have been implicated in a variety of functions including budding and scission of vesicles into endosomes also known as a multivesicular bodies (MVB) that aid in cargo-recognition/sorting of proteins (Hurley, 2010; Henne et al., 2015). These MVBs contain intraluminal vesicles (ILVs) that form from the inward budding and scission of the endosome membrane and contain cargo such as; protein, RNA, and lipids (Colombo et al., 2014). These MVB can fuse with the lysosome, degrading the MVBs content, or fuse with the plasma membrane releasing the ILVs into the extracellular space as exosomes (Colombo et al., 2014). Proteins associated with the ESCRT pathway are also essential in the final stages of mitosis, where their ability to regulate membrane expansion and scission controls cytokinesis (Hurley, 2010; Henne et al., 2015). Lastly, during a viral infection the ESCRT complex is hijacked in order for the virus to bud and release viral particles into the extracellular space (Hurley, 2010; Henne et al., 2015). Viruses that contain L-domains, which are proline rich motifs, will sequester ESCRT or ESCRT accessory proteins (Chudak et al., 2013).

The L-domain PT/SAP motif interacts with TSG101 (tumour susceptibility gene 101), which is a part of the ESCRT-I complex (Chudak et al., 2013; Martin-Serrano et al., 2003). When TSG101 is suppressed by small interfering RNA, HIV-1 viral release is severely interrupted (Garrus et al., 2001). The YPX₅L motif used by equine infectious anaemia virus (EIAV) as well as other retroviruses directly associates with ALIX also known as, Programmed Cell Death 6 Interacting Protein (PCD6IP), or AIP1 (Wang et al., 2014; Strack et al., 2003). The N-terminal Bro1 domain of ALIX interacts with ESCRT-III while its C-terminus interacts with
TSG101, bringing ESCRT-III and ESCRT-I together (Gottlinger, 2007; Sette et al., 2012). Other viruses like Rous sarcoma virus (RSV) use a PPXY motif which recruits Nedd4 (neuronal precursor cell expressed developmentally downregulated 4) (Freed, E. 2002). Nedd4 is involved in ubiquitination of proteins, which drives budding of proteins in the ESCRT pathway (Yang & Kumar, 2010). Several retroviruses like Mason Pfizer monkey virus (MPMV) have more than one of these L-domain that are close together on Gag (Chudak et al., 2013). For HIV-1 Gag and potentially HERV-K Gag, these L-domains work together to hijack host proteins facilitating the release of the nascent viral particles from the plasma membrane (Lee et al., 2007).

Figure 3. Representation of HERV-K (HML-2) provirus. A full-length HERV-K provirus encodes; gag, pol, pol, and env and is flanked by two LTRs. Gag main domains are: MA, CA, NC, smaller peptides spacer peptide (SP1), QP1 and QP2. L-domains are indicated with an arrow. YPXnL has the potential to bind ALIX while PTAP can bind TSG101. Zn fingers on the NC domain are also indicated which have the potential to bind viral RNA. Figure adapted from Chudak, C. et al. Retrovirology. 2013. 10(140), 1–14. © 2013 Chudak et al.; licensee BioMed Central Ltd.
**Physiological Implications of HERVs**

Recent investigations have begun to unravel the impact of HERVs on disease and pathology. HERV transcripts and proteins originating from HERV-K (HML-2) insertions have been linked to various diseases such as; autoimmune diseases, multiple sclerosis, inflammatory disease, and breast cancer to name a few (Gifford & Tristem, 2003; Kassiotis & Stoye, 2016). HERV-K mRNA, reverse transcriptase and antibodies recognizing HERV-K are increased in the serum of patients with breast cancer (Wang-Johanning et al., 2014; Golan et al., 2008). HERV transcripts and proteins have also been shown to be increased in prostate cancer (Reis et al., 2013), schizophrenia (Slokar & Hasler, 2016), lymphoma (Contreras-Galindo et al., 2008), germ cell tumor cell lines (Ruprecht et al., 2008), leukemia (Depil et al., 2002), melanoma (Buscher et al., 2005), teratocarcinoma (Boller et al., 1983; Bieda et al., 2001), and HIV infections (Contreras-Galindo et al., 2012; Mulder et al., 2017).

In breast cancer, HERV-K Env transcripts and proteins were shown to be upregulated in breast cancer tissue compared to healthy controls (Wang-Johanning et al., 2003; Zhao et al., 2011; Zhou et al., 2016). As well Zhou *et al.*, (2016) showed that immunodeficient mice xenografted with various breast cancer cell models stably transduced with short hairpin RNA (shRNA) targeting HERV-K Env had a significant reduction of tumor weight and metastasis to the lungs. It should be noted that Zhou *et al.*, (2016) also did the same technique with shRNA targeting HERV-K Gag which decreased tumor weight, but was not as effective as targeting HERV-K Env. Data on the effect of HERV-K Gag on metastasis to the lungs was not presented (Zhou et al., 2016). This suggest that HERV elements, especially Env might be linked to tumor growth and metastasis of cancer. In another example, HERV-K transcripts; *gag, pol* and *env* were increased in the tissue of patients with Amyotrophic Lateral Sclerosis (ALS) compared to
healthy control (Li et al., 2015). Strikingly, expressing HERV-K Env in motor neurons in mice caused death of motor neurons and motor deficits, which are symptoms of ALS. Taken together, this suggests that pathology in some diseases may be attributed to over-expression of HERV-K.

While HERVs are implicated in a variety of diseases there are also studies showing that HERVs may be vital in healthy tissue. For example, Syncytin-1 and Syncytin-2 are two proteins that evolved from the Env protein of ERVs. Syncytins are highly expressed in the trophoblast, which will eventually develop into the placenta. It has been suggested that Syncytin-1 plays a role in implantation of the trophoblast to the uterine wall (Soygur & Moore, 2016; Vargas et al., 2009). This hypothesis implicates ERVs in the formation of the placenta and suggests ERVs may have been necessary for the evolution of mammals (Lokossou et al., 2014). Recently, two papers showed how the protein Arc, which is involved in neuroplasticity, is derived from the Gag protein of Ty3/gypsy retrotransposon family. Arc was able to form viral-like particles and deliver mRNA from one neuron to another (Pastuzyn et al., 2018; Ashley et al., 2018). Also, Monde et al., 2012 reported that HERV Gag can co-assemble with HIV Gag at the plasma membrane. This co-assembly of HERV-K Gag and HIV-1 Gag reduced the infectivity and spread of HIV (Monde et al., 2012). These examples show that HERVs, over time, can be evolutionarily advantageous.

Endogenization of HERVs

It is hard to study how HERVs became “tamed” to exist symbiotically in the human genome since there is currently no evidence suggesting active insertion events are happening in humans (Kassiotis & Stoye, 2016; Stoye, 2012). Fortunately, research can study other animal models where active endogenization is happening (Garcia-Etxebarria et al., 2014). The sheep retrovirus enJSRV (endogenous Jaasiekte sheep retrovirus) inserted into the genome 5-7 million
years ago and insertions have been accumulating with time with the latest one estimated to be 200 years old (Dewannieux & Heidmann, 2013). Some research suggests that enJSRV is a key part of sheep reproductive biology (Dunlap et al., 2006; Arnaud et al., 2008). Probably the most striking example of endogenization is currently happening in koalas. Tarlinton et al., 2006 showed that koala retrovirus (KoRV) was endogenized in some animals from northern Australia that were suffering from leukemia and lymphosarcoma. KoRV was transmitted vertically as DNA in the sperm of infected animals. These infected animals had a high viral load in their plasma and high copy number of KoRV in the genome. This was in contrast to the koalas in southern Australia which had a lower copy number of KoRV and lower viral load in the plasma (Simmons et al., 2012). Taken together this could suggest that we are seeing real-time endogenization of KoRV (Dewannieux & Heidmann, 2013). There are no current insertion events happening in humans (Bannert & Kurth, 2006; Young et al., 2013), though there is indirect evidence for HERV activity such as HERV-K (HML-2) insertions having almost intact ORF for; gag, pro, pol, and env and in rare cases having a fully intact genome that is usually suppressed (Wildschutte et al., 2016). Of the HERV-K transcripts able to be transcribed, gag is of particular interest (Bhardwaj et al., 2015). HERV-K Gag has the ability to form viral-like particles and potentially interact with the ESCRT machinery through L-domains (Ruprecht et al., 2008; Monde et al., 2012). Based on all of the evidence presented one pathway we theorize HERV-K Gag may impact is the biogenesis of extracellular vesicles.

**Extracellular Vesicle Definition and Biogenesis Pathways**

Extracellular vesicle (EV) is the term given to any vesicle released from the cell into the extracellular space, including viral-like particles (VLPs) (Figure 4; Raposo & Stoorvogel, 2013;
Microvesicles are 100-1000 nm particles that form from the budding and fission of the plasma membrane (Tkach & Théry, 2016). This has been suggested to be the result of interplay between phospholipid redistribution and cytoskeleton protein contraction (Akers et al., 2013). There is emerging evidence that microvesicles can also form with the aid of ESCRT-I (Hurley, 2016; Gan & Gould, 2011). When RNA interference (RNAi) is used against ESCRT-I or ESCRT-0 there is suppression of particles shedding from the plasma membrane (Wehman et al., 2011). Another EV population forms when cell undergo apoptosis in the form of apoptotic bodies (Akers et al., 2013). These EVs are usually larger in size ranging 800-5000 nm, but can be as small as 50 nm (Crescitelli, R. et al. 2013; Simpson & Mathivanan, 2012).

Exosomes are another type of EVs 40-120 nm in size that arise from vesicles budding into an early endosome (Hanson & Cashikar, 2012). Once these vesicles have budded into the endosome (also known as multivesicular bodies (MVBs)) they are called intraluminal vesicles (ILVs). These ILVs contain cargo that is sorted in a non-random fashion (Colombo et al., 2014; Guo et al., 2018). These MVBs can either fuse with a lysosome, degrading its contents and is involved with regulation of cell surface receptors like EGFR (Epidermal Growth Factor Receptor) (Hurley, 2010; Madshus & Stang, 2009). The MVB could also fuse with the plasma membrane (Colombo et al., 2014). When the MVB fuses with the plasma membrane the ILVs are released into the extracellular space as exosomes (Colombo et al., 2014; Akers et al., 2013; Alenquer & Amorim, 2015). The fusion of the MVB with the plasma membrane has been shown to be typically controlled by Rab27a and Rab27b (Ostrowski et al., 2010; Guo et al., 2018). The sorting and formation of the ILVs are widely believed to be dependent on thirty plus proteins that come together to form the endosomal sorting complex required for transport (ESCRT); although some groups have shown that exosomes can also form in ESCRT-independent pathways. 
Biogenesis of extracellular vesicles. Apoptotic bodies form when the cell undergoes apoptosis and can range 50-5,000 nm in size. Microvesicles range 100-1000 nm in size and mostly form on the plasma membrane. Exosomes are 40-120 nm in size and form in the endosomes and are heavily regulated by the ESCRT pathway. The endosome/MVB will fuse with the plasma membrane releasing the particles into the extracellular space. Lastly, viral-like particles (VLPs) arising from HERV-K Gag range 90-120 nm in size and have been theorized to bud from the plasma membrane and/or arise from the ESCRT pathway. Note the overlap in sizes between all of the EV.

The biogenesis of exosomes in an ESCRT dependent fashion is a complex mechanism still being elucidated. What is known is that the ESCRT complexes (ESCRT-0, -I, -II, -III) and the Vps4 complexes are involved in the budding and formation of ILVs (Schmidt & Teis, 2012; Hurley & Emr, 2006; Babst, 2011). Protein can be recruited to early endosomes in a non-random fashion usually through ubiquitination, but some protein can be recruited to the MVB without
ubiquitination (McGough & Vincent, 2016). Once recruited, the membrane of the MVB starts to bud through the help of ESCRT-I and ESCRT-II forming a bottleneck (Babst, 2011; Schmidt & Teis, 2012). ESCRT-III will start to narrow the neck of the vesicle and together with the recruitment of Vps4 complexes will initiate scission of the bottleneck and release of the ILV into the MVB (Babst, 2011; Schmidt & Teis, 2012). This process is summarized below for ubiquitinated proteins (Figure 5).

As mentioned not all cargo needs to be ubiquitinated and recruited by ESCRT-0, -I, and –II to be enriched in exosomes (Hurley & Odorizzi, 2012). Some proteins can be recruited by ALIX through the L-domains, which are proline rich motifs (PRM) (Ren & Hurley, 2011). G protein-coupled receptor PAR1 directly binds to ALIX by the YPXₙL motif (Dores et al., 2012). ALIX which is an accessory protein for the ESCRT pathway will recruit ESCRT-III for budding into MVBs (Dores et al., 2012). Syndecans (transmembrane heparan sulphate proteoglycans) which binds Syntenin can also be recruited to the early endosomes by Syntenin binding ALIX at the YPXₙL motif (Hurley & Odorizzi, 2012; McGough & Vincent, 2016; Baietti et al., 2012). As well both TSG101 and ALIX have been shown to associate with centrosomal protein CEP55 at the midbody, interacting with these PRM (Hanson & Cashikar, 2012). The recruitment of the ESCRT machinery through TSG101 and ALIX is essential for cytokinesis (Ren & Hurley, 2011). During a retroviral infection, these same L-domains can be utilized to recruit ESCRT machinery and promote viral budding (Ren & Hurley, 2011; Hanson & Cashikar, 2012).
Models for ESCRT-dependent and independent mechanisms of ILV formation. The deformation of the membrane is mainly driven by the formation of a membrane domain with a unique lipid composition and is supported by the pH gradient across the endosomal membrane. (a) ESCRT-0 sorts ubiquitinated cargo proteins into the lipid domain. The neck of the forming vesicle is first stabilized by ESCRT-I and ESCRT-II. The neck is further narrowed by the formation of ESCRT-III. The recruitment of the Vps4 complex to ESCRT-III initiates the scission of the vesicle neck and the disassembly of the ESCRT-III complex.

**Figure 5. Recruitment of ubiquitinated proteins into MVBs through ESCRT-dependent mechanism.** ESCRT-III is recruited with ALIX acting as the bridge to ESCRT-I. Figure from Babst, M. *Curr Opin Cell Biol.* 2011, 23(4), 452–457. Copyright © 2011 Elsevier Ltd. All rights reserved.
The last type of EVs are viral-like particles ranging 90-120 nm in size (Akers et al., 2013). VLPs arising from endogenous retroviruses like HERVs are believed to form from the plasma membrane; but during a retrovirus infection such as with HIV or MLV there are studies showing viral particles also budding into the MVBs (Sherer et al., 2003). This suggests that there is a possibility for HERVs to form VLPs through multiple biogenesis pathways, including through MVB. As well, retroviral Gag can often be detected in Western blots of isolated EVs (Trajokovic et al., 2008; Akers et al., 2013) and it was only recently that a subset of VLPs produced using MLV were able to be distinguished by flow cytometry through targeting the Env protein (Tang et al., 2017). This suggest that VLPs will pellet with other EVs of similar size, for example, with exosome-like vesicles during ultracentrifugation.

While relatively enriched populations of different sized vesicles can be prepared, isolating these different types of vesicles completely free from each other is nearly impossible due to their overlapping size, density, and shared markers (Gould & Raposo, 2013; Colombo et al., 2014). This has caused confusion in the nomenclature used to describe EVs and the nomenclature is still a matter of debate and has not been standardized (Colombo et al., 2014; Akers et al., 2013; Gould & Raposo, 2013). The origin, function, and features of these EVs are diverse and because of this, multiple names have been used to describe the same vesicles. Some researchers defined the vesicles based on where in the body they were isolated, thus giving rise to terms including; epididymosomes (Sharma et al., 2016), oncosomes (Al-Nedawi et al., 2008), archaeosomes (Krishnan et al., 2000), promininosomes (Radha et al., 2013), etc. Other groups named vesicles differently, even if the methods of isolation and biogenesis were similar. This lead to names such as exosomes (Valadi et al., 2007; Kamerkar et al., 2017; Hoshino et al.,2015), ectosomes (Hess et al., 1999), microvesicles (Skog et al., 2008) and even some groups calling
them “exosomes/microvesicles” (Shen, 2011; Miranda et al., 2010) for similar populations of EVs.

**Extracellular Vesicle Isolation and Quantification**

There are currently twenty documented methods for isolating EVs which are summarized in Konoshenko et al., 2018. The most common method of isolating exosome-like EVs is differential ultracentrifugation (Konoshenko et al., 2018; Gould & Raposo, 2013; Gardiner et al., 2016). For collection of EVs in this thesis, the ultracentrifugation protocol used to isolate exosome-like EVs was used with the full protocol outlined in materials and methods (Théry et al., 2006). There are multiple methods to characterize EVs, the most common being Western blotting, single-particle tracking, and electron microscopy (Gardiner et al., 2016). For single-particle tracking the most common method is nanoparticle tracking analysis (NTA) using NanoSight or ZetaView instruments which allows the user to measure the size and concentration of EVs (Gardiner et al., 2016; Konoshenko et al., 2018). In these experiments nanoparticle tracking analysis (NTA) by ZetaView® (Particle Metrix) was used due to access, ease of use, and acceptance in the field (Konoshenko et al., 2018). Nanoparticle tracking analysis (NTA) allows the user to visualize particles in a suspension using a focused laser beam (Steppert et al., 2016). This non-invasive method captures the movement of every single particle in solution. The Brownian motion of these particles is dependent on their size and allows the concentration and size distribution of EVs to be calculated (Steppert et al., 2016).

In summary, while it is obvious that there are different biogenesis pathways it is difficult, if not impossible, to currently separate the populations from each other due to their similarities. Whether these vesicles were collected by ultracentrifugation or any other method, there will
always be residual contamination with an array of EVs (microvesicles, exosomes, VLPs) present in the final sample (Konoshenko et al., 2018; Gould & Raposo, 2013). Due to this, the terminology used in this thesis to encompass the vesicles studied will be extracellular vesicles (EVs). The population is expected to be enriched in exosomes, exosome-like vesicles and VLPs while containing smaller amounts of microvesicles or apoptotic bodies. EVs in this thesis were isolated by ultracentrifugation and characterized by NTA and Western blotting.

**Extracellular Vesicles Role in Pathology**

There is growing interest in EVs due to their role in disease and cell-to-cell communication (Colombo et al., 2014). For example, EVs released from primary tumor cells can determine the location of future metastases by permeabilizing the vasculature and establishing a niche (Hoshino et al., 2015). HERV-K transcripts can also increase metastasis to the lungs in a breast cancer model (Zhou et al., 2016). Both HERV-K and EVs have been linked to neurological disorders. HERV-K transcripts have been found in patients with Amyotrophic Lateral Sclerosis (ALS) (Li et al., 2015), while EVs have been shown to spread misfolded SOD1 and TDP43 proteins that contribute to ALS (Grad et al., 2014; Smethurst et al., 2016). EVs are also required for spread of misfolded proteins like Tau and prion proteins between cells and throughout the brain (Alais et al., 2008; Fevrier et al., 2003; Asai et al., 2015). Additionally, EVs have been shown to deliver tRNA to the epididymis to help in the maturation of sperm (Sharma et al., 2016) and EVs in blood can act as biomarkers for renal disease and glioblastoma (Miranda et al., 2010; Skog et al., 2008). Lastly, EVs are able to transfer mRNA and microRNA from one cell to another and are being exploited to deliver therapeutics to target diseased tissue (Kamerkar et al., 2017; Alvarez-Erviti et al., 2011; Valadi, H. et al. 2007). By engineering EVs to package
short interfering RNA or hairpin RNA, researchers have shown the ability to inhibit expression of disease-causing genes such as oncogenic $Kras^{G12D}$ in pancreatic cancer (Kamerkar et al., 2017) and $BACE1$ in Alzheimer’s disease (Alvarez-Erviti et al., 2011). Understanding the extent of HERV Gag’s role in EV production and function will help gain a better understanding of EVs role in disease and aid in efforts to develop EV derived therapeutics.

**Objective and Overview**

HERVs, are derived from retroviruses that infected the inheritable germline and some families such as HERV-K are still able to produce mostly intact transcripts, including Gag. HERV-K Gag can produce VLPs that are similar to exosome-like vesicles in size, density, and markers making it difficult to separate these two populations with currently technology. As mentioned, HERV-K Gag contains late-binding domains that can potentially recruit ALIX and TSG101, hijacking the ESCRT proteins in order to be released into the extracellular space. Exosome-like vesicles use the ESCRT machinery to bud into the endosome. Both VLPs and exosomes are able to be isolated by ultracentrifugation at 100,000 x g and retroviral Gag can often be detected in Western blots of isolated exosome-like EVs. Due to the difficulty of isolating these populations from each other we aim to study the EV population as a whole. We hypothesize that Human Endogenous Retroviral Gag impacts the formation and content of extracellular vesicles. There are three possibilities whereby HERV-K Gag could affect EVs. The first is that HERV-K Gag is required for the production of some exosome-like EVs and shaping their content. A second possibility is that HERV-K VLPs formed by Gag are a major contaminant of EV preparations and account for many functions attributed to EVs. Lastly, HERV-K Gag might compete for ESCRT proteins, affecting EV content and number. There was
a lot of trial and error to determine an effective method for studying Gag’s effect on the EV population. Initially ALIX was studied in relation to ERV Gag in mouse cell lines. After determining that it was ineffective we then tried to knockout HERV-K Gag with CRISPR-Cas9 technology in MSC immortalized with TERT. This method could potentially have worked but no knockouts were determined by Syrveyor method and the PCR data remains inconclusive. Finally, by designing HERV-K Gag expression vectors and siRNA targeting HERV-K Gag we effectively expressed and knockdown Gag. We then studied the formation of EVs by nanoparticle tracking analysis (NTA) to test for changes in particle size and concentration. As well we used Western blotting to determine if there were any changes in the EV content that may be affected (ALIX and TSG101). Extracellular vesicles (EVs) are attributed to a growing variety of important biological functions in disease and have attracted an incredible amount of scientific investigation. By understanding HERV-K Gag’s role in EV biogenesis we can gain a better understanding of these ancient viral symbionts and potentially their effects on EVs.
Materials and Methods

Cell culture and transfections

The following cell lines were obtained from ATCC (American Type Culture Collection); SH-SY5Y (ATCC CRL-2266), HEK293T (ATCC CRL-1573), MSC TERT (ATCC SCRC-4000), C8-D30 (ATCC CRL-2534), C8-D1A (ATCC CRL-2541), Neuro2a (ATCC, CCL-131), BV2 (have since been discontinued). NSC34 cell line was obtained from Cedarlane (CLU140). MEF cell line was gifted by D. Philpott (University of Toronto, Canada). MSC primary cells were gifted by Dr. David Courtman (Ottawa Hospital Research Institute, Canada). The cells were derived from the bone marrow obtained at the Ottawa Hospital from healthy CTO screened donors under good manufacturing production (MSC GMP, REB protocol #20140809-01H).

All cell lines, expect MSCs, were cultured in Dulbecco’s Modified Eagle Medium (DMEM; 319-015-CL, Wisent) containing 10% fetal bovine serum (FBS; 080-150, Wisent) and 1% penicillin-streptomycin (450-201-EL, Wisent) in 10cm dishes (10062-880, vwr). MSCs lines were cultured in MSCBM hMSC Basal Medium (PT-3238, Lonza) with 10% Mesenchymal cell growth supplements (PT-4106E, Lonza), 2% L-Glutamine (PT-4107E, Lonza), and 0.1% Gentamicin sulfate (PT-4504E, Lonza).

For transient transfection with DNA, Lipofectamine2000 (11668-019, Invitrogen) was used for all cell lines except MSCs which used jetPRIME® (114-07, Polyplus). Silencer Select siRNA (Life Technologies) were transfected at 10 nM concentration with RNAiMAX (13778150, Invitrogen). After transfected cells were harvested for analyses 48 hours or 72 hours respectively.
**Extracellular vesicles isolation and quantification**

Extracellular vesicles (EVs) were isolated by differential centrifugation as previously described (Théry et al., 2006). Briefly, cells were cultured in media containing 10% FBS previously depleted of EVs by centrifugation at 100,000 x g for 16 hours. To purify the EVs, the media from the cells was centrifuged at 300 x g (10 mins, 4 °C), 2,000 x g (10 mins, 4 °C) and 10,000 x g (30 mins, 4 °C). At each step the supernatant was transferred to a new 15 mL conical tube, combining technical replicates before the 10,000 x g spin. After the 10,000 x g spin the media was centrifuged at 100,000 x g (2 hrs, 4 °C). The supernatant was removed and the pellet was re-suspended in 1 mL PBS. The pellet was then concentrated by a final centrifugation at 100,000 x g (40 mins, 4 °C). The pellet was re-suspended in 20 uL of PBS and stored in -80 °C for further analyses by Western blot.

After the 2,000 x g spin an aliquot of the supernatant was diluted 1/50-1/100 in PBS for EV quantification. By collecting at the 2,000 x g spin, more technical replicates were able to be analyzed since samples of similar conditions were combined before the 10,000 x g spin. The size distribution and concentration of particles was determined by nanoparticle tracking analysis (NTA) on the ZetaView® (Particle Metrix). The concentration of the EV sample was corrected by subtracting the number of particles in the EV-depleted media. The concentration was then multiplied by the volume of media that was collected to obtain total number of particles. The number of cells at harvest were counted in parallel with EV collection by hemocytometer. Particles/cell was calculated by dividing total number of particles by total number of cells for each technical replicate. It should be noted that all samples measured by nanoparticle tracking by ZetaView® were collected after the 2,000 x g spin.

The ZetaView® has camera settings that were adjusted to specifically focus on smaller
EVs. For these experiments, we used the following camera settings; 640 x 480 Mono - 30 frames per second, sensitivity = 85, shutter = 40 which are adapt for capturing smaller EVs that move quicker and have a weaker scatter. The post-acquisition parameters were set as; Max Size = 200 pixels, Min Size = 10 pixels, Min Brightness = 15 pixels.

**Whole cell extracts, western blotting, and antibodies**

Cells were washed in cold PBS and lysed in 5 mM Tris pH 7.4, 75 mM NaCl, 0.5 mM EDTA, 0.5% Triton-X-100 with protease inhibitors (4693159001, Sigma). Lysate were then spun down at 10,000 × g, 5 min at 4 °C. The supernatant was kept in order to estimate the protein content.

For western blotting, equal amounts of protein samples were resolved on 10% (w/v) acrylamide (BP1410-1, Fisher) Tris-glycine gels, hand-casted, then transferred to PDVF membrane (IPVH00010, EMD Millipore), blocked with 5% milk (sc-2325, Santa Cruz) in TBST (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 hour and probed with primary antibody in TBST overnight at 4 °C. All antibodies used are summarized in Table 1. Blots were then washed in TBST, probed with HRP labelled secondary antibodies (goat anti-mouse and goat anti-rabbit), washed again in TBST and imaged with HRP substrate (WBLUR0100A, EMD Millipore) on an ImageQuant LAS 4000 system (GE Healthcare). Mean intensity levels were measured for densitometry-based normalization to Flotillin-2 levels for EVs. For each protein in an experiment, an area encompassing the maximum band size was selected and mean intensity of this an area with this size was measured for each band using the Photoshop ‘Histogram’ function. Mean intensity of an identically-sized area without any bands was subtracted from these values.
as a measurement of background. Adobe Photoshop CC 2017 was used for all Western blot quantifications.

**PCR and Reverse Transcription (RT)-PCR**

Total RNA was collected from cells using TRIzol® (Life Technologies) according to manufacturer’s protocols. For RT-PCR, cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen). PCRs were then set up using Promega GoTaq Green DNA Polymerase (M791A) according to manufacturer’s instructions. PCR conditions were as follows for validation of HERV-K101 and HERV-K109 Gag expression: 95 °C for 2 min, (95 °C for 15 sec, 55 °C for 30 sec, 72 °C for 1min 30 secs) × 39 cycles, 72 °C for 10 min. For screening CRISPR knockouts the PCR conditions were as followed: 95 °C for 2 min, (95 °C for 15 sec, 55 °C for 30 sec, 72 °C for 30 sec) × 39 cycles, 72 °C for 10 min. List of primers and their application are listed in Table 2. Amplicons were run on a 2% agarose gel (containing ~2% EtBr 20 mg/mL) and visualized under UV light.

**CRISPR design, cloning, transfection, and screening**

The CRISPR-Cas9 vectors used for targeting HERV-K Gags were designed and created as previously described (Ran et al., 2013). Full sequences of the HERV-K Gag were used to design CRISPR vectors are summarized in Figure 8. Briefly, the 20-nt guide sequence were chosen by cross-matching top hits of guide sequences determined by CRISPR Design Tool (http://tools.genome-engineering.org) and sgRNA Designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). From the top matches, it was narrowed down to at least two guide DNA sequences targeting each of the HERV-K Gags.
Table 3. The 20-nt guide sequences were cloned into the pSpCas9(BB)-2A-puro vector (gift from Feng Zhang, Cambridge, Massachusetts [Addgene plasmid #62988]) using the protocol outlined in Ran et al., 2013. The vectors were validated by sequencing at The Ottawa Hospital Research Institute sequencing facility.

MSC TERTs in a 6-well plate were transiently transfected with 2 ug of DNA using jetPRIME® (114-07, Polyplus). Puromycin was added at 0.75 ug/mL concentration to each well 24 hours after cells had been transfected. After three days, the puromycin was removed and the cells were able to recover in complete media for 24 hours. After 24 hours, the cells were monoclonally selected by limited dilution into 96-well plates. Single cell colonies were expanded and potential knock-outs were screened by PCR. Colonies that appeared to be knockouts were expanded to a 75 cm flasks and re-screened by PCR. DNA obtained for PCR was extracted by QuickExtract (QE09050, Lucigen) following manufacture’s protocol.

**Surveyor Assay**

Surveyor Mutation Detection Kit (706020, Transgenomic) was used to validate CRISPR-Cas9 knock-outs in colonies. The primer that was used is listed in Table 2. and the assay was followed according to manufacturing protocol (Qiu et al., 2004).

**Cloning of HERV-K Gag**

The pcDNA3.1 HERV-K Gag expression vectors were constructed by cloning the designed DNA G-blocks (Figure 7) into a pcDNA3.1 SOD1 wild-type vector via Kpn-I/XhoI restriction sites. To validate proper cloning of the G-block into the pcDNA3.1 plasmid and the successful removal of the SOD1 gene a restriction digest was done (Figure 11B). The digest with
only XhoI was done to make the linear form of the vector and show that only one G-block was inserted into the vector. If two G-blocks were cloned into the vector there would be a band at 2 Kb. The double digest was to show that the SOD1 gene had been removed and that the HERV-K Gag gene was properly cloned into the vector. The sequence of the newly inserted HERV-K Gags were validated by Sanger sequencing at the Ottawa Hospital Research Institute. Both vectors were 100% identical to the original G-block design.

**Design of siRNA targeting HERV-K Gag**

Using the list of HERV-K Gag provided by Dr. Patric Jern, Uppsala University and siDirect (http://sidirect2.rnai.jp/) siRNA targeting multiple HERV-K Gags were designed (Figure 8 for full list of HERV-K Gags and Table 4 for siRNA sequences). An siRNA was chosen if it could target ten or more HERV-K Gag sequences. Also, if the siRNA had off-targets effects they all targeted other HERV-K transcripts.

**Protein identification by LC-MS/MS**

Samples destined for mass spectrometry were prepared in a sterile work environment and were run part way down a 4-20% Mini-PROTEAN TGX Pre-cast Protein Gels (BioRad) using polyacrylamide gel electrophoresis. Proteins were visualized using the PlusOne Silver Staining Kit following the manufacturer's protocol (GE Healthcare Life Sciences). All protein bands were excised from gels in three parts, stored in 1% acetic acid, and sent to the Ottawa Hospital Research Institute Proteomics Core Facility for proteomics analysis. Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko (Nat Protocols 2006; 1(6):2856-60). Peptide extracts were concentrated by Vacufuge (Eppendorf). LC-MS/MS was
performed using a Dionex Ultimate 3000 RLSC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). MASCOT software version 2.6 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The observed spectra were matched against virus and human or mouse sequences from SwissProt (version 2016-09) and against an in-house database of common contaminants. The results were exported to Scaffold PTM Viewer (Proteome Software, USA) for further validation and viewing. Peptides with 1 or more spectral counts were included in the analysis.

**Live cell imaging**

IncuCyte ZOOM® Instrument (Essen BioScience) was used for live-cell imaging. Cells treated with siRNA were placed in the IncuCyte ZOOM® and imaged at objective 10x every 2 hours, then after 48 hours the cells were imaged every 15 minutes and removed after 64 hours. All images were analyzed by Fiji - Image J (Schindelin et al., 2012).

**Statistical Analysis**

All statistical tests were done using GraphPad Prism version 7 (www.graphpad.com). The following tests were done, unless stated elsewhere. One-way ANOVA followed by Dunnett’s multiple comparisons test was performed where the mean of the test columns was compared individually to the mean of the control column. Two-way ANOVA followed by Dunnett’s multiple comparisons test was performed where matched values are stacked into a sub-column. A paired two-tail student t-test. Normal distribution was assumed for all tests. p<0.05, *; p<0.01, **; p<0.001, ***.
Table 1. Summary of antibodies used and their manufacture.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution Used</th>
<th>Source</th>
<th>Identifier</th>
<th>Used</th>
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<tr>
<td>ALIX (Host: mouse)</td>
<td>1: 1,000</td>
<td>BD Transduction Laboratories</td>
<td>Cat#: 611620; Clone: 49/AIP1</td>
<td>Probing ALIX in mouse cell lines</td>
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<tr>
<td>ALIX (Host: mouse)</td>
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<td>Cell Signalling Technology</td>
<td>Cat#: 2171; RRID: AB_2299455</td>
<td>Used in combination to probe for ALIX in human cell lines</td>
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<td>ALIX (Host: mouse)</td>
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<td>Santa Cruz</td>
<td>Cat#: sc-53538; Clone: 3A9</td>
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<tr>
<td>Flag (Host: mouse)</td>
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<td>Sigma</td>
<td>Cat#: F1804; Clone: M2</td>
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<td>Flotillin-2 (Host: rabbit)</td>
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<td>Cell Signalling Technology</td>
<td>Cat#: 3436; RRID: AB_2106572; Clone: C42A3</td>
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<tr>
<td>HERV-K Gag (Host: mouse)</td>
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<td>AUSTRAL Biologicals</td>
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<td>HIV1 p55+p24+p17 (Host: rabbit)</td>
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<td>MLV p30 Gag (Host: rabbit)</td>
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<td>Origene</td>
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<td>TSG101 (Host: mouse)</td>
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<td>Genetex</td>
<td>Clone: 4A10; RRID: AB 373239</td>
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<td>Tubulin (Host: mouse)</td>
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<td>Sigma</td>
<td>Cat#: T9026; Clone: DM1A</td>
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Table 2. Summary of primers used and their application. HERV-K Gags are designated with an ERV ID number generated by GRCh38 used to identify that unique HERV-K Gag. HERV-K Gag 5858+ represents HERV-K Gag 5858, 5863, 5954 which all have identical sequences. See Figure 8 for full list of HERV-K Gag sequences.

<table>
<thead>
<tr>
<th>Target gene/locus</th>
<th>Sequence (5’-3’)</th>
<th>Application</th>
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<tbody>
<tr>
<td><strong>FLAG-HERV-K primers</strong></td>
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<tr>
<td>HK5 P7 Fwd</td>
<td>aataagacccaacgccgagt</td>
<td>RT-PCR of HERV-K101 and HERV-109 Gag transcripts</td>
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<tr>
<td>HK5 P7 Rev</td>
<td>ctgtcatactgcatcctgtt</td>
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<td><strong>HERV-K Gags</strong></td>
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<td>Detection of HERV-K Gag CRISPR Knock-out</td>
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**Figure 6.** Example of CRISPR (pSpCas9(BB)-2A-puro) vector with the custom 20-nt guide DNA (blue) inserted at the BbsI restriction sites (429.1 is shown). Both the 20-nt guide DNA and sgRNA scaffold (red) are under the U6 promoter (green) that is transcribed and attaches to the Cas9 complex. SpCas9 (yellow) encodes the Cas9 protein while the Puro (blue) is the puromycin resistance marker under the CBh (orange) promoter. Figure adapted from Ran *et al.*, 2013.
Table 3. Summary of custom 20-nt guide DNA for CRISPR-Cas9 system targeting HERV-K Gags. Highlighted sequences in yellow or blue are identical to each other. HERV-K Gags are designated with an ERV ID number generated by GRCh38 used to identify that unique HERV-K Gag. HERV-K Gag 5858+ represents HERV-K Gag 5858, 5863, 5954 which all have identical sequences. See Figure 8 for full list of HERV-K Gag sequences.

<table>
<thead>
<tr>
<th>Target gene/locus</th>
<th>20-nt Sequence (5’-3’)</th>
<th>PAM Sequence</th>
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<tbody>
<tr>
<td>429.1</td>
<td>CCATCAGAGTCTAAACCACG</td>
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<tr>
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<td>AAGATTACAACCTCAAACGC</td>
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<td>1295.1</td>
<td>CCATCAGAGTCTAAACCACG</td>
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<td>1295.2</td>
<td>AACATTACAACCTCAAACGC</td>
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<td>2276.1</td>
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<td>CCATCAGAGTCTAAACCACG</td>
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<td>3238.1 (HERV-K101)</td>
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<tr>
<td>5858.2+</td>
<td>TGACTATAATCAATTACAGG</td>
<td>AGG</td>
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</table>
Figure 7. HERV-K101 and HERV-K109 G-block sequence and design. Each G-block contains a 3x FLAG tag (in green) which is in the same reading frame as the HERV-K Gag. M13 forward and reverse primers flank the restriction site so multiple G-block can be made through PCR.

Legend:
M13 Primers – Pink
Kpn 1 restriction site – Orange
Kozak Sequence – Blue
Start sequence – Red
HERV-K Gag Gene – Black
3x FLAG Tag – Green
Stop Codon – Purple
Xhol restriction site – Grey

HERV-K101 Gag G-Block (2,120nt)

GTAAACGCAGGCGCCAGGgtaccgccacatgggggagcaactaagaagatatatatggtcattttttttctgatcgtttaaaaactttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
HERV-K109 Gag G-Block (2,118nt)

GTAAAAACGACGCCAGgtaccgccactatggggccaaactaaagttgtaaatggtgctcttatctcagcttaattaatctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Figure 8. List of HERV-K Gag open reading frames. HERV-K Gags are designated with an ERV ID number generated by GRCh38 used to identify that unique HERV-K Gag. HERV-K101 and HERV-K109 are noted and highlighted. Chromosome position is indicated beside ERV ID.

| 429 | chr1: 75,377,086-75,384,951 |
|--------------------------------|
| atggggccatagctaaaccagaggcacaaggtctctctcagcaggtgctaggtgcccctgtaagattcaacctcaacaagcaggttaaag|
| aaataaagcccaacgcagctatgctttatataactgtgccccgcgcgtgaactatcagatgcccccccaagaaagcagtagtggctac|
| aagtagtccccccatcagcaggtgtcgtactccatcagctgtcaccacagctagactaagtctgtgagctgagtcaggtgaagggc|
| caggtgcccctgtaagattcaacctcaacaagcaggttaaagaaaataagacccaaccgccagtagcttatcaatactg|
| gcccgcggctgaacttcagtatcggccacccccagaaagtcagtatggatatccaggaatgcccccagcaccacagggcagggcg|
| cctaggagactgaagacctcactcattcattcatgagcctatatccctatcattcattcattcattcattcattcattcattcattc|
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Results

ALIX and Endogenous Gag

In an initial exploratory study, wild-type EVs from various human and murine cell lines were sent for mass spectrometry and a positive correlation was seen between the levels of ALIX and endogenous Gag-Pol polyprotein in EVs (Figure 9A). This suggests that Gag packaging into EVs may depend on ALIX. The strongest correlation was seen in murine cell lines BV2 and NSC34. As previously mentioned, HERVs are related to the betaretrovirus MMTV (Hanke et al., 2016). If there seemed to be an effect of ALIX on packaging of murine endogenous retroviral Gag (ERV Gag) into EVs, then perhaps something similar could be seen with HERVs and ALIX.

A variety of murine cells were treated with siRNA targeting murine ALIX. There was successful knock-down of ALIX in cell lysate and EVs, but in all cell lines, the ERV Gag amount remained unchanged in both cell lysate and EVs (Figure 9B). N2a had a significant increase in the number of particles/cell when treated with siRNA targeting ALIX while BV2 and NSC34 had a similar trend to increasing particle number that did not reach significance (Appendix, Figure 3C). As well there was a significant decrease in the total number of cells at harvest for BV2, N2a, and NSC34 (Figure 9C). When the cells were collected, 72 hours after initial transfection, it was noted that there was a change in cellular morphology. Cell size appeared to increase (Figure 10A).
Figure 9. Murine ALIX has an effect on EV concentration and cell proliferation but not endogenous Gag. 

A) Mass spectrometry data showing a correlation between Gag-Pol polyprotein and ALIX (panel A) but not with Floitllin-2 (panel B) (n=1). 

B) Five different murine cell lines were transiently transfected with siRNA targeting ALIX. Equal amounts of protein were loaded onto a 10% SDS-PAGE (CL – cell lysate, EV – extracellular vesicles). No change in endogenous Gag was seen. 

C) Total number of particles, determined by nanoparticle tracking analysis (NTA) on the ZetaView®, was used to calculate particles/cell. The concentration of the EV sample was corrected by subtracting the number of particles in the EV-depleted media. The number of cells at harvest were counted in parallel with EV collection. The error bars represent the standard deviation of three independent experiments performed in duplicate. A paired two-tail student t-test was performed for each cell lines. p<0.05, *; p<0.001, ***.
We theorized that the reason for this phenotypic change was due to ALIX being involved in cell division (Morita et al., 2007). BV2, which had the most drastic and consistent change in cell morphology, was used to test this theory. BV2 cells treated with siRNA targeting ALIX were observed using the IncuCyte ZOOM®, a live cell imager. It showed that as cells started to increase in size there was a drastic drop in the total number of cells (Figure 10B&C). Upon further inspection, it was seen that cell division often failed after chromosomal replication (Figure 10D bottom row). It was also seen that after 48 hours, cells started to die more frequently (Figure 10D top row). This could explain why there was an increase in the number of particles/cell observed (Figure 9C). The increase of particles could actually be a result of the cells dying or undergoing apoptosis and cellular debris or apoptotic bodies at the size of small EVs may be measured as exosome-like vesicles by NTA. Following this we determined it was best to study ERV Gag directly by knocking-out Gag in MSC TERT cell lines using CRISPR-Cas9 technology.
Figure 10. Murine ALIX effect on cell proliferation. A) Selected pictures of BV2, N2a and NSC34 72 hours post-transfection of siRNA targeting ALIX showing increased size morphology. B) BV2 cells were analyzed by live cell imaging (IncuCyte ZOOM®) for 64 hours after being treated with siRNA targeting ALIX. Average size of cells (top panel) and average number of cells (bottom panel) are shown. Equal amounts of cell lysate were loaded onto a 10% SDS-PAGE gel to confirm knock-out. C) Selected pictures from IncuCyte ZOOM® of BV2 cells treated with siRNA targeting ALIX at time points over the 64 hours. D) Selected picture of BV2 cells treated with siRNA targeting ALIX with a close up on one cell undergoing cell death (top panel) or remaining multinucleated (bottom panel). In B) the error bars represent the standard deviation of four independent experiments. A two-way ANOVA followed by Dunnett’s multiple comparisons test was performed where matched values are stacked into a sub-column. p<0.001, ***.
Using CRISPR-Cas9 to knock-out HERV-K Gag

Our collaborator Dr. Patric Jern from Uppsala University, Sweden provided us with 13 ORFs (open-reading frame) of HERV-K Gag that express RNA and are in principal capable of expressing Gag protein endogenously in humans. These were determined by RetroTrector software (Sperber et al., 2007) to identify ERVs with intact ORFs of Gag in the human genome (GRCh38, Genome Research Consortium human build 38). They are denoted by ERV ID numbers generated by this software (i.e. 429, 3276, 3238, 5067, etc.; Figure 8). Mesenchymal stem cells (MSC) are multipotent stem cells traditionally isolated from the umbilical cord that are capable of self-renewal and have the potential to differentiate (Pelekanos et al., 2016). MSC are promising candidates for cell-based therapy in cell repair and immune regulation (Pelekanos et al., 2016). The role of MSC extracellular vesicles (EVs) is also starting to be studied more closely in their relation to disease, for example, stroke (Li et al., 2018). MSC cells are able to be immortalized by the transduction of TERT (telomerase reverse transcriptase) (Wolbank et al., 2009). In our initial attempt to study HERV-K Gag’s effects on EVs we wanted to produce knockouts of the 13 ORFs of HERV-K Gag using the CRISPR-Cas9 system in MSC TERT cells.

The CRISPR-Cas9 system is part of the microbial adaptive immune system that has been harnessed for biological research to edit the mammalian genome, for example to knockout genes in a specific location using a complementary guide RNA (Ran et al., 2013). In short, a guide DNA is transcribed to give a guide RNA that associates with the Cas9 protein forming an RNA-protein complex. The Cas9 complex will scan the genomic DNA until it finds a complementary sequence for the guide RNA at which point it will make a double strand break 3-nt upstream of the Protospacer Adjacent Motif (PAM) sequence (NGG) (Shin et al., 2016). After the break, the cell can naturally undergo non-homologous end joining (NHEJ), which often creates short
deletions, insertions, point mutations and occasionally large deletions (Shin et al., 2016). These mutations can disrupt protein expression by introducing a frame-shift, stop codon or other mutations. For these experiments, two 20-nt guide DNAs were designed for each of the 13 ORFs of HERV-K Gag that would be cloned into the pSpCas9(BB)-2A-puro expression vector (Table 3). These guide DNAs were designed to target the specific HERV-K Gag and chosen if they had minimal off-target effects. Two of the guide DNAs that were designed targeted multiple HERV-K Gags in an attempt to knockout multiple active HERV-K Gag sequences. The full summary of the guide DNAs designed and cloning are located in Materials and Methods.

The CRISPR-Cas9 system introduces a double-strand break between the 3rd and 4th-nt upstream of the PAM sequence. The double-strand breaks will be repaired by the precise gene editing method Homology Directed Repair (HDR) or more commonly NHEJ which distributes the gene by insertions or deletions also called indel mutation (Figure 1A; Doudna & Charpentier, 2013). When designing the PCR primers to detect knockouts, one of the primers 3’ end overlapped the area where the double-strand break would be introduced. If the gene was not mutated the primer would bind across its whole length and amplification would happen, resulting in a PCR product. If a mutation was introduced, the primer would not bind in an optimized stringent PCR reaction and amplification would be unable to occur (Figure 11B). This is due to the specificity of PCR primers at the 3’ end and even if there is a single-nucleotide polymorphism there will be a drastic reduction of PCR detection especially with a stringent PCR condition (Stadhouders et al., 2010). On a gel, this translated to wild-type cells having a band at the predicted target length and potential knock-outs not having a band. β-actin was used as a control to show that DNA was present and PCR amplification occurred. A summary of the
primers used can be found in Table 2 and the predicted size of the product for each PCR reaction are listed in Table 5.

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*same primers were used for both CRISPR guides
**primers were not able to be designed to cover the desired area and therefore could not be tested
MSC immortalized with TERT were transiently transfected with the 16 different CRISPR vectors targeting HERV-K Gag. Puromycin was used to eliminate cells that didn’t express the CRISPR plasmid. After drug selection, the cells underwent monoclonal selection by limiting dilution into a 96-well plate to obtain a single cell in each well. This was an important step since the CRISPR mutation introduced by NHEJ (non-homologous end joining) could be different in each knock-out cell. As well, not all cells that exhibited puromycin resistance would be mutated, so it was critical that colonies grew up from a single cell to have a consistent knockout. Once cells grew up in a 96-well they were screened by PCR. Of the 16 conditions, only 10 survived to be screened (Figure 1C). β-actin was used as a control for each clone tested to ensure equal amounts of DNA were used and the PCR reaction was successful. For each primer set, wild-type DNA was used as a positive control to ensure that the primers amplified one target HERV-K Gag at the specific predicted length. Most colonies screened appeared to be wild-type. Some of the colonies for example; 2538.1 clone #2, 3276.2 clone #9, and 5067.1 clone #3 appeared to have decreased band intensity, or no bands for the HERV-K Gag compared to wild-type and could potentially have mutations in one or more copies of the HERV-K Gag gene (Figure 1C). The PCR was run twice in order to confirm results were real and not just an error in the PCR.

Colonies that still appeared to be heterogeneous or knockouts were expanded to 75 cm flasks. They were rescreened using the same PCR primers. Wild-type DNA with the specific primers was used as a positive control. DNA samples from the original 96-well plate were also used to compare to the cells expanded in the 75 cm flasks (Figure 1D). At this stage, it appeared that colonies were no longer knockouts. This was probably due to contamination of knockouts with wild-type cells during cell expansion. As well since there are multiple HERVs in the genome it is possible there is a knockout at the gene of interest but the PCR primers have
multiple off-target effects, masking the knockout. This would make my PCR method of detecting HERV-K Gag knockouts difficult if not impossible. To try and overcome this problem, the surveyor assay was used which can detect single nucleotide changes and amplifies the entire region of interest (Figure 1E&F).

The surveyor assay kit purchased from Transgenomic provides positive controls; G and C which are identical plasmid sequences except for a single nucleotide base change. The 3276.2 CRISPR guide had knockouts in the 96-well stage and had potential heterozygotes in the 75 cm dish as detected with the PCR assay, suggesting that it could represent a heterogeneous population. The first step of the surveyor assay was to amplify the area of interest containing the mutation for both wild-type cells and potential knockouts to confirm only one product is made at the correct predicted size (Figure 1E). The wild-type DNA and potential knockout DNA was then mixed in a 1:1 ratio (G and C are also mixed in 1:1 ratio) and hybridized together. Surveyor Nuclease which is a DNA endonuclease is added, in order to cut the DNA at any mismatching base pairs. The product after the endonuclease step was run on a gel (Figure 1F). The assay worked because control G and C had the full-length 633 bp product of self-hybridized DNA, while hybridized G + C generated two endonuclease products at 217 bp and 416 bp. For the HERV-K Gag CRISPR knockouts, the wild-type DNA + HERV-K Gag CRISPR clone DNA hybridized together were predicted to produce bands at 200 bp and 400 bp when cleaved by endonuclease. Only wild-type bands were detected and no endonuclease cleavage bands at 200 bp and 400 bp were detected indicating that all clones were wild types (Figure 1F). Potential problems with this method are that the PCR primers used could be conserved between multiple HERV Gags, so even if there was a mutation in the predicted location it was masked by the abundance of other HERV Gags that were amplified by the PCR primers. Therefore, it was
determined to study HERV-K Gag’s effect on EVs with an expression vector for HERV-K Gag and siRNA targeting HERV-K Gags.
Figure 11. Construction and testing of CRISPR knockouts targeting HERV-K Gag. A) Summary of CRISPR-Cas9 model. In brief, the CRISPR-Cas9 protein carries the 20-nt guide RNA (blue) with a scaffold sequence (red). It will scan the gene until there is a complementary sequence with the guide RNA where Cas9 will make a precise double stranded break (orange triangles) upstream of a PAM sequence. From here the double stranded break is either repaired by Homology directed repair (HDR) which leads to a precise gene editing or non-homologous end joining (NHEJ) which can lead to random mutations. B) Design of PCR primers to detect CRISPR knockouts by overlapping one primer with the intended mutation site. Left panel: if there is no mutation the primer will bind and extension from the 3’ end will happen normally resulting in a PCR product. Right panel: if there is a mutation, the primer might not bind at the 3’ end and extension will not happen, resulting in no PCR product. C) Initial screen of potential knock-outs from 96-well. Top band (indicated by arrow) in all gels is β-actin which acts as a loading control to ensure equal amounts of DNA were used in the PCR. The bottom band is the respective band amplified by the HERV-K Gag primers (i.e. 5067.2 primers screened clones #1–#6 that were treated with the 5067.2 CRISPR plasmid). D) Clones that appeared to be knock-downs at the 96-well stage were grown out to 75 cm dishes and retested with the same primers in C). Surveyor assay was done on clones from 75 cm dish to see if a more sensitive method could pick up more subtle mutations. E) Initial PCR to ensure one product is being produced with primers that flank the site targeted by the CRISPR vector. F) Endonuclease treated DNA after combining equal amounts of wild-type and CRISPR treated clone in a 1:1 ratio. Control G and control C differ by 1-nt. When mixed together Control G+C duplexes are formed and the endonuclease will cleave the strands, resulting in the two bands 217 bp and 416 bp seen in lane 4.

Validation of EV collection method and presence of endogenous retroviral Gag in EVs

As outlined in the materials and methods the extracellular vesicles (EVs) were collected and analyzed by ZetaView® which is a nanoparticle tracking analysis (NTA) instrument. The samples of interested were collected after the 2,000 x g spin and diluted 1/50-1/100 in PBS. This method obtained the number of particles and size distribution of the particles. After the 2,000 x g
spin all cells and dead cells have been removed, leaving behind the EV population (Théry et al., 2006). The EVs population of interest, the exosome-like vesicles, pellets at 100,000 x g and ranges from 40 to 130 nm in size (Figure 12A). It was first necessary to validate that samples taken after the 2,000 x g spin accurately represented the EV population pelleted at 100,000 x g. To do this EVs from wild-type HEK293T and SH-SY5Y were collected after the 2,000 x g spin and were measure by ZetaView®. The size distribution of wild-type EVs in both samples were not distinguishable (Figure 12A,B,C). To validate pure EV populations were being obtained by differential ultracentrifugation, equal amounts of cell lysate and EVs were loaded onto a Western and probed for ALIX, TSG101, Flotillin-2, and Tubulin (Figure 12B&C). Flotillin-2 is a common EV marker that acts as a loading control that was enriched in the wild-type EVs (Figure 12B&C). As expected, ALIX and TSG101 were both enriched in the EVs compared to cell lysate (Figure 12B&C). Lastly, Tubulin was used as a loading control for cell lysates and was not present in the EVs serving as a negative control (Figure 12B&C). Taken together, this suggests that the isolation of EVs by ultracentrifugation worked and were free of cellular contamination.

In parallel with validating the EV collection method, wild-type EVs pelleted at the 100,000 x g spin were sent for proteomic analysis at The Ottawa Hospital Research Institute. Around 800 proteins were identified in the EV population, including common markers such as tetraspanin transmembrane family proteins (CD81, CD63), Flotillin, and ALIX. Gag-Pol polyprotein was among the most abundant protein identified in EVs produced by all cell lines, ranging from 0.1%-6% of the total peptide counts. All of the cell lines, except C8-D30, had some amount of Gag-Pol polyprotein in the EVs. Most of the Gag-Pol polyproteins from both human and murine cells lines originated from murine endogenous retroviruses (MLV and MMTV) (Figure 12E). For the human cell lines, all other proteins were identified as human in origin so it
is possible that proteomics identified them as mouse ERVs but the sequences are identical to human ERVs. Another possibility is mouse ERVs can infect human cell lines, suggesting that the cell lines could be infected with a mouse retrovirus (Kozak, 2015). Lastly, there is a possibility that the human cells lines are actually mouse cell lines, but this seems unlikely due to all other proteins being originated as human.
Figure 12. ERV Gag present in wild-type EVs. Wild-type EV were collected from various cell lines and purified by differential ultracentrifugation. 

A) Size distribution of purified EV particles (pelleted at 100,000 x g) diluted 1/1,000 in PBS obtained by nanoparticle tracking analysis (NTA) on the ZetaView® (n=3). Wild-type EVs collected from B) HEK293T and C) SH-SY5Y were purified by differential ultracentrifugation. An aliquot of supernatant after the 2,000 x g spin was diluted 1/50-1/100 in PBS and size distribution was calculated by nanoparticle tracking analysis (NTA) on the ZetaView® (top panel). Equal amounts of protein were loaded onto a 10% SDS-PAGE gel. Flotillin-2, ALIX, and TSG101, probed to confirm EV population with tubulin as the negative control (bottom panel; CL – cell lysate, EV – extracellular vesicles). D) The same EVs used to calculate size distribution in B) & C) were also used to determine the concentration of particles in the sample by nanoparticle tracking analysis (NTA) on the ZetaView®. The concentration of the EV samples were corrected by subtracting the number of particles in the EV-depleted media. The concentration was then multiplied by the volume of media that was collected. The number of cells at harvest were counted in parallel with EV collection. Particles/cell was calculated by dividing total number of particles by total number of cells for each technical replicate. The error bars represent the standard deviation of three independent experiments performed in duplicate. A paired two-tail student t-test was performed. p<0.05, *; p<0.001,**. E) Total spectral counts of Gag-Pol polyproteins from Proteomics data from purified human (panel 1) and murine (panel 2) EVs (n=1).
**Exogenous Gag is enriched in EVs but does not affect EV abundance**

Retroviruses have the ability to hijack host proteins in order to be released from the cell (Henne et al., 2015). MLV Gag and HIV Gag, both from exogenous retroviruses, have been shown to do this through the late-binding domains on the Gag protein that recruits TSG101 and ALIX, both proteins involved in the ESCRT pathway (Chudak et al., 2013). MLV (Gag/Pol) and HIV (Gag/Pol) vectors were transiently transfected into HEK293T. Both MLV and HIV Gag levels increased in the EV population as compared to GFP control (Figure 13A). There was no difference in the amount of ALIX or TSG101 in EVs normalized to Flotillin-2 when either MLV (Gag/Pol) and HIV (Gag/Pol) were expressed. Unfortunately, only one of the three replicates had a blot that was able to be properly quantified, because other blots either didn’t transfer properly or controls were not imaged at the same time as treated samples (Figure 13B). When analyzed by ZetaView®, EVs produced by cells expressing MLV (Gag/Pol) had a smaller percentage of particles in the range of 105 nm - 135 nm compared to GFP control (Figure 13C). The total number of particles measured by NTA and cell number measured by haemocytometer showed no difference when either MLV (Gag/Pol) or HIV (Gag/Pol) were expressed (Figure 13D, panel 1&2). Particles/cell was calculated but showed no difference for both conditions (Figure 13D, panel 3). Taken together this suggests that while both exogenous Gags can be expressed effectively and are enriched in the EV population, there is no change in EV concentration and only a small change in size distribution for MLV (Gag/Pol) overexpression.
Figure 13. Exogenous Gag increased in EVs but EV abundance unaffected. MLV (Gag/Pol) and HIV (Gag/Pol) were transfected into HEK293T cells with GFP as a transfection control. Media was changed to EV-depleted media 24 hrs post-transfection. The EVs were collected 14-18 hours later and purified by
differential ultracentrifugation. A) Equal amounts of protein were loaded onto a 10% SDS-PAGE gel (CL – cell lysate, EV – extracellular vesicles). Arrows represent the main Gag proteins recognized by the MLV Gag and HIV-1 Gag antibodies. 

B) EV protein quantification of ALIX/Flotillin-2 and TSG101/Flotillin-2 (n=1). After the 2,000 x g spin an aliquot of the supernatant was diluted 1/50-1/100 in PBS. C) Size distribution and D) total number of particles was determined by nanoparticle tracking analysis (NTA) on the ZetaView®. The concentration of the EV sample was corrected by subtracting the number of particles in the EV-depleted media. The number of cells at harvest were counted in parallel with EV collection. The error bars represent the standard deviation of three independent experiments performed in duplicate. One-way ANOVA followed by Dunnett’s multiple comparisons test was performed. For size distribution of EVs a two-way ANOVA followed by Dunnett’s multiple comparisons test was performed where matched values are stacked into a sub-column. p<0.01, **; p<0.001, ***.

Design, construction and validation of HERV-K101 and HERV-K109 Gag vector

Of the 13 HERV-K Gag ORFs only 2 were used to make expression vectors. To determine which of the 13 ORFs to express, multiple criteria were used. Lee & Bieniasz, 2007 reconstructed a fully infectious HERV and it was decided that the HERV-K Gag sequence chosen must be at least 90% similar to this reconstructed Gag. From Ruprecht et al., 2008 and the genomic location of HERV-K Gag_3238 and HERV-K Gag_5067 provided, we were able to determine that HERV-K Gag_3238 was HERV-K101 and HERV-K Gag_5067 was HERV-K109. Both HERV-K101 and HERV-K109 are common names used throughout the literature to denote these two HERV-K Gag at their respective genomic location. Both are 98.65% similar to the reconstructed Gag and have intact protease cleavage sites (George et al., 2011). It was also considered important that the HERV-K Gags chosen had conserved late-binding domains to interact with the ESCRT machinery. Both HERV-K101 and HERV-K109 Gag had PT/SAP and YPX₃_L motifs that could, in theory, bind TSG101 and ALIX, respectively. Similarly, HERVs were selected if there was a conserved zinc fingers which are involved in binding RNA (Chudak et al., 2013; Kraus et al., 2011). The next criterion was to confirm the location of the HERV-K Gag in the genome using NCBI Basic Local Alignment Search Tool (BLAST) and UCSC Genome Browser. The last criterion was to validate that the HERV-K Gags chosen were
expressed naturally. Both HERV-K101 and HERV-K109 have been detected in the blood of HIV-1 patients (Contreras-Galindo et al., 2012). HERVs RNA and protein expression have also been used as a marker for hESC (Human embryonic stem cells) and iPSC (Induced pluripotent stem cells), where HERV-K101 Gag and HERV-K109 Gag were among the highest expressed (Fuchs et al., 2013). Overall, HERV-K101 and HERV-K109 Gag were chosen because they met all the criteria by having the most conserved sequence with a fully infectious virus and are highly expressed in healthy and disease models. These sequences were the best options for studying the role of HERV-K Gag.

With these criteria, DNA encoding HERV-K101 and HERV-K109 Gag was synthesized with a 3x FLAG tag on the C-terminal of the Gag protein to ensure that matrix domain at the N-terminal was not interrupted and proper binding to the membrane could occur (Figure 14A, see Figure 7 for full design of HERV-K Gag G-blocks). The DNA was cloned into a pcDNA3.1 expression vector. The pcDNA3.1 vector was chosen since it had been used by previous groups to clone HERV-K constructs (George et al., 2011).

We next wanted to validate that the expression vector could produce mRNA. A RT-PCR was done on HEK293T and SH-SY5Y cells transiently transfected with HERV-K101 Gag and HERV-K109 Gag expression vectors, using GFP as a transfection control. The PCR primers were designed to overlap the FLAG sequence as well as the HERV-K Gag sequence, making it unique to these vectors. A no template control was used to show that there was no extraneous nucleic acid contamination. As well, a no reverse transcription (RT) control was done to show that the RNA isolation was pure and the primers were binding cDNA instead of genomic DNA still in the isolated RNA. Both HERV-K101 Gag and HERV-K109 Gag plasmids were used as positive controls to show where the intended product size would be. β – actin was used as a
control to show equal amounts of cDNA were used for each experiment (Figure 14C). The results showed that both HERV-K101 and HERV-K109 were able to express HERV Gag-FLAG transcript in both cell lines, although at very low levels.
Figure 14. Validation of HERV-K Gag vector cloning and expression. A) HERV-K Gag G-block design in the pcDNA3.1 expression vector. Note that a 3x FLAG tag is in the same reading frame as Gag protein. B) pcDNA3.1 SOD1 plasmid was used to clone in desired HERV-K Gag G-block. Validation of cloning was done by restriction digest (LD – ladder, -ve – no template control, XhoI – single digest with only XhoI, DD – double digest with XhoI and Kpn-1). C) mRNA collected from HEK293T and SH-SY5Y transiently transfected with HERV-K101 Gag and HERV-K109 Gag. An RT-PCR was performed with primers overlapping HERV-K Gag and FLAG (FLAG-HERV-K primers). β-actin was used as a control to show equal amounts of cDNA were used. Arrows indicate HERV-K Gag cDNA from expression vectors (top arrows) and β-actin (bottom arrows) for HEK293T (top gel) and SH-SY5Y (bottom gel).
Effect of HERV-K Gag expression on EV and cell number

Once validated, HERV-K Gag vectors were transiently transfected into HEK293T and SH-SY5Y cells using GFP as a transfection control (Figure 15A). The predicted length of the protein was estimated to be 76 kDa with the 3x FLAG tag. When FLAG was probed, there was a band in the EV population at the predicted mass. This suggests that the constructs were expressing Gag protein with the FLAG tag attached. ALIX/Flotillin-2 and TSG101/Flotillin-2 were quantified and normalized to the GFP control (Figure 15A). For HEK293T and SH-SY5Y there was no difference in the amount of ALIX or TSG101 in the EVs.

There was no change in the size distribution of EVs for HEK293T when either construct were expressed (Figure 15B). For SH-SY5Y there was a higher percentage of particles between the sizes of 135 nm – 195 nm when HERV-K Gag was expressed compared to GFP control (Figure 15B). This suggests that different cell types might utilize HERV-K Gag differently and in some cell types Gag may produce changes in the size of EVs released. It was also expected that when HERV-K101 Gag and HERV-K109 Gag was expressed the total number of particles/cell would increase. When expressed, there was no significant difference in particles/cell for both cell types though it appeared there could be a small decrease that may become significant with an increased number of replicates (Figure 15C&D).

There was a slight increase in the total number of HEK293T cells at collection when HERV-K109 Gag was expressed but not when HERV-K101 Gag was expressed (Figure 15C). There was no significant difference in total SH-SY5Y cell number when either HERV-K109 or HERV-K101 Gag was expressed. (Figure 15D).
**Figure 15. Effect of HERV-K Gag expression on EV concentration and cell proliferation.** HEK293T and SH-SY5Y were transiently transfected with HERV-K101 Gag (HERV-K101) and HERV-K109 Gag (HERV-K109) expression vectors. Media was changed to EV-depleted media 24 hrs post-transfection. EVs were collected 14-18 hours later and purified by differential ultracentrifugation. A) Equal amounts of protein were loaded onto a 10% SDS-PAGE gel (CL – cell lysate, EV – extracellular vesicles). FLAG was used to validate successful transfection of HERV-K Gag constructs (left panel). ALIX/Flotillin-2 and TSG101/Flotillin-2 protein levels were quantified in the EVs and normalized to GFP control (right panel). After 2,000 x g spin of EV purification, an aliquot of supernatant was diluted 1/50-1/100 in PBS. The size distribution and total number of particles for C) HEK293T and D) SH-SY5Y was determined by nanoparticle tracking analysis (NTA) on the ZetaView®. The concentration of the EV samples were corrected by subtracting the number of particles in the EV-depleted media. The number of cells at harvest were counted in parallel with EV collection. The error bars represent the standard deviation of three independent experiments performed in duplicate. One-way ANOVA followed by Dunnett’s multiple comparisons test was performed. For size distribution of EVs a two-way ANOVA followed by Dunnett’s multiple comparisons test was performed where matched values are stacked into a sub-column. p<0.05, *; p<0.01, **.

**Effect of knocking down HERV-K Gag on EV and cell number**

Three siRNA targeting multiple ORF of HERV-K Gag were designed and named Gag 1, Gag 2, and Gag 3. Each of the siRNA designed were selected because they (i) targeted ten or more of the ORFs of HERV-K Gag given to us by our collaborator and (ii) major off-targets consisted of other HERV-K Gag genes. Since each siRNA targeted different combinations of the HERV-K Gags each siRNA was transiently transfected individually and combined to determine which siRNA combination had the biggest effect on each cell line (i.e. Gag 1, Gag 2, Gag 3, and Gag 1-3 which contained all three siRNA). The siRNAs that seemed to have effect on HERV-K
Gag protein levels in EVs are shown (Figure 16). All other siRNA that did not have an effect on HERV-K Gag protein level in EVs are shown in Appendix, Figure 1. When probing for HERV-K Gag, there were certain bands in the EV lane that were detected in SH-SY5Y lysates that were not detected in HEK293T cells (Figure 16A&B). The band at ~70 kDa is likely the full length HERV-K Gag protein, while the smaller processed bands in SH-SY5Y are most likely MA, p15, and/or NC domains of Gag. In HEK293T there was a 60% decrease in the full length HERV-K Gag protein when Gag 1-3 siRNA was used. In SH-SY5Y the 10kDa band decreased 50% and 85% when treated with siRNA Gag 2 and Gag 3 respectively. When ALIX and TSG101 were quantified in there EVs there was no difference for cells treated with HERV-K Gag siRNAs compared to cells treated with control siRNA. (Figure 16A&B). There was also no difference in size distribution of particles and number of particles/cell for HEK293T and SH-SY5Y (Figure 16C,D,E).

When HEK293T was treated with the Gag 1-3 siRNA cocktail an interesting phenotype was seen. They displayed a stacking morphology rather than a monolayer typically seen in wild-type conditions and total number of cells was decreased by 40% at the time of collection (Figure 16D&F). This suggests that HERV-K Gag might play a role in cell proliferation. When HERV-K Gag is knocked down cell death might be occurring or HERV-K Gag may have an effect on cell number by preventing apoptosis or other cell death pathways.

In several experiments, it appeared that Gag expression may have small effects on the number of EV released or cell proliferation or survival, but these effects were not significant. More replicates and experiments will be required to determine conclusively if HERV-K Gag has any small effect on EV release or cell number.
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#### HEK293T

**Amount of HERV-K Gag/Flotillin-2 in EV normalized to average of Ctrl siRNA**

- Ctrl: 1.00
- Gag 1-3: 1.50

**Amount of ALIX/Flotillin-2 in EV normalized to average of Ctrl siRNA**

- Ctrl: 1.00
- Gag 1-3: 1.00

**Amount of TSG101/Flotillin-2 in EV normalized to average of Ctrl siRNA**

- Ctrl: 1.00
- Gag 1-3: 1.00

### B) siRNA

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#### SH-SY5Y

**Amount of HERV-K Gag/Flotillin-2 in EV normalized to average of Ctrl siRNA**

- Gag 2: 1.50
- Gag 3: 1.00

**Amount of ALIX/Flotillin-2 in EV normalized to average of Ctrl siRNA**

- Gag 2: 1.00
- Gag 3: 1.00

**Amount of TSG101/Flotillin-2 in EV normalized to average of Ctrl siRNA**

- Gag 2: 1.00
- Gag 3: 1.00

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*Processed HERV-K Gag proteins*
Figure 16. Effect of siRNA targeting HERV-K Gag on EV concentration and cell proliferation. A) HEK293T and B) SH-SY5Y were transiently transfected with siRNA targeting multiple HERV-K Gags. Equal amounts of protein were loaded onto a 10% SDS-PAGE gel where the arrows indicate the main HERV-K Gag bands that were quantified (CL – cell lysate, EV – extracellular vesicles). Quantification of ALIX/Flotillin-2, TSG101/Flotillin-2 and the major HERV-K Gag/Flotillin-2 bands are shown normalized to Ctrl siRNA protein levels. After the 2,000 x g spin an aliquot of the supernatant was diluted 1/50-1/100 in PBS. The C) size distribution and total number of particles was determined by nanoparticle tracking analysis (NTA) on the ZetaView® for D) HEK392T (top panel) and E) SH-SY5Y (bottom panel). The concentration of the EV sample was corrected by subtracting the number of particles in the EV-depleted media. The number of cells at harvest were counted in parallel with EV collection. All error bars represent the standard deviation of three independent experiments performed in duplicate. F) HEK293T 72 hours post-transfection after being treated with siRNA Ctrl or a cocktail of three siRNA targeting multiple HERV-K Gags. A paired two-tail student t-test was performed for comparison of HEK293T cells treated with Ctrl siRNA or Gag 1-3 siRNA. p<0.05, *. One-way ANOVA followed by Dunnett’s multiple comparisons test was performed for SH-SY5Y cells treated with Ctrl siRNA, Gag 2 or Gag 3 siRNA. p<0.05, *. For size distribution of EVs and Gag protein quantification for SH-SY5Y a two-way ANOVA followed by Dunnett’s multiple comparisons test was performed where matched values were stacked into a sub-column. p<0.05, *; p<0.01, **.
Discussion

Human endogenous retroviruses (HERVs), are derived from retroviruses that infected the inheritable germline (Young et al., 2013). HERVs have been implicated in a variety of disease models, but also have been theorized to be involved in the fusion of trophoblasts, implicating them in the evolution of mammals (Soygur & Moore, 2016; Vargas et al., 2009; Subramnian et al., 2011). HERV-K is the most recent HERV family to insert into the genome and is still able to produce mostly intact transcripts, including Gag (Bhardwaj et al., 2015). When Gag is expressed, it can oligomerize and produce viral-like particles (VLPs), contributing to the EV population (George et al., 2011). These VLPs do not contain the viral genome and resemble an immature virion (George et al., 2011). One way HERV-K Gag is theorized to be released as a VLP is through late-binding domains that can bind ALIX and TSG101 which recruits the ESCRT machinery (Chudak et al., 2013). The ESCRT machinery is also a key component in the formation of exosomes, a sub-class of EVs. The VLPs produced from HERV-K Gag are very similar to exosomes sharing similar; size, density, protein and lipid components (Gould et al., 2003; Colombo et al., 2014; Trajokovic et al., 2008). EVs have also been implicated in a variety of biological functions including; the metastasis of tumors, spread of prion proteins, and intercellular communication (Hoshino et al., 2015; Colombo et al., 2014). Separating the various groups of EVs from each other is extremely difficult due to lack of technology and similarities in size, density, and composition making. Also, it very likely that EVs isolated by ultracentrifugation are a composition of vesicles from multiple biogenesis pathways that share an overlapping set of markers. The possible connection between HERV-K Gag and its potential effects on the EVs population has yet to be explored. In this study, this lead us to investigate the role HERV-K Gag might have in the formation and content of extracellular vesicles.
After analyzing the data there was an insignificant difference in particles/cell when HERV-K Gag was overexpressed or knocked down in HEK293T and SH-SY5Y. As well, there was no difference in the amount of ALIX and TSG101 enrichment in the EVs when normalized to Flotillin-2. The most intriguing observation made, was the subtle differences in cell number for HEK293T cells which decreased when siRNA targeting HERV-K Gag was used and increased when HERV-K109 Gag was expressed. A similar trend was seen in SH-SY5Y and may become significant with more replicates. It is important to note that additional studies are needed to make any definite conclusions on HERV-K Gag effects on EVs and cell proliferation, if any. The techniques in this thesis helped gain a better understanding of the potential impact of HERV-K Gag on EVs. More in-depth investigation should be done to distinguish between the different EV sub-populations, potentially using biochemical methods or flow cytometry, to determine which EV sub-populations HERV-K Gag affects. Testing the effect of Gag on the comprehensive proteome of EVs using mass spectrometry, would be beneficial in determining any EV content that might change when HERV-K Gag is manipulated. We speculate that HERV-K Gag might affect cell number through competition with ESCRT machinery which could indirectly affect receptor tyrosine kinases that are recycled through the endosomal pathway, such as EGFR (Epidermal Growth Factor Receptor).

The biggest pitfall in this project was not having optimized overexpression and knockdown. This is probably the reason why there were only subtle changes, if any, in all areas measured. To truly see if HERV-K Gag plays a significant role in EV production/content or cell proliferation, we would need to optimize both HERV-K Gag plasmids and siRNA targeting HERV-K Gag. HERV-K101 Gag and HERV-K109 Gag vectors both have weak expression of mRNA (Figure 14C) and protein (Figure 15A) compared to optimized vectors such as MLV.
(Gag/Pol) and HIV (Gag/Pol) (Figure 13A). This may be due in part to cellular pathways that repress HERV Gag expression (Hanke et al., 2016). It could also be caused by accumulation of sub-optimal codons due to mutations in HERV-K that reduce the rate of translation. One way to overcome this could be to optimize codons to improve translation, which has been done in a previous study (Lee & Bieniasz, 2007). When designing the siRNA to target the 13 ORFs provided to us, there could potentially be more Gag transcripts that are being endogenously expressed. Another explanation for not having a 100% knockdown is that different cell lines could express variants of these ORFs of HERV-K Gags making it difficult to isolate a specific HERV to target as well as select a single siRNA to see an effective knockdown in multiple cell lines. Lastly, the antibody used might predominantly recognize HERV-K Gags that are not targeted by the siRNA, or cross-react with other proteins in the EV population. Lastly, future steps should be taken to discriminate between the different EV populations, through a combination of capture beads to better understand which EV sub-population HERV-K Gag is affecting. In parallel with codon optimization, repeating the experiments in more cell lines and more than three times will strengthen statistical analysis and confidence in the observed trends.

Using CRISPR to target HERV-K Gag

Our initial attempt to study HERV-K Gag involved using the genome editing CRISPR-Cas9 system. While the design and cloning of CRISPR-Cas9 vectors targeting individual or multiple HERV-K Gag was a success, the application proved to be more difficult. The MSC TERT cell line used was not adapted to growing in single cell colonies efficiently and made monoclonal selection difficult (Salzig et al., 2016). Even if the colonies survived to be screened, the screening method of PCR to detect potential knockouts was not ideal. Of the primer set...
designed, one of the primers 3’ end would sit on top of the predicted cleavage site of the Cas9 protein. This region was between the 3rd and 4th - nt upstream of the PAM sequence (Ran et al., 2013). The rationale for this was when there was an indel mutation in this site the primer binding would be sufficiently weakened that no PCR product would be made in carefully optimized PCR conditions (Figure 11B). If this region is conserved among other HERV Gags it is possible that the PCR primers bind to multiple HERV Gags and the potential knockout is masked. This could potentially have given us multiple clones that appeared wild-type but were actually knockouts at the genes of interest. A better way to approach this in the future would be to target as many HERV-K Gags as possible instead of individually targeting them. Yang et al., 2015 was able to completely eradicate 62 copies of porcine endogenous retrovirus (PERVs) using the CRISPR-Cas9 technology. They had two guide RNAs that would target a highly-conserved region in the pol gene. Following with this idea, the HERV-K Gag has a highly-conserved region in the CA domain (George et al., 2011). By targeting this region with two guide RNAs, there could be a better chance to observe a more drastic effect and study the global effect of HERV-K Gag.

**HERV-K Gag in EV production and content**

Our main goal was to investigate the role of HERV-K Gag in the formation of EVs through measuring size, concentration, and potential content that could be effected (ALIX, TSG101). When expressing HERV-K101 Gag and HERV-K109 Gag we were able to probe for the FLAG tag attached to the construct confirming that expression occurred and Gag was being packaged into EVs (Figure 15A). There was no change in ALIX and TSG101 levels in EV proteins when these constructs were expressed and no change when siRNAs targeting HERV-K Gag were used (Figure 15A, Figure 16A&B). There was however, a significant increase in the
number of larger particles (135 nm – 195 nm) in SH-SY5Y but no change in the HEK293T even though both cell lines expressed the HERV-K Gag constructs compared to GFP transfection control (Figure 15B). When HERV-K Gag was targeted with multiple siRNA there was no change in the particle size for either HEK293T or SH-SY5Y (Figure 16C). This suggests that when HERV-K Gag is expressed, and depending on the cell line used, there might be different size particles that form. The average size of a VLP typically ranges 90-120 nm while exosomes are 40-120 nm (Bachmann & Jennings, 2010; Hanson & Cashikar, 2012; Akers et al., 2013). Perhaps SH-SY5Y forms larger VLPs while HEK293T form exosome-like vesicles. Another possibility is exosomes have been shown to have subpopulations that vary in size and potentially when HERV-K Gag is expressed in SH-SY5Y it produces larger exosome-like vesicles while HEK293T produces smaller ones (Willms, E. et al. 2016). As with many phenomena, seeing different results in different cell lines with the same treatment is something that can be seen when studying EVs. When Colombo et al., 2013 knocked out ALIX in HeLa cells expressing the CIITA transcription factor (HeLa-CIITA) and dendritic cells (DCs), MHC-II expression was enhanced. When EVs were isolated from both cell lines, EVs from HeLa-CIITA had an enrichment of MHC-II while the DCs did not, despite expressing endogenous CIITA. This indicates that depending on the cell type there could be a difference in the results of the EVs, even if the conditions are the same (Colombo et al., 2013). One reason could be different types of vesicles are released depending on the cell line. This is seen in wild-type cells where each cell line releases subpopulations of exosome-like vesicles that each have their own unique protein and RNA composition (Willms et al., 2016).

When calculating particle concentration, particles/cell was used rather than particles/mL, because it was observed that HERVK Gag was affecting cell number. Both HEK293T and SH-
SY5Y showed a trend to decreased number of particles/cell when HERV-K Gag was expressed (Figure 15C&D). Expressing HERV-K Gag was expected to give an increase in the amount of particles/cell, similar to the trend of increasing particles/cell for MLV (Gag/Pol) or show no difference like HIV (Gag/Pol) (Figure 13D). This suggest that the trend to lower numbers of particles/cell might be unique to HERV-K Gag expression or that there is similar particle release but a greater increase in cell number making particles/cell decrease. Regardless, this deserves to be investigated further.

Certain siRNAs were more effective in reducing the amount of HERV-K Gag protein in the EVs (Figure 16A&B). The more effective siRNAs (Gag 1-3 for HEK293T and Gag 3 for SH-SY5Y) did show a trend to increasing the number of particles/cell (Figure 16D&E). This was the opposite of what was seen when HERV-K Gag was expressed in both cell lines, building confidence that these trends could be due to manipulation of HERV-K Gag levels, and may become significant with an increased number of experiments.

We saw a similar outcome of an apparent increase in the number of particles/cell when we knocked down ALIX in murine cell lines to see if there was an effect on murine endogenous retroviral (ERV) Gag recognized by the murine leukemia virus (MLV Gag) antibody (Figure 9). Even with a successful knockdown of ALIX, the amount of murine ERV Gag did not change in cell lysate or EVs, but there was a change in particles/cell, cell number, and cell size. In particular; N2a showed a significant increase in the number of particles/cell and BV2 and NSC34 showed a similar trend when the cells were treated with siRNA targeting ALIX (Figure 9C). When knocking down ALIX there is a mix of researchers who saw an increase in the number of EVs (Colombo et al., 2013) and others who saw a decrease (Baietti et al., 2012). Results in research on EVs rely heavily on the cell line used. In Baietti et al., 2012 EVs were purified from
MCF-7 (human breast cancer cell line) by high speed centrifugation and analyzed by NTA where only the sizes 30-110 nm were measured. Colombo et al., 2013 followed the same differential ultracentrifugation that was used in this thesis (Théry et al., 2006) and obtained similar results of increasing number of EVs in HeLa-CIITA and dendritic cells. Even though the protocols are similar, they resulted in drastically different outcomes. This could indicate that ALIX has a different role depending on the cell line used. Even within our experiments MEF cells had no difference in the cell number or particles/cell when ALIX was knocked down, providing support for the argument that the effect of ALIX on EV numbers could be cell dependent (Figure 9C).

In this set of experiments there was also a change in the size of BV2, N2a, and NSC34 cells 72 hours’ post-transfection with siRNA targeting ALIX (Figure 10A). Upon further investigation with BV2 it was noted that as the size of the cells started to increase, the total number of cells started to decrease significantly (Figure 10B). ALIX is not only part of the ESCRT pathway but also has a critical role in cytokinesis (Mercier et al., 2016, Christ et al., 2016). By analyzing the images of BV2 cells treated with siRNA targeting ALIX, it was obvious that the cells were becoming multinucleated (Figure 10D). It appeared that the cells were stalled after DNA replication and were unable to complete mitotic division, which has been reported previously for in vitro experiments (Morita et al., 2007; Christ et al., 2016). In my experiments, this led to cells dying more frequently than cells only treated with the control siRNA (Figure 10B). When in vivo models were made of ALIX knockout mice, they noted that they didn’t see any defects in cytokinesis, but rather the brain and testis of the knockout mice were smaller (Laporte et al., 2017). This was interesting because of where we saw the biggest effects; N2a, BV2, and NSC34, which are murine cell lines derived from different brain regions, while MEF which are derived from fibroblasts didn’t change.
For my experiments, when knocking down ALIX with siRNA, one potential reason for an increase in the number of particles/cell is that during cell death the cell releases its contents into the extracellular space. EVs were collected after the 2,000 x g spin for NTA meaning that smaller pieces of cell debris had not been removed. This debris could be at the same size/density as EVs and be counted as a particle using Nanoparticle tracking making it seem that there are a greater number of particles when it is actually cell debris (Théry et al., 2006). A similar situation might be happening in a smaller scale when HERV-K Gag is knocked down, leading to a trend toward an increased number of particles/cell. This could be due to cells undergoing cell death and the particles measured have an increased amount of cell debris, skewing the results. This could be tested by skipping the 10,000 x g step of the ultracentrifugation protocol and pelleting all of the material after the 2,000 x g step for control and treated cells. Then comparing both on a Western blot probing for cellular markers like tubulin, calnexin, GM130, and Tom20.

**HERV-K Gag and Cell Proliferation**

When some CRISPR vectors, such as 429.1, were transfected there was massive cell death and the cells were not able to be monoclonally selected. The 429.1 vector which in theory targeted 8 of the 13 HERV-K Gags would cause massive cell death unlike other HERV-K Gag CRISPR vectors such as 3276.2 which were transfected in MSC TERT cells at the same time but in a different plate. The most probable explanation is that off-target mutations were happening in critical genes causing cell death. Another possibility is HERVs are ~8% of the genome and if the CRISPR vector targets a conserved region between many of these HERVs there could be massive amounts of double strand breaks in the genome leading to cell death. Lastly, it is
possible that these HERV-K Gags play a critical role in cell survival and when mutated causes cell death.

When HERV-K Gag was expressed there is an increase of HERV-K Gag in EVs suggesting that our construct was expressed properly and formed EVs as expected (Figure 15A). As well, for HEK293T when HERV-K109 Gag was expressed there was a slight increase in the total number of cells at collection (Figure 15C). HERV-K101 Gag expressed in HEK293T, and SH-SY5Y expressing both HERV-K101 and –K109 Gag showed a trend to an increased total number of cells, which may become significant with more replicates (Figure 15C&D).

We also attempted to knockout the 13 ORFs provided to us from our collaborator with siRNA instead of CRISPR-Cas9. We used three different siRNA designed to target as many HERV-K Gag ORFs as possible. By western blot analysis HERV-K Gag was enriched in control EVs and HERV-K Gag appeared to have different banding patterns between HEK293T and SH-SY5Y (Figure 16). For HEK293T there was a band around ~70 kDa which was expected to be the full length HERV Gag. In SH-SY5Y there was the band at ~70 kDa corresponding to full-length HERV-K Gag as well as smaller processed proteins could be seen between 10-17 kDa. These bands are most likely the MA, p15, and NC domains of Gag that were cleaved by endogenous HERV-K protease (Mueller-Lantzsch et al., 1993; Kraus et al., 2011). When siRNA targeting HERV-K Gag were transfected into HEK293T and SH-SY5Y, Gag 1-3 siRNA gave a significant reduction in the 70kDa band in HEK293T cells while Gag 2 and Gag 3 siRNA gave a significant reduction in the 10kDa band in SHSY cells (Figure 16). What was interesting to note was when HEK293T was treated with siRNA Gag 1-3, there was a 40% decrease in the number of cells at collection (Figure 16D). As well, the cells displayed an interesting morphology of stacking onto each other (Figure 16F). One reason for this could be that the siRNAs have off-
target effects that are interfering with cell proliferation. Another reason could be that the siRNAs targeting HERV-K Gag were effective and we are seeing a phenotype caused by knocking down HERV-K Gag. It was a similar effect to that seen in the CRISPR vectors that targeted multiple HERV-K Gags as well as the opposite effect seen in cell number when expressing HERV-K Gag, suggesting that we could be seeing a potential effect but further investigation is needed.

To date, no papers have indicated a role for HERV-K Gag in cellular proliferation, but some papers have provided evidence that HERV-K transcripts and proteins are involved in the proliferation of cancer models (Wang-Johanning et al., 2003; Zhao et al., 2011; Zhou et al., 2016; Depil et al., 2002; Buscher et al., 2005; Boller et al., 1983; Bieda et al., 2001). Of particular interest, Zhou et al., 2016 showed that HERV-K Env was essential for promotion of tumorigenesis in breast cancer. When an shRNA was used to target HERV-K Env (shRNAenv) in breast cancer there was a decrease in cell proliferation and metastasis to the lungs. This effect was rescued by expression of a mutant HERV-K Env that was completely functional but not targeted by the shRNAenv. Stable transduced breast cancer cells for shRNAenv were further investigated by RNA-seq. Their results showed a significant reduction in Ras expression compared to cancerous controls, a common misregulated protein in breast cancer (McCubrey et al., 2007). Zhou et al., 2016 proposed a model in which HERV-K Env plays an undefined role in activation of the Ras pathway, which leads to the progression of breast cancer. Based on our data, we speculate that HERV-K Gag may also have an indirect role in this pathway.

Ras is a small GTP-binding protein involved in the Ras/Raf/MEK/ERK signalling pathway (Steelman et al., 2011). Various stimuli can activate cell surface receptors, that will in-turn propagate this signal transduction pathway (Martinelli et al., 2017). The ultimate goal of the Ras/Raf/MEK/ERK signalling pathway is to activate transcription in the nucleus which will
control cell proliferation, differentiation, embryogenesis and apoptosis (Akula et al., 2004; Madshus & Stang, 2009; Roepstorff et al., 2009). EGFR (Epidermal Growth Factor Receptor) is part of a family of transmembrane proteins present on the plasma membrane that can activate this signalling pathway (Roepstorff et al., 2009). The other receptors in this family are ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4 (Singh et al., 2016). Multiple ligands can bind and active EGFR including EGF (epidermal growth factor), TGF-α (Transforming growth factor alpha), Amphiregulin (AREG), heparin-binding EGF-like growth factor (HBEGF), betacellulin (BTC), epiregulin (EREG), and epigen (EPGN) making it a very dynamic receptor (Singh et al., 2016; Roberts & Der, 2007). Upon ligand-binding, EGFR dimerizes and becomes a functional kinase that can transphosphorylate (Madshus & Stang, 2009). While all of these ligands have been shown to have an interaction with EGFR; EGF, TGF-α, AREG, and EPGN only interact with EGFR and are among the most common studied (Singh et al., 2016). These ligands activate EGFR which will phosphorylate GRB2 (growth factor receptor binding protein 2) that will then activate SOS (son of sevenless) which will propagate the Ras cascade signalling, increasing transcription factors activity leading to cell proliferation (Figure 17; Wee & Wang, 2017; Madshus & Stang, 2009). Mutations in the Ras pathway are a common cause of many cancers and antibodies targeting this family of receptors, including EGFR, are a common therapeutic (Zhou et al., 2016; Roberts & Der, 2007).

Keeping EGFR balanced is key as hyperactivity has been linked to various cancers, while too little can cause apoptosis (Hyatt & Ceresa, 2008.; Rush et al., 2012). The current consensus among researchers is activated EGFR is ubiquitylated and recruited into clathrin-coated pits, where it is partially deubiquitylated after internalization (Madshus & Stang, 2009; Wegner et al., 2011). If the EGFR ligand dissociates or EGFR is deubiquitylated, EGFR becomes inactive and
is recycled to the plasma membrane (Figure 17; Roepstorff, K. et al. 2009; Longya et al., 2002; Wegner et al., 2011). Ligand-bound EGFR can also be reubiquitylated, recruiting the ESCRT machinery and budding active EGFR into vesicles in the MVB lumen (Longya et al., 2002; Tomas et al., 2014; Wegner et al., 2011). The MVB will fuse with the lysosome and EGFR will be degraded (Figure 17). While only speculative, we theorize that HERV-K Gag might play a role in this dynamic pathway influencing cell proliferation.
Figure 17. Potential effect of HERV-K Gag on EGFR degradation. A) Upon binding of a ligand EGFR is now activated and can phosphorylate downstream proteins. GRB2 becomes activated and in turns activates SOS which will propagate the Ras/Raf/MEK/ERK signalling pathway, activating
transcription in the nucleus promoting cell proliferation (blue arrows). It can also become ubiquitylated and recruit into clathrin-coated pits, where it is partially deubiquitylated after internalization. The ligand can dissociate or EGFR becomes deubiquitylated effectively inactivating EGFR. Inactive EGFR will be recycled to the plasma membrane to become re-activated (black arrows). Ligand-bound EGFR can also reubiquitylate, recruiting the ESCRT machinery and bud into the MVB. The MVB will fuse with the lysosome and EGFR will be degraded (red arrows). B) When HERV-K Gag is expressed, Gag can compete with ESCRT machinery when forming VLPs on the plasma membrane or endosome. This could prevent ESCRT from degrading EGFR, and an increase in the signalling cascade resulting in increased cell proliferation.

Hypothetically, HERV-K Gag could compete for the ESCRT proteins preventing EGFR signalling from being turned off in the endocytic degradation pathway. This would lead to continuous EGFR stimulation and increased cell proliferation seen when HERV-K109 is expressed in HEK293T cells (Figure 15C). There are papers showing that internalization of too much EGFR, can suppress its signalling and cause apoptosis (Rush et al., 2012; Hyatt & Ceresa, 2008). In regards to this proposed model, if HERV-K Gag is silenced, more ESCRT would be available to bud EGFR into MVB for degradation leading to suppression of cell proliferation, or possibly causing cell death. To study this in more depth, it would be interesting to use live cell imaging to capture the number of cells over the course of treatment in order to analyze cell proliferation. The small effect on cell proliferation in HEK293T cells is interesting and should be investigated further but at this time we can’t make any conclusion on the effect of HERV-K Gag on cell proliferation.

**Conclusion**

It is still important to continue to study the role of HERV-K Gag in EV production. Especially in light of the recent discoveries concerning the Arc protein (Ashley et al., 2018; Pastuzyn et al., 2018). Arc is a vital protein in neuronal plasticity and has been implicated in Alzheimer’s disease, neurodevelopmental disorders, and schizophrenia. Arc is homologous in
sequence and function to Group-specific antigen (Gag) polyproteins that may be derived from the Ty3/gypsy retrotransposon family (Ashley et al., 2018; Pastuzyn et al., 2018). These two papers show that Arc is able to mediate intercellular communication between neurons through EVs (Ashley et al., 2018; Pastuzyn et al., 2018). Arc assembles into a capsid structure similar to immature virion and delivers its own mRNA from one neuron to another. This shows that over time these retroviral elements can incorporate into the genome and become a vital part of normal cellular function. By continuing to study the basics of HERV-K Gag and its effects on cellular function, it is possible that we can uncover other symbiotic pathways that are similar to Arc.

In summary, we explored the effects of HERV-K Gag in the formation and content of EVs. Upon investigation, we discovered HERV-K Gag might affect cell growth and survival. HERV-K Gag might also have a different role in production of extracellular vesicles compared to exogenous Gag, like MLV or HIV. Further research is necessary to optimize expression and investigate new leads. For example, HERV-K Gag may recruit cellular proteins or RNAs into EVs that we did not detect with the EV markers we tested by western blot. Other EV markers should be examined and a larger analysis of EVs should be done, including proteomics and RNA-seq to uncover the potential impacts of HERV Gag on EV content. This study begins to outline the potential effects HERV-K Gag might have on EV contents, release and cell proliferation.
References


Appendix

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HERV-K Gag
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ALIX
TSG101

B) siRNA

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HERV-K Gag
Flotillin-2
Tubulin
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Appendix, Figure 1. siRNA targeting HERV-K Gag that were not effective. A) HEK293T and B) SH-SY5Y were transiently transfected with siRNA targeting multiple HERV-K Gags. Equal amounts of protein were loaded onto a 10% SDS-PAGE gel where arrows indicate the main HERV-K Gag bands that were quantified (CL – cell lysate, EV – extracellular vesicles). Quantification of ALIX/Flotillin-2, TSG101/Flotillin-2 and the major HERV-K Gag/Flotillin-2 bands are shown normalized to Ctrl siRNA protein levels. After the 2,000 x g spin an aliquot of the supernatant was diluted 1/50-1/100 in PBS and C) Size distribution was determined by nanoparticle tracking analysis (NTA) on the ZetaView (Particle Metrix). For D) HEK392T and E) SH-SY5Y the total number of particles was also determined by nanoparticle tracking analysis (NTA) on the ZetaView (Particle Metrix). The concentration of the EV sample was corrected by subtracting the number of particles in the EV-depleted media. The number of cells at harvest were counted in parallel with EV collection. All error bars represent the standard deviation of three independent experiments performed in duplicate. A One-way ANOVA followed by Dunnett’s multiple comparisons test was performed. p<0.05, *. For size distribution of EVs and SH-SY5Y Gag protein quantification, a two-way ANOVA followed by Dunnett’s multiple comparisons test was performed where matched values were stacked into a sub-column. p<0.05, *; p<0.01, **.
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