The HERV-K Np9 Protein Regulates Viability of Teratocarcinoma Cells

by

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Dedication

I dedicate this dissertation to my parents, whose endless sacrifices have given me the opportunities that I could have never imagined. Their endurance, dedication, and perseverance in the face of adversity and obstacles are my motivation to always strive to better myself in every aspect of life. I am fortunate to call them my parents.

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Table of Contents

Dedication .... **ii**

Acknowledgements .... **iii**

List of Tables .... **ix**

List of Figures .... **x**

List of Abbreviations .... **xii**

Abstract .... **xiv**

Chapter 1 .... **1**

INTRODUCTION **1**

Part I. Discovery and identification of endogenous retroviruses 1

Discovery of Murine Mammary Tumor Virus 1

Retrovirus and endogenous retrovirus 4

Mouse Mammary Tumor Virus and the discovery of Human Endogenous Retroviruses 7

Human Endogenous Retroviruses 9

Part II. Human Endogenous Retroviruses and their implications in human disease 14

HERV-K (HML-2) subfamily 14

HERVs and autoimmune disease and psychiatric disorders 16

HERVs and skin diseases 18

HERVs and solid tumors 18

HERVs and placental development 19

Discovery of a previously unknown HERV-K (HML-2) type 1 virus termed K111 20

HERV-K accessory proteins and their interaction with PLZF and/or TZFP 22

Np9 interacts with the Numb protein in the Notch signaling pathway 23

Np9 role in leukemogenesis 24

THESIS FOCUS AND HYPOTHESIS 25

Figures 27

Tables 32

Chapter 2 35

THE HERV-K ACCESSORY PROTEIN NP9 CONTROLS VIABILITY AND MIGRATION OF TERATOCARCIONOMA CELLS 35

Abstract 35

Introduction 36

Results 41

Np9 controls cellular viability when teratocarcinoma cells are stressed 41

Decreased Np9 expression increases sensitivity of teratocarcinoma cells to chemotherapeutic agents 43

Decreasing Np9 levels severely affects migration of NCCIT cells 44

Discussion 45

Materials and Methods 48

Cell lines and cell culture 48

CRISPR/Cas9 plasmid construct and transfection 48

Immunoblot analysis of Np9 knockdown 49

Starvation and MTT assay 50

Bleomycin and cisplatin treatments and MTT assay 51

Cell Cycle Analysis 51
Flow cytometry for activated caspase 3/7 apoptosis assay 52
Wound closure assay 52
Np9 complementation plasmid construct 53
Figures 55
Supplementary Figures 65
Chapter 3 67
DISCUSSION 67
HERV expression beneficial for development of the placenta 69
HERV expression linked to neurological and autoimmune diseases 70
HERV expression and cancer 72
HERV-K 111 74
The implication of Np9 in cancer and its disruptive influence on cell signaling pathways 75
The role of Np9 in teratocarcinoma cell viability 76
Conclusion 82
Figures 84
Appendices 87
The role of Np9 in brain development 87
Figures 91
Bibliography 93

List of Tables

Table 1.1 Classification of the *Retroviridae* family 32

Table 1.2 Classification of HERVs 33

Table 1.3 HERVs and associations in MS and schizophrenia 34

Table 1.4 HERV-K (HML-2) activation in solid tumors 34

List of Figures

Figure 1.1 Simplified retroviral life cycle 27

Figure 1.2 Structure of a retrovirus particle 27

Figure 1.3 The retroviral genome 28

Figure 1.4 Proviral organization of HERV-K (HML-2) and RNA transcripts 28

Figure 1.5 Identification and genomic organization of K111 proviruses 29

Figure 1.6 Expansion of K111 proviruses in humans took place after the Homo-Pan divergence.... 30

Figure 1.7 Identification of K111 proviruses in individual human chromosomes 31

Figure 2.1 Reduced expression of Np9 in NCCIT teratocarcinoma cells increases sensitivity to environmental stress 57

Figure 2.2 Reduced expression of Np9 in NCCIT teratocarcinoma cells increases sensitivity to chemical stress 59

Figure 2.3 Reduced expression of Np9 in NCCIT teratocarcinoma cells increases sensitivity to chemical stress, resulting in apoptotic cells 60

Figure 2.4 Np9 is essential for cell migration and wound closure 62

Figure 2.5 Re-introduction of Np9 into teratocarcinoma cells rescues the migration phenotype 64

Figure 3.1 Quantitative RT-PCR of *np9* mRNA in different teratocarcinoma cells. 84

Figure 3.2 The reduced expression of Np9 in teratocarcinoma had no effect on the c-myc and Wnt/ß-catenin signaling pathways 84

Figure 3.3 The reduced expression of Np9 in teratocarcinoma cells downregulated the MAPK/Erk signaling pathway 85

Figure 3.4 The reduced expression of Np9 resulted in the altered regulation of Numb, a Notch repressive element 86

Figure A.1 Relative Np9 expression in post-mortem adult human brain samples 91

Figure A.2 Forced expression of Np9 affects Notch activity in the developing cortex 92

Supplementary Figure 2.1 A map of pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid 65

Supplementary Figure 2.2 An illustration of the PiggyBac Transposon system 66

Supplementary Figure 2.3 HERV-K 101 Np9 66

List of Abbreviations

ALS	Amyotrophic Lateral Sclerosis
ALV	Avian Leukosis Virus
CSL	CBF1-Suppressor of Hairless-Lag1
DNA	Deoxyribonucleic Acid
ENV	Envelope
ERV	Endogenous retrovirus
HTDV	Human Teratocarcinoma-derived Virus
HERV	Human Endogenous Retrovirus
HERV-K	Human Endogenous Retrovirus Type-K
HIV	Human Immunodeficiency Virus
HK2	HERV-K (HML-2)
HML-2	Human Mouse Mammary Tumor-like Group 2
HTLV	Human T-cell Leukemia Virus
IN	Integrase
KO	Knock out
KD	Knock down
LINE	Long interspersed nuclear element
LNX	Ligand of Numb protein X
LTR	Long terminal repeat

MAML-1	Mastermind-like-1
MLV	Murine Leukemia Virus
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger RNA
MS	Multiple Sclerosis
MSRV	Multiple Sclerosis Retrovirus
NICD	Notch intracellular domain
NC	Nucleocapsid
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PBMC	Peripheral blood mononuclear cells
PBS	Primer binding site
PLZF	Promyelocytic zinc finger
PPT	Polypurine tract
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
RT	Reverse transcriptase
SINE	Short interspersed nuclear element
SLE	Systemic Lupus Erythematosus
Solo LTR	Solitary long terminal repeat
TGCT	Testicular Germ Cell Tumor
TZFP	Testicular zinc finger protein
VLP	Viral-like particles

Abstract

Human endogenous retroviruses are remnants of ancient germline infections that make up approximately 8% of the modern human genome. Most HERVs entered primates approximately 40 million years ago, and have been rendered inactive due to the accumulation of mutations. However, one of the most recent entrants into the human germline is the HERV-K (HML-2) subfamily, most having only integrated between 200,000 and 5 million years ago. The HML-2 subfamily is the most conserved and transcriptionally active, and some members have retained functional open reading frames (ORFs) to code for all of their viral proteins. HERV elements exist in the human genome as retroviral genes (*gag*, *pol*, and *env*) flanked by two long terminal repeats (LTRs). There are approximately 117 full-length copies and around 2500 solitary LTRs of HERV-K (HML-2) spanning multiple chromosomes. Even though no infectious HERV particle has been detected to date, HERV-K (HML-2) have been found to produce viral like particles in breast cancer, leukemia, lymphoma, melanoma, and teratocarcinoma. HERVs have been implicated in human biology, autoimmune diseases, and malignancies. There are two types of HERV-K (HML-2): type I is characterized by a 292 bp deletion at the boundary of the *pol* and *env* genes, while type II contains the full sequence for *pol* and *env*. The deletion in the type I virus causes an alternative splicing event that results in the expression of the accessory protein Np9, while type II expressed the accessory protein Rec.

In our previous study, we have detected the activation of HERV-K type I proviruses in the blood of patients with HIV-1 infection. The HIV-1 infection activates the expression of a novel HERV-K (HML-2) provirus, which we termed K111, and it is present in multiple copies in the centromeres of chromosomes throughout the human genome. At the time of detection, the K111 virus was not annotated in the most recent human genome assembly. While a few copies of the K111 provirus appear in the genomes of the extinct Neanderthal and Denisovan, modern humans have a least 100 copies of K111 spread across the centromeres of fifteen chromosomes, which suggests the expansion of K111 during the evolution of hominins. Most of the viral genes of K111 are mutated and cannot produce viable viral products, with one exception: the K111 virus contains an intact ORF for the Np9 protein and variants of Np9. For this reason, we investigated the effects of retaining the coding sequence for this accessory protein.

To this end, we decided to study the role of HERV-K Np9 in teratocarcinoma cells. Teratocarcinoma cells have been shown to express HERV-K mRNA, proteins and produce viral like particles. Also, NCCIT teratocarcinoma cells have been shown to express K111 in their viral particles. We show that decreasing the expression of Np9 in NCCIT teratocarcinoma cells increases the sensitivity of NCCIT cells to chemical stresses (bleomycin and cisplatin) and environmental stress (serum starvation). Further, we determined that Np9 is essential to the migration of NCCIT teratocarcinoma cells. These findings support the implication that the HERV-K accessory protein Np9 has oncogenic potential.

Chapter 1

INTRODUCTION

Part I. Discovery and identification of endogenous retroviruses

Discovery of Murine Mammary Tumor Virus

In 1933, a study published from the Jackson Memorial Laboratory suggested that the incidence of mammary tumors in laboratory mice was most likely a result of spontaneous development rather than the result of a genetic correlation. The study concluded that it is possible that the development of breast tumors have genetic and non-genetic influences, but that further experiments were necessary to determine the cause of breast cancer in laboratory mice (Jackson Memorial Laboratory 1933).

In a later study published in 1936, researchers at the Jackson Memorial Laboratory investigated the possible effect of nursing on laboratory mice developing breast tumors. They noticed that a strain of their laboratory mice (inbred A strain) had an about 88% incidence of mammary gland tumors, while strain X (Strong's CBA race) only had about a 10% incidence of developing mammary gland tumors. Combined with the previous study, investigators reasoned that laboratory mice perhaps developed

breast tumors as a direct result of contracting a milk-borne pathogen through nursing or the physical act of nursing (Bittner 1936).

These investigators, led by John J. Bittner, thus wanted to determine if nursing from mothers with a high incidence of breast tumors had any influence on the offspring developing mammary tumors. For the study, the scientists took three litters of pups (at around or less than twenty-four hours old) from A stock mothers (high incidence of mammary tumors), and the pups were nursed by strain X foster mothers (low incidence of mammary tumors). There was a total of nine females in the foster litters. The fostered female pups reached adulthood, were bred, and nursed their subsequent progeny; the nine females produced a total of 40 pups. Therefore, the nine strain A female pups were foster nursed by strain X mothers, and had undergone all the necessary roles that were thought to be essential to the development of breast tumors. Three of the fostered females developed mammary tumors (33%), while four developed primary lung tumors (44%), and two never developed any tumors (23%). Further, investigators noted tumors in the subsequent progeny of the fostered female pups: twelve developed breast tumors (30%), two developed breast and pulmonary tumors (5%), thirteen developed primary lung tumors (33%), and thirteen never developed any tumors (33%). The study led researchers to believe that the incidence of mammary gland tumors was affected by nursing rather than a chromosomal influence (Bittner 1936). However, there were critical flaws in the experimental design and execution that rendered inconclusive results. For example, investigators separated the pups from their biological mothers at up to 24 hours old, meaning that these pups had already been nursed and might have

had contracted a milk-borne pathogen from their birth mothers; these pups should have been separated from their birth mothers at birth. Furthermore, if there was no genetic correlation to the incidence of mammary gland tumors, nursing the pups by the foster mothers should have resulted in the pups' developing mammary gland tumors at the same percentage as the foster mothers. Also, if the physical act of nursing influenced the development of mammary gland tumors, the percentage of mice that developed mammary gland tumors would not fluctuate. The errors in experimental design are reasonable given how complex the etiology of mammary gland tumor is in laboratory mice.

A study published in 1942, led by Howard B. Andervont at the National Cancer Institute, found that milk is not essential for the development of mammary gland tumors, and the incidence of tumors is due to the milk-borne pathogen present in the milk of the mice with high incidence of mammary gland tumors. Also, investigators determined that the incidence of tumor development in mice depends on whether they were fed or subcutaneously injected with milk from mothers with a high incidence of mammary tumors, and the amount of milk administered (Andervont, Shimkin, and Bryan 1942).

From the 1930s to 1960s, investigators performed many studies to identify the infectious milk-borne particle responsible for the high incidence of mammary tumors in laboratory mice (Nandi and McGrath 1973). The infectious milk-borne particle was given the name mouse mammary tumor virus (MMTV). A major breakthrough occurred in the late 1960s and early 1970s, when investigators identified MMTV as an endogenous

retrovirus (reviewed in Weiss et al. 2006). During that time, two other endogenous retroviruses were identified: the Avian Leukosis Virus (ALV), and the Murine Leukemia Virus (MLV) (Robin A. Weiss 2006). The discovery of these three endogenous retroviruses sparked the beginning of our understanding that the transmission of such viruses was not exclusive to infection (exogenous) but could also be transmitted genetically (endogenous), and that endogenous retrovirus had tumorigenic properties (Cohen, Majors, and Varmus 1979; Bentvelzen and Daams 1969; Bentvelzen et al. 1970; Bentvelzen and Hilgers 1980).

Retrovirus and endogenous retrovirus

Retroviruses are infectious organisms belonging to the *Retroviradae* family, classified by their genetic material and life cycle. The *Retroviradae* family of viruses differ from other families in that most of these viruses contain genetic material consisting of Ribonucleic Acid (RNA), with the exception of Spumaviruses, which contain genetic material of consisting of Deoxyribonucleic Acid (DNA). Interestingly this family of viruses have distinct biological features as part of their viral life cycle:

1. The ability to convert their viral genetic material from RNA to DNA;
2. The ability to insert their converted genetic material (RNA to DNA) into the host genome (Gifford and Tristem 2003; Gifford et al. 2005).

These viruses received their name from the Latin *retro*, from their ability to defy the central dogma of molecular biology, in that genetic coding starts from DNA, with transcription into RNA, and then ultimately translation into proteins. However, in the

case of retroviruses, which are generally RNA viruses, the viruses can reverse transcribe their RNA into DNA, then DNA is transcribed into RNA, and then finally translated into protein. As part of the retroviral life cycle (Figure 1.1), the virus must integrate the viral genetic material into the host, ultimately taking permanent residence within the host genome (reviewed in Balvay et al. 2007).

A retroviral particle, also known as a virion, packages all components necessary to infect a host (Figure 1.2), complete the retroviral life cycle and propagate the next generation. On the surface of the virus is a lipid bilayer consisting of envelope proteins, a surface and a transmembrane glycoprotein. The inner layer of the virus contains three protein shells: the gag matrix protein surrounding the gag capsid protein, and the nucleocapsid (NC). The gag nucleocapsid protein encapsulates the viral genome (2 single stranded RNA molecules), the reverse transcriptase (RT), and the viral integrase (IN) enzyme, each component playing an integral part in the life cycle of a RNA virus (Figure 1.3) (reviewed in Voisset, Weiss, and Griffiths 2008). In addition, complex retroviruses produce accessory proteins, for example the human immunodeficiency virus (HIV) expresses the Tat and Rev proteins, while the human endogenous retroviruses type-K (HERV-K) human mouse mammary tumor virus like-2 (HML-2) expresses the Np9 and Rec accessory proteins (discussed below) (reviewed in Cavallari et al. 2011).

The *Retroviradae* family contains two subfamilies: *Spumaretrovirinae* and *Orthoretrovirinae* (Table 1.1). While *Spumaretrovirinae* has only one genus

(Spumavirus), *Orthoretrovirinae* contains six genera: alpha, beta, delta, epsilon, gamma, and lentiviruses. Alpha, beta, and gamma retroviruses have simple genomes, while delta, epsilon, and lentiviruses are retroviruses with complex genomes (see ICTV database https://ictvonline.org/index.asp). Most virus transmission comes about when an infected individual infects other individuals (horizontal transmission), such as it occurs with the Human Immunodeficiency Virus (HIV) and the Human T-cell Leukemia Virus (HTLV) (Coffin, Hughes, and Varmus 1997). The retrovirus and host dynamic can either be parasitic or symbiotic (Ryan 2004). Currently, we know that mammalian retroviruses can be parasitic in nature, but are also associated with tumors, immunodeficiency, autoimmunity and neurological disorders (Weiss 1996). For example, HIV is a retrovirus that is responsible for the depletion of $CD4^{+}$ T cells in the host, ultimately resulting in the Acquired Immunodeficiency Syndrome (AIDS) (Okoye and Picker 2013).

The endogenous retroviruses (ERVs) are a subset of retroviruses that share similar properties; however, they differ in one important aspect in that ERVs can and did infect a germ line cell (Nelson et al. 2003). Retroviruses are unique among the viruses because they can reverse transcribe their RNA genome and insert their genome into the host cellular DNA as part of the viral replication cycle (Gifford and Tristem 2003; Gifford et al. 2005). Normally, retroviruses are only capable of infecting somatic cells and, while the cell is proliferating, the virus can insert its retroviral genome into the host (Coffin, Hughes, and Varmus 1997). The inserted retroviral sequence exists in all progeny cells; this retroviral element inserted in the host genome is termed a provirus (Baltimore

1975). However, in the case of an endogenous retrovirus, they can effectively become "fixed" by infecting a germ cell and integrating their proviral elements into the host DNA. The offspring of an infected host would inherit the proviral element and any subsequent descendants would inherit the proviral elements in a Mendelian fashion (Gifford and Tristem 2003; Subramanian et al. 2011; Jern and Coffin 2008; Feschotte and Gilbert 2012). Therefore, the mode of transmission of an endogenous retrovirus is considered "vertical", meaning from parent to child (generation to generation), while the exogenous retroviruses are transmitted "horizontally", from somatic cells to other somatic cells (within the same generation). Endogenous retroviruses have been found to be present in plants and vertebrates, from mice to humans (Bannert and Kurth 2006; Feschotte and Gilbert 2012; Herniou et al. 1998).

Mouse Mammary Tumor Virus and the discovery of Human Endogenous Retroviruses

Since its initial discovery, the mouse mammary tumor virus has been classified as a betaretrovirus, and is responsible for the incidence of mammary tumors in mice (see ICTV database https://ictvonline.org/index.asp). This virus exists as both an endogenous and exogenous virus, and replicates initially in lymphocytes (Nandi and McGrath 1973; Finke and Acha-Orbea 2001; Golovkina, Dudley, and Ross 1998). The infected lymphocytes carry the virus to the mammary gland, where they transmit the virus to the mammary epithelial cells (Golovkina, Dudley, and Ross 1998; Finke and Acha-Orbea 2001). The virus replicates within the mammary tissues, maximizing the

viral load within the mammary epithelial cells to maximize virion production, and ultimately inducing mammary tumors. The mammary tumorigenesis occurs after the proviral DNA integrates itself near host cellular proto-oncogenes, thus activating transcription (Callahan and Smith 2000). However, the MMTV integration is not site specific but, with more virion production, the likelihood that the proviral DNA will integrate near a proto-oncogene increases (Faschinger et al. 2008).

The MMTV genome is approximately 9 kb, and the viral genome transcribes five different viral genes: *gag*, *dut-pro*, *pol/in*, *rem* and *env* (Ross 2008). The envelope (Env) protein is transcribed from the *env* gene, and functions in viral entry into host cells (Coffin, Hughes, and Varmus 1997). In addition, the Env protein is involved in MMTV-mediated transformation, thus promoting tumorigenesis (Katz et al. 2005; Ross et al. 2006).

Shortly after the discovery of MMTV, researchers began looking for a human equivalent. At first, they detected MMTV-like proteins in human breast cancer biopsies by probing with antibodies against the mouse viral proteins (Mesa-Tejada et al. 1982; Day et al. 1981), but that proved to be inconclusive. During the search for a MMTV-like sequence in human cellular DNA, Callahan et al. (1982) described finding a family of human endogenous retroviruses with sequence similarity to MMTV, one of the first descriptions of a human endogenous retrovirus in the human genome. Nonetheless, subsequent studies showed that there was no reactivity between human antibodies and MMTV proteins (Goedert, Rabkin, and Ross 2006). In 1986, Ono et. al cloned the full

sequence of the first human endogenous retroviruses related to MMTV, the provirus was termed HERV-K101 (Ono et al. 1986). Other investigators attempted to identify MMTV-like sequences in humans, and reported identifying MMTV-like sequences in 30% of human breast cancers (Liu et al. 2001; Pogo et al. 1999), although a full MMTV-like sequence has yet been identified. Investigators also reported finding MMTV-like *env* sequences by PCR in the DNA from human breast cancer tissue and, unlike MMTV, where *env* sequences can be found in normal mouse cells as well as mouse transformed mammary tissue, in humans the MMTV-like *env* sequences were tumor specific, and not detected in healthy tissue (Wang et al. 1995; Etkind et al. 2000). However, it is still unclear if a full MMTV sequence is present in human tumors or that the MMTV sequence in humans are the result of mouse contamination. Nonetheless, the use of MMTV probes led to the discovery of Human Endogenous Retroviruses (HERVs).

Human Endogenous Retroviruses

The commencement of the Human Genome Project resulted in the sequencing of 3.2 billion base pairs in the human genome, giving us a vast amount of information about our DNA sequences. From this large collaborative project, we now know that only 1.1% of our DNA sequence codes for protein, and the remaining 98.9% was considered "junk" DNA (Lander et al. 2001; Collins et al. 2004; Chial 2008). These sequences were referred to as "junk" DNA because at the time it seemed that they either had no biological function or their function was not yet known. However, it is becoming clear

that many of the these sequences are not “junk” after all and can serve as regulatory elements that are integral to the workings of cells, functioning as enhancers, promoters, silencers and insulators (Maston, Evans, and Green 2006; Plank and Dean 2014). These “junk” DNA sequences also consists of repetitive elements, such as Alu elements, LINE and SINE elements (Long and Short Interspersed Nuclear Elements, respectively) and Human Endogenous Retroviruses (HERVs) (Subramanian et al. 2011; Weiss 2006; Bannert and Kurth 2006; Lander et al. 2001).

One of the major insights from the Human Genome Project was that 8% of the human genome consisted of human endogenous retroviruses (Weiss 2006; Bannert and Kurth 2004; Subramanian et al. 2011). These exogenous retroviruses began to infect primates about 60 million years ago (about the same time that primates emerged) (Martin 1993), and subsequently these retroviruses became endogenized through integration of the viral genomes into the germ line. About 40 million years ago there was a divergence leading to the evolution of New World and Old World monkey lineages (Glazko and Nei 2003). Hominoidea (humans and great apes) emerged and separated from Old World monkeys about 35 million years ago. The last common ancestor of humans and chimpanzees lived about 6 million years ago (Martin 1993; Goodman 1999). Since the initial infection and integration of retroviruses into the primate genome, new retroviruses have infected and integrated into the germ line, thus explaining the quantity of endogenous retroviruses in our genome today (Nelson et al. 2003; Jern and Coffin 2008; Subramanian et al. 2011).

It is estimated that there are more than 30 HERV families or lineages, however there are still HERVs that have not been classified due to fragmented or incomplete sequences (Gifford and Tristem 2003; Gifford et al. 2005). Since HERVs were once exogenous retroviruses, these viruses share the same genetic organization and structure, and are classified by their sequence similarities to other retroviruses. Human endogenous retroviral structure consists of the following: a 5' LTR, PBS (primer binding site), *gag*, *pro* (protease), *pol* (polymerase), *env* (envelope), PPT (polypurine tract) and a 3' LTR (Bannert and Kurth 2004). However, over the course of human evolution and subsequent re-infections and integrations, HERVs have accumulated insertions, mutations and deletions, rendering them as incomplete viruses (Hughes and Coffin 2004). Therefore, the classification and grouping of HERVs is based on the *pol* sequence homology to other exogenous retroviruses, and each HERV family is denoted by a letter that signifies the resemblance to a specific primer binding site (Table 1.2) (Gifford and Tristem 2003; Cohen and Larsson 1988; Larsson, Kato, and Cohen 1989). For example, the primer binding site for HERV-K resembles that of the t-RNA binding site for lysine.

Not all HERVs have been classified into retrovirus taxonomy due to their incomplete or fragmented sequences (Gifford and Tristem 2003; Gifford et al. 2005). Another major obstacle in finalizing a complete list of HERVs and their copy number is that the copy number within each group varies greatly and proviral sequences can also exist as solo LTRs. The advancement in bioinformatic tools has facilitated the detection and classification of retroviral elements in the genome. A recent study identified 3173

proviral sequences in the human genome, and upon further analysis of the HERV sequences they grouped 38% of them into specific groups (Class-I gamma-like or epsilon-like, Class-II beta-like, or Class-III spuma-like), whereas 62% of the HERVs remained unclassified (Vargiu et al. 2016). Even within each class there is heterogeneity in sequence, so most HERVs considered to be part of the same group almost never share exact homology.

The discovery of endogenous retroviruses and their implication in diseases has sparked an interest in studying human endogenous retroviruses, especially as they make up a large quantity of our genome. To date, there is no known replication competent human endogenous retrovirus (Löwer et al. 1993; Seifarth et al. 1998; Bieda, Hoffmann, and Boller 2001), meaning there is no known HERV that maintained its ability to undergo all processes necessary to complete the viral life cycle and re-integration. However, the more recently integrated families of HERVs have been shown to express some of their viral proteins (Tönjes et al. 1996; Seifarth et al. 1998; Johnston et al. 2001; Sugimoto et al. 2001; Wang-Johanning et al. 2001; Yi et al. 2001; Ruda et al. 2004). Since the initial infection of humans by exogenous retroviruses and their subsequent integration into germ line cells, these endogenous retroviruses have undergone mutations, deletions and insertions that have rendered them unable to replicate and form infectious viruses (Hughes and Coffin 2004). For some time, it was thought that these germ line infections were just remnants of ancient infections and did not have any biological purpose. However, in the 1970s it was discovered that some teratocarcinoma cell lines produce viral like particles (VLPs). The viral like particles

were later called human teratocarcinoma-derived viruses (HTDV) and it was later determined that a subfamily of Class-II HERVs were responsible for coding HTDV (Löwer et al. 1981; Löwer et al. 1993; Bieda, Hoffmann, and Boller 2001; Bhardwaj et al. 2015). This later finding was a major milestone in the study of HERVs, as it was the first evidence that some family of HERVs were still capable of coding for their viral proteins and even produced particles, although they are perhaps incapable of replication. In the recent years, our laboratory demonstrated that VLPs produced by some teratocarcinoma and breast cancer cells lines can be passaged from cell to cell (Contreras-Galindo et al. 2015).

HERV-K Human mouse mammary tumor-like group 2 (HML-2) (HK2) is a subfamily belonging to the Class-II beta-like group of HERVs (May and Westley 1986). To date, this subfamily of HERVs is known to be the most recent one to infect and integrate into the human genome with a few exceptions (Barbulescu et al. 1999; Okahara et al. 2004). Most of the HERV-K (HML-2) subfamily integrated itself between 200,000 and 5 million years ago, it is the most conserved subfamily of retroviruses, is transcriptionally active, and has potentially functional open reading frames (ORFs) for all of its viral proteins (Tönjes et al. 1996; Seifarth et al. 1998; Johnston et al. 2001; Sugimoto et al. 2001; Wang-Johanning et al. 2001; Yi et al. 2001; Ruda et al. 2004). There are approximately 3000 HK2 sequences in the modern human genome (Paces et al. 2004). Of these, about 2500 exist as solo LTRs; however, about 117 HK2 full-length proviral sequences have been identified (Subramanian et al. 2011; Babaian and Mager 2016; Wildschutte et al. 2016; Marchi et al. 2014; Contreras-Galindo et al. 2012;

Contreras-Galindo et al. 2013; Turner et al. 2001). It is the HERV-K (HML-2) subfamily upon which I focus on in this thesis.

Part II. Human Endogenous Retroviruses and their implications in human disease

HERV-K (HML-2) subfamily

The class II HERV-K group family consists of subfamilies (HML-1 to HML-11), each a result of ancient germline infections (reviewed in Hohn, Hanke, and Bannert 2013). The HERV-K (HML-2) subfamily is one of the most intensely studied subfamilies because, as one of the most recent entrants into the human genome (between 200,000 to 5 million years ago), some of these viruses have undergone the least number of mutations, insertions and deletions. As a result, the HML-2 subfamily contains proviruses that have maintained complete or near complete open reading frames for all viral polyproteins. To date, there are 117 known HML-2 proviruses and more than 2500 solitary LTRs in the genome (Subramanian et al. 2011; Babaian and Mager 2016; Wildschutte et al. 2016; Marchi et al. 2014; R. Contreras-Galindo et al. 2012; Contreras-Galindo et al. 2013; Turner et al. 2001). Even though currently there are no known intact viruses capable of reinfection, there has been substantial evidence of HERV-K viral particles in certain cancers, and these viruses have maintained genome flexibility in that within the viral particles exists both potentially infectious RNA and DNA genomes exist (Seifarth et al. 1998; Büscher et al. 2005; Wang-Johanning et al. 2001; Bieda, Hoffmann, and Boller 2001; Löwer et al. 1993; Muster et al. 2003; Contreras-Galindo et

al. 2012; Contreras-Galindo et al. 2013; Contreras-Galindo et al. 2008; Contreras-Galindo et al. 2007; Dube et al. 2014).

There are two types of HERV-K HML-2 present in the genome, type I and type II, with the type I virus being characterized by the 292 bp deletion at the boundary of *pol* and *env* genes; type II does not contain such a deletion (N. Bannert and Kurth 2004). HERV-K have all the similar genes (*gag*, *pro, pol*, *env*) as other retroviruses, and much like complex retroviruses, it codes two accessory proteins: the type I virus encodes Np9, and type II encodes Rec (Figure 1.4) (Bannert and Kurth 2004).

Complex retroviruses use intricate transcriptional regulation systems to control the expression of their viral mRNAs (Coffin, Hughes, and Varmus 1997). For instance, HIV produces primary transcripts that are unspliced to express the structural proteins Gag and Pol (Kim et al. 1989; Pomerantz et al. 1990). The viral Env glycoprotein is expressed from a singly spliced mRNA, while the accessory proteins Tat and Rev are expressed by mRNAs that are spliced multiple times. Complex retroviruses are not inherently efficient at splicing mRNAs, which results in nuclear retention or degradation of primary transcripts within the nucleus (Schneider et al. 1997; Maldarelli, Martin, and Strebel 1991; Nasioulas et al. 1994). However, the HIV Rev accessory protein functions as a regulatory protein and transports unspliced or partially spliced mRNA out of the nucleus and into the cytoplasm.

Interestingly, HERV-K also encodes an accessory protein to aid in the shuttling of unspliced or partially spliced mRNAs (Yang et al. 1999; Magin, Löwer, and Löwer 1999). The HERV-K Rec accessory protein was first named as the central open reading frame (cORF), but later renamed to Rec due to its functional and sequence similarity to both HIV Rev and HTLV Rex (Lower et al. 1995). The Rev and Rex proteins are transported into the nucleus through the interaction of the nuclear localization signal and cellular import factors, and within the nucleus they function to suppress splicing, stabilize full length and singly spliced transcripts, and export transcripts to the cytoplasm (Magin, Löwer, and Löwer 1999). However, the expression of the other HERV-K accessory protein Np9 and its physiological importance or function is still not well understood, as is discussed below.

HERVs and autoimmune disease and psychiatric disorders

HERVs have long been implicated in multiple sclerosis and psychiatric disorders (Perron and Lang 2010; Antony et al. 2011; Slokar and Hasler 2016). Published work on HERVs describes an association with multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system (Trapp et al. 1998). The cause of the disease is highly debated and not well understood, but it is thought to be immune-mediated, with association with genetic and possible viral factors. Several HERV transcripts and proteins have been detected in the central nervous system of patients with MS and patients with schizophrenia. Of the HERV families, HERV-W, HERV-H, HERV-F, and HERV-K have been reported to be associated with MS (Table 1.3)

(reviewed in Slokar and Hasler 2016). These HERV families exist as multiple copies in the genome, with some copies retaining coding potential for viral genes, some of which are activated in MS. Also, the multiple sclerosis retrovirus (MSRV), a member of the HERV-W family, has been reported in MS patients as a biomarker and a regulator of aberrant immune responses (Antony et al. 2011). Studies showed that serum and cerebral spinal fluid from patients with MS have detectable levels of MSRV in 50% of the French population and 100% of the Sardinian population, in 6% the blood of control groups, and 40% of patients with other neurological diseases (Garson et al. 1998; Serra et al. 2001; Dolei et al. 2002). Studies show that patients with MS with enhanced expression of MSRV in the brain and increased copy number in the blood have a poorer prognosis (Dolei et al. 2002; Serra et al. 2003).

HERVs have also been associated with other autoimmune diseases such as systemic lupus erythematosus (SLE). Patients with SLE produce autoantibodies against components of the cell nucleus and double-stranded DNA; these autoantibodies can induce inflammation that can lead to arthritis, vasculitis, neurological disorders, and hypertension (Nakkuntod et al. 2013). Studies reported that T-cells from patients with active SLE contain global hypo-methylation and demethylation at promoters of many genes and contribute to the pathogenesis of the disease (Nakkuntod et al. 2013). Also, peripheral blood mononuclear cells (PBMCs) from SLE and rheumatoid arthritis patients show an elevated expression of HERV-E and HERV-K *gag* genes, respectively (Okada et al. 2002; Piotrowski, Duriagin, and Jagodzinski 2005; Ejtehadi et al. 2006). The implication is that hypo-methylation in SLE patients inadvertently activates the

expression of HERVs (Nakkuntod et al. 2013). Further, the rise of epigenetics has somewhat spurred a level of acceptance that the expression of certain HERVs is controlled or maintained by epigenetic mechanisms (Lavie et al. 2005).

HERVs and skin diseases

HERVs are also associated with skin diseases such as psoriasis, and viral like particles have been detected in skin lesional plaques, urine, and lymphocytes in individuals with psoriasis (Dalen et al. 1983; Iversen 1983; Guilhou, Vannereau, and Theunynck 1982). It is estimated that 2-3% of the world's population is affected by psoriasis (Gupta et al. 2014). Psoriasis is an inflammatory skin disease in which keratinocytes proliferate in an uncontrolled manner and T-lymphocytes are recruited into the skin (Bessis et al. 2004). The expression of the mRNA of HERV-K, HERV-W and HERV-E has been shown to be elevated in lesional psoriatic skin when compared to non-lesional skin (Molès, Tesniere, and Guilhou 2005; Hohenadl et al. 1999), and HERV-E Env protein has been detected in psoriatic skin samples (Molès, Tesniere, and Guilhou 2005).

HERVs and solid tumors

HERVs have been implicated in solid tumors, and there is mounting evidence of HERV-K (HML-2) subfamily activation in germ cell tumors, ovarian cancer, melanoma, breast cancer, prostate cancer, lymphomas, leukemias, and sarcomas (Iramaneerat et

al. 2011; Wang-Johanning et al. 2007; Reiche, Pauli, and Ellerbrok 2010; Büscher et al. 2005; Serafino et al. 2009; Ishida et al. 2008; Wang-Johanning et al. 2008; Golan et al. 2008; Dehm 2008; Goering, Ribarska, and Schulz 2011; Contreras-Galindo et al. 2008; Depil et al. 2002; Schiavetti et al. 2002). Considering that 8% of the human genome consists of these ancient HERV infections, and the levels of viral mRNA, proteins and virions in various cancers, it is important to consider whether these ancient germline infections play roles in human malignancy (Table 1.4). The general understanding is that HERVs have the potential to cause disease or cancer through an array of mechanisms, such as the expression of oncogenes, the inactivation of tumor suppressor genes through mutational insertions, homologous recombination, and the transcription of oncogenes and growth factors through the LTRs (Gonzalez-Cao et al. 2016). Many studies identified the expression of HERV proteins in cancer tissues, but the causative role of HERVs in cancer development remains controversial; however, data from animal models demonstrated that endogenous retroviruses are potentially oncogenic (Gonzalez-Cao et al. 2016).

HERVs and placental development

The expression of HERVs can also be beneficial for human development and expression of their genes has been detected in normal tissues. The mammalian placenta has elevated expression of endogenous retroviruses, and HERVs have been shown to play a critical role in placental development. The proper formation and expansion of the villous syncytiotrophoblast is crucial for the function of the human

placenta; the syncytiotrophoblast layer expands through intracellular fusion with the differentiating mononuclear villous cytotrophoblast. Certain HERV envelopes have been associated with placental development, including those from the families HERV-W and HERV-FRD, termed syncytin-1 and syncytin-2, respectively (reviewed in Bolze, Mommert, and Mallet 2017). These HERV envelope proteins are crucial for the evolution of the placental development and are thought to facilitate the retroviral transmission from mother to child. The syncytin proteins appear to be involved in maternal immune system suppression against the placenta, promote membrane fusion, and participate in the syncytiotrophoblast differentiation. Therefore, the retention of HERVs is essentially a double-edged sword, and their expression in human biology can either be beneficial or detrimental.

Discovery of a previously unknown HERV-K (HML-2) type 1 virus termed K111

In recent years, our research group identified a phylogenetically distinct HERV-K (HML-2) viral RNA sequences termed K111 in the blood of individuals afflicted with HIV-1 but not in the blood of healthy individuals or patients with breast cancer or lymphoma (Figure 1.5A) (Contreras-Galindo et al. 2012; Contreras-Galindo et al. 2013). At the time, mining through the version of the annotated human genome we could not find the corresponding sequence. However, when we looked at the genome of one of our closest relatives, the chimpanzee, we located a virus similar to that of K111 in the telomere, close to the q arm of chromosome 7. These HERV-K sequences were only about 95% similar to those of the closest known HERV-K (HML-2) proviruses (HML-2),

but they were 98% similar to one provirus found in the genome of chimpanzee (Contreras-Galindo et al. 2013). Integration of HERV-K (HML-2) in human DNA produced 5-6 bp target site duplication sequences on each side of the provirus. The target site duplication is not apparent in all HERV-K (HML-2) proviruses, as homologous recombination between different HERV-K (HML-2) proviruses created hybrid proviruses with different flanking target site sequences (Hughes and Coffin 2005). Using this information, we designed primers that target the flanking regions and the internal viral genes of the K111 provirus to amplify K111 in humans by PCR (Figure 1.5B), and found that although K111 RNA was particularly expressed in the blood of patients with HIV (Figure 1.5A), K111 exists at the genomic DNA level in all the 189 human samples that we tested, including healthy subjects. K111 exists in the human genome as a full-length virus as well as a solo LTR. K111 integrated into the centromeric repeat CER:D22Z3, which has been assigned to the centromere of chromosome 22 (Dunham et al. 1999; Metzdorf, Göttert, and Blin 1988) and created a characteristic GAATTC target site duplication flanking each side of the proviruses after integration (Figure 1.5B).

From our study, we discovered that during the course of evolution, the hundred to a thousand copies of K111 expanded by a mechanism similar to homologous recombination, and this virus is now detected in chromosomes 1, 4, 7, 9, 12, 13, 14, 15, 17, 18, 20, 21, 22, X and Y, but not in the other human chromosomes; solo LTRs were detected in chromosomes 15 and 22 (Figure 1.6A and Figure 1.7). Furthermore, we determined that the integration of K111 took place around the time of the Homo-Pan divergence based on the molecular analysis of the LTRS of K111, and thus K111

insertions were only detected in chimpanzee and modern day humans, but not in other primates or monkeys (Figure 1.6B). Through the course of many recombination events, the K111 virus has accumulated mutations in the all of its viral genes with the exception of *np9*, suggesting that K111 is not capable of replication, but can still produce the viral Np9 accessory protein (discussed further in Chapter 2). Also, the NCCIT teratocarcinoma cell line has been shown to express the K111 provirus (Contreras-Galindo et al. 2017). This further suggested that due to its abundance, Np9 might play an important role in human health and disease. Indeed, Np9 has been linked to oncogenesis, as we now will discuss.

HERV-K accessory proteins and their interaction with PLZF and/or TZFP

When Np9 or Rec is co-expressed with the promyelocytic zinc finger tumor suppressor protein (PLZF), which normally functions to repress the expression of the proto-oncogene c-*myc*, the interaction between Np9/PLZF or Rec/PLZF results in the sequestering of PLZF's normal biological function, resulting in the de-repression of c-*myc*. This de-repression promotes cell proliferation, which is particularly important considering that one of the hallmarks of cancer is unregulated cell proliferation (Denne et al. 2007). Much like PLZF, the testicular zinc finger protein (TZFP) acts as a transcriptional repressor of genes and, while the biological function of TZFP and the target genes is still not well understood, one of its functions is the interaction with and repression of the androgen receptor. A study showed that the interaction between HERV-K Rec and TZFP results in de-repression and transcriptional activation

(Kaufmann et al. 2010), and the inducible expression of Rec in the testis of transgenic mice resulted in the development of lesions similar to that of testicular germ cell tumors (Galli et al. 2005). Further, Rec supports germ cell tumor growth in mice through its interaction with PLZF. PLZF is a spermatogonia specific transcription factor that is essential for the regulation and maintenance of the sperm pool, and the improper control of spermatogenesis and maturation of gonocytes is thought to promote the development of germ cell tumors (Boese et al. 2000).

Np9 interacts with the Numb protein in the Notch signaling pathway

The Notch signaling pathway is highly conserved functions in developmental and homeostatic processes, and is known to be a pro-proliferative pathway; perturbations in the pathway can lead to oncogenesis (Aster, Pear, and Blacklow 2017). In simple terms, the canonical Notch pathway is activated through extracellular interactions between Notch ligands (Jagged-1, Jagged-2, Delta-like 1, 3 and 4) and Notch receptors (Notch 1-4). The Notch receptors consist of an extracellular domain, a transmembrane portion, and an intracellular domain. Once activated, the Notch intracellular domain (NICD) undergoes a series of proteolytic cleavages by ADAM17, ADAM10, and γ-secretase, thus releasing the NICD and translocating to the nucleus. In the nucleus, the NICD interacts with the DNA-binding protein CBF1-Suppressor of Hairless-Lag1 (CSL) and the co-activator Mastermind-like-1 (MAML-1) to promote gene transcription. However, the notch pathway is inhibited by the Numb protein (Chillakuri et al. 2012). Mammalian Numb interacts with the RING-type E3 ubiquitin ligase and together tether the NICD

prior to its translocation to the nucleus, resulting in the proteasomal degradation of the NICD (Kopan and Ilagan 2009). Interestingly, the HERV-K Np9 accessory protein has been shown to interact with the RING-type E3 ubiquitin ligase LNX (ligand of Numb protein X), and alters the normal biological function of Numb, thus promoting the activation of the Notch signaling pathway (Armbruester et al. 2004). It is still unclear if the Np9, LNX and Numb interaction forms a trimeric complex, or if Np9 can sequester LNX from Numb.

Np9 role in leukemogenesis

A recent study investigated the role of HERV-K Np9 in the growth of leukemia, and the possible mechanisms of action. HERV-K Np9 was shown to be highly expressed in approximately 56% of leukemia patients, while it was hardly detected in CD34+ hematopoietic stem cells and healthy blood cells (Chen et al. 2013). The findings were that Np9 played a role in promoting the growth of leukemia cells, and immunocompromised NOD-SCID mice injected subcutaneously with Np9 over-expressing Raji cells developed larger tumors than the control population. To further elucidate the function of Np9 in leukemia, the authors investigated the role of Np9 in different cell signaling pathways known to be abnormally activated in human leukemia, and Np9 activated the Wnt/ß-catenin, ras/ERK, and the c-myc/AKT signaling pathways, in addition to that of Notch1 (Chen et al. 2013). These findings have prompted us to search for a role for Np9 over-expression in another important malignancy that has

been particularly linked to over-expression of HERV-K virus and the production of viral particles, teratocarcinoma.

THESIS FOCUS AND HYPOTHESIS

In recent years, our group discovered a previously unknown HERV-K (HML-2) type 1 virus termed K111. We determined that there are hundreds to thousands of copies of the K111 provirus in the modern-day human genome, and the K111 provirus is present in many human chromosomes. Although this virus is highly mutated and is incapable of replication, it still contains the open reading frame for the viral accessory protein Np9. For this reason, we decided to study the function of the HERV-K accessory protein Np9 in teratocarcinoma cells.

Teratocarcinoma is the ideal model for our studies, as teratocarcinoma cell lines over-express HERV-K virus and have been shown to produce viral like particles that contain K111 RNA. Treatment for teratocarcinoma has progressed since its discovery, with improved prognosis for patients, and since the introduction of platinum based therapy first year survival has greatly improved even with disseminated disease. However, it is estimated that 20% to 30% of patients with metastatic germ cell tumors relapse following initial treatments, and toxicity associated with the use of chemotherapeutic agents is still a major concern.

We hypothesized that the Np9 protein functions as an oncoprotein in teratocarcinoma, and could be targeted for therapeutics. We further found that the Np9 accessory protein has oncogenic potential controlling viability and migration of teratocarcinoma, both of which are hallmarks of cancer. Our data presented in this thesis would suggest that targeting Np9 in teratocarcinoma will lead to better outcomes if proper drugs/delivery mechanisms can be obtained.

Figures

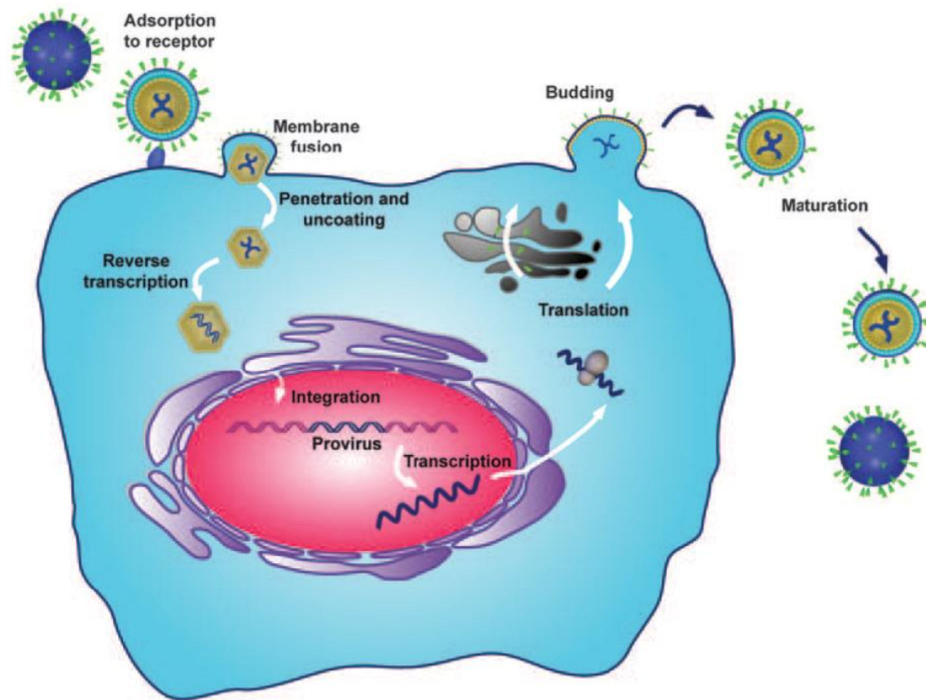


Figure 1.1 Simplified retroviral life cycle. Adapted from Voisset et al. 2008.

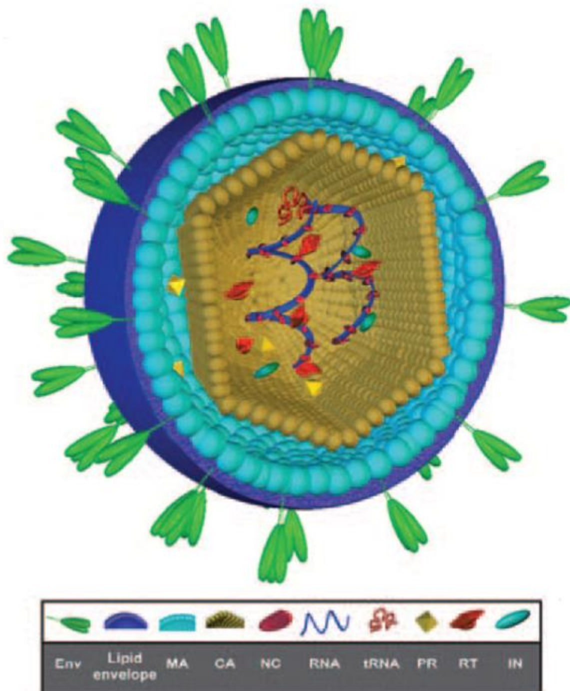


Figure 1.2 Structure of a retrovirus particle. The virion is surrounded by a lipid bilayer derived from the host during the budding of a mature virus, and also contains the envelope glycoproteins (Env). Inside the lipid bilayer are three protein shells: the viral Gag matrix protein (MA) surrounding the Gag capsid protein (CA), and the nucleocapsid (NC). Encapsulated in the protein core is the viral genome (either as 2 copies of single-stranded RNA molecules or a double-stranded DNA molecule in the case of Spumaviruses, the viral reverse transcriptase (RT), viral protease (PR), viral integrase enzyme (IN), and cellular tRNA molecule. Adapted from Voisset et al. 2008.

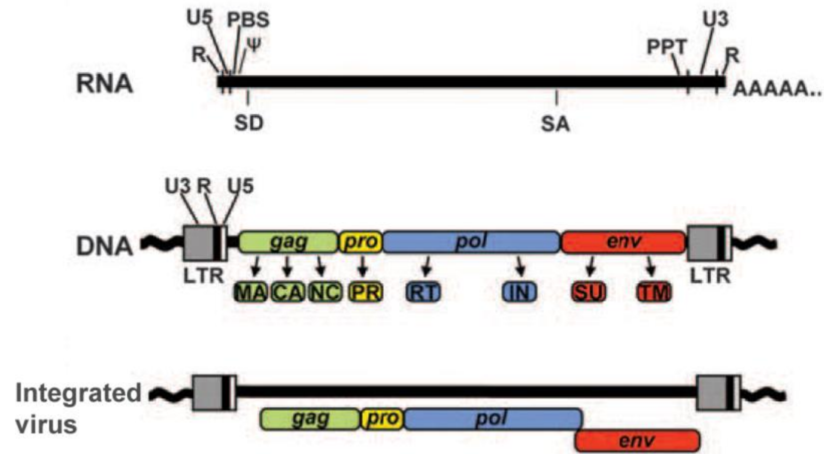


Figure 1.3 The retroviral genome. Generalized retroviral genome in the RNA, DNA, and integrated forms. The conserved features of a retrovirus: repeated region (R), U3 and U5 unique elements, primer binding site (PBS) (used for initiation of reverse transcription), encapsidation signal (Ψ), polypurine tract (PPT). All infectious retroviruses have at least one splice donor (SD) and splice acceptor (SA) site used for the expression of a spliced transcript encoding env. During reverse transcription, the LTR is formed, which contains promoter and enhancer elements. At least four genes are present in all infectious retroviruses (*gag*, *pro*, *pol* and *env*). Additional retroviral proteins were described in Figure 1.2. Adapted from Voisset et al. 2008.

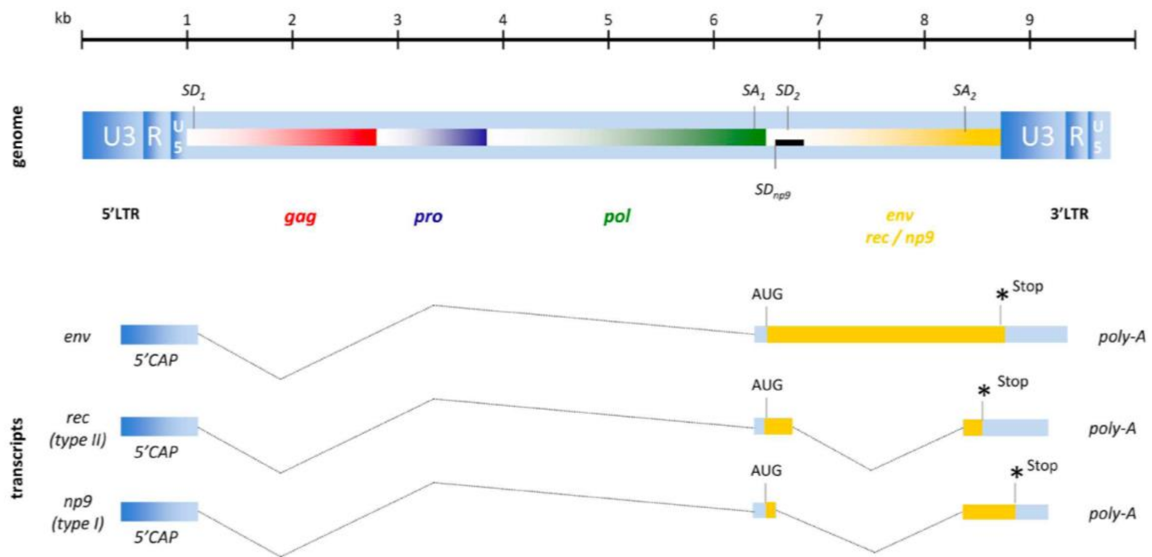
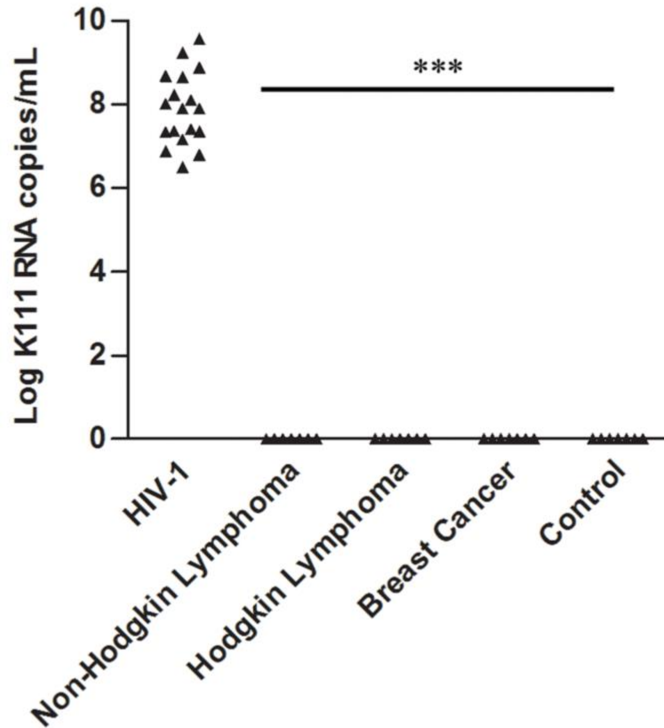


Figure 1.4 Proviral organization of HERV-K (HML-2) and RNA transcripts. Adapted from Hohn et al. 2013.

A)



B)

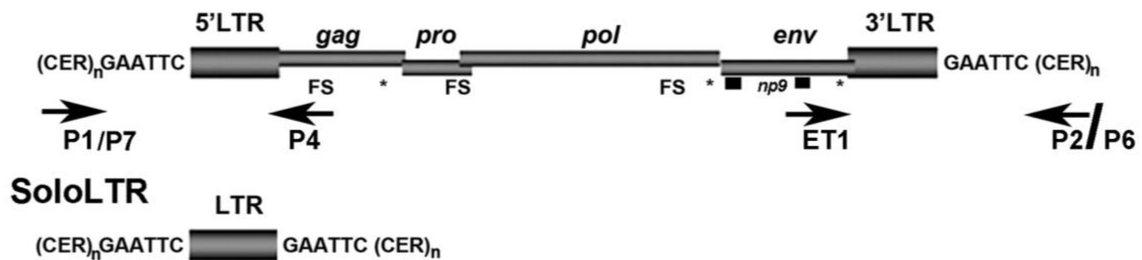


Figure 1.5 Identification and genomic organization of K111 proviruses. (A) Quantitation of K111 *env* titers by qRT-PCR in the plasma of patients with HIV-1 and other diseases. The K111 *env* titers were measured by qRT-PCR using the probe K111P that specifically discriminates the K111 *env* gene from other HERV-K (HML-2) *env* sequences due to a 6 bp mutation. K111 titers were detected in the plasma of patients with HIV infection but not in the plasma of healthy individuals or the plasma of patients with lymphoma or breast cancer. (B) Genomic organization of K111 full-length and Solo LTR, target site duplication "GAATTC", and centromeric flanking sequences (CER:D22Z3). Frame shift (FS) and stop codon (asterisks) mutations are indicated. The positions of the primers used to amplify K111 5'LTR and 3'LTR insertions are indicated by arrows. Taken from Contreras-Galindo et al. 2013.

A)

B)

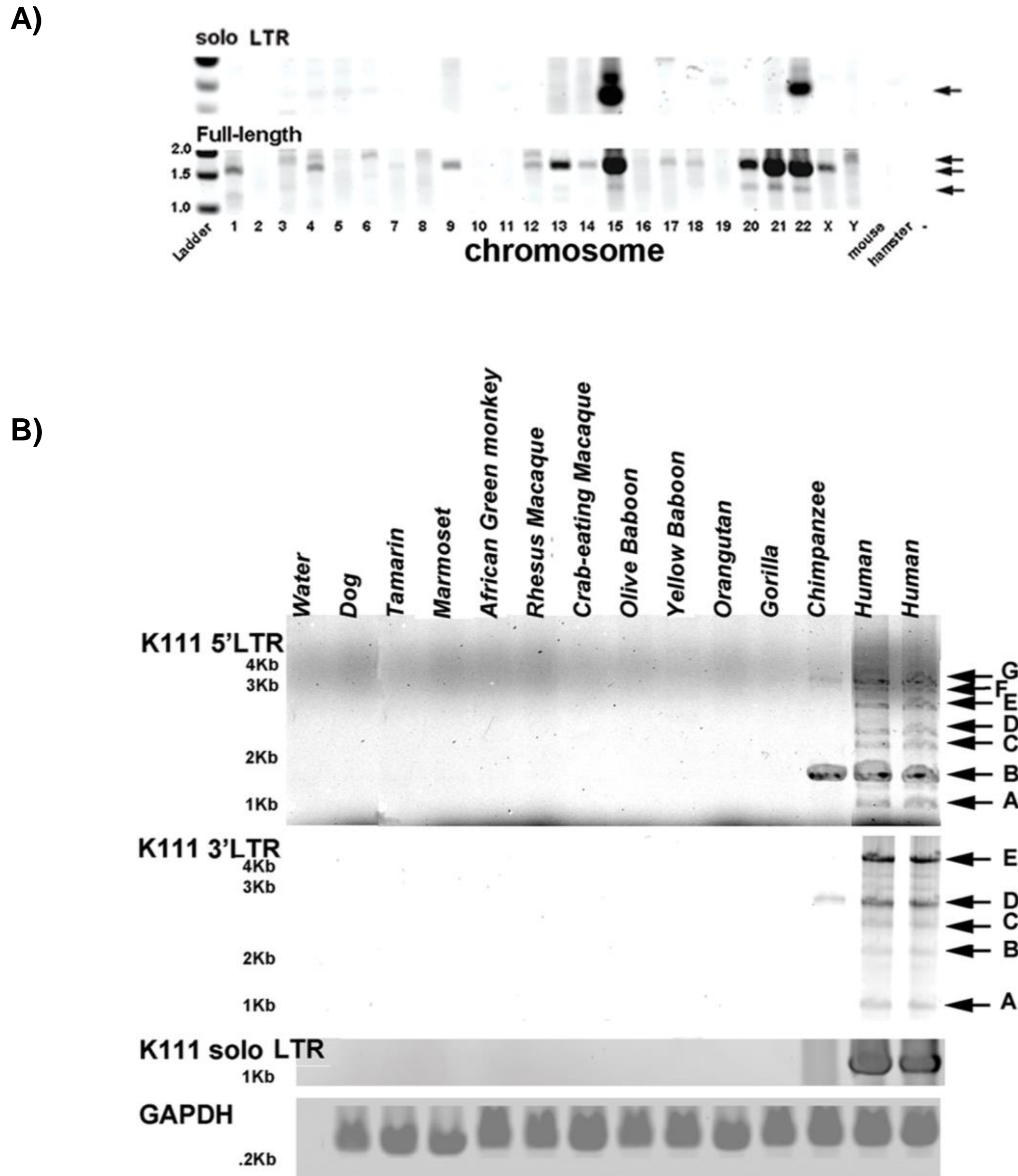


Figure 1.6 Expansion of K111 proviruses in humans took place after the Homo-Pan divergence. (A) Detection of K111 insertions in human chromosomes. DNA from human-hamster hybrid cell lines, which carry only one specific human chromosome, were analyzed by PCR for the presence of K111 using the set of primers that amplify the 5'LTR insertion as described. Other bands (for example the PCR products detected in chromosomes 3, 6, and 8) were shown by sequencing to be the result of non-specific PCR amplification. (B) Detection of K111 full-length and Solo LTR insertions from DNA of New and Old-World primates. Full-length K111 proviruses were detected by PCR only in the human and chimpanzee. The 5' flanking K111 insertions were amplified with the primers P1/P7 and P4 and the 3' flanking K111 insertions were amplified with the primers ET1 and P6. Solo LTRs were amplified by PCR using the primers P1 and P2 and were seen only in humans. Arrows indicate individual insertional polymorphisms. Taken from Contreras-Galindo et al. 2013.

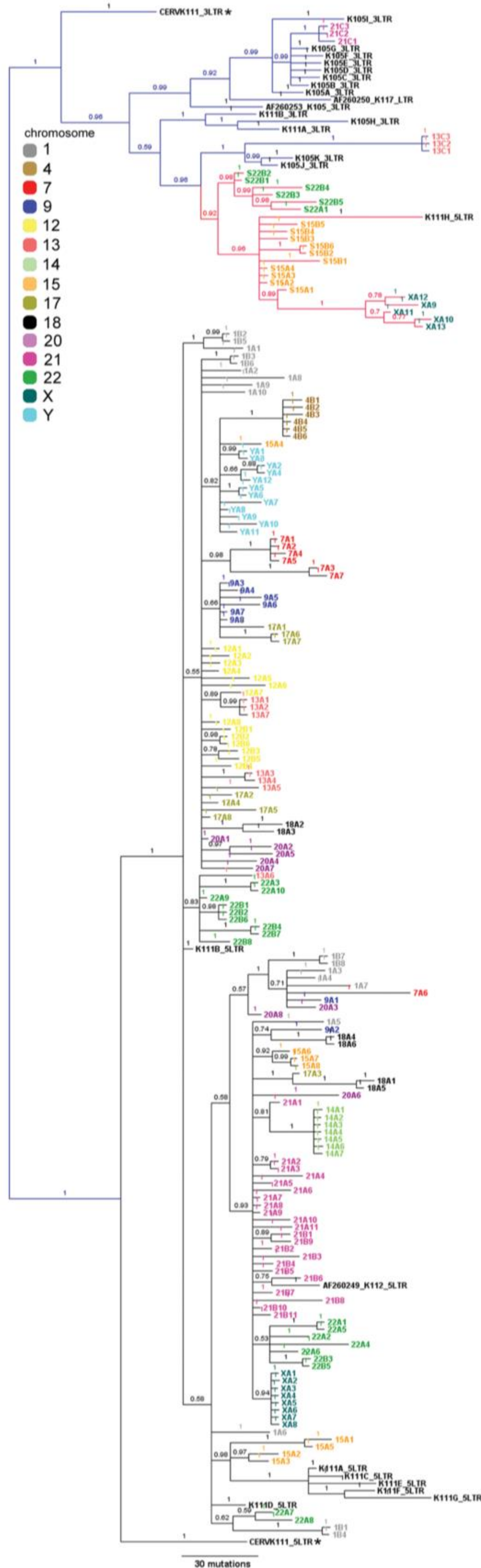


Figure 1.7 Identification of K111 proviruses in individual human chromosomes. Bayesian inference tree of the 5'LTR and 3'LTRs, and flanking CER:D22Z3 sequences, of K111 proviruses amplified from human chromosomes. Sequences are colored to indicate from which human chromosome they arise. Note that each color tends to cluster to specific evolutionary branches, indicating that individuals K111s often spread within an individual chromosome. Posterior probability values > 70 are shown for an unrooted tree. The tree was generated using Bayesian inference with four independent chains run for at least 1,000,000 generations until sufficient trees were sampled to generate more than 99% credibility. 5' (black), 3' (blue) LTRs, and Solo LTR (red) lineages are shown along with the chimpanzee LTRs (CERV-K111). Informative nucleotide sequence substitutions were found that are specific for the K111 group of sequences found in each chromosome. Taken from Contreras-Galindo et al. 2013.

Tables

Subfamily and genus	Species infected	Example(s)
Orthoretrovirinae		
Alpharetrovirus	Avian	Avian Leukosis Virus Rous Sarcoma Virus
Betaretrovirus	Mice Sheep Primates	Mouse Mammary Tumor Virus
Gammaretrovirus	Avian Cats Reptiles Primates	Murine Leukemia Virus
Deltaretrovirus	Cattle Primates	Human T-lymphotropic virus
Epsilonretrovirus	Fish	Walleye dermal sarcoma virus
Lentivirus	Primate Sheep Cats Horses	Human immunodeficiency virus type-1
Spumaretrovirinae		
Spumavirus	Primates Cats Cattle	Human foamy virus

Table 1.1 Classification of the *Retroviridae* family. Adapted from Voisset et al. 2008 and the ICTV database https://ictvonline.org/index.asp.

HERV Family	Primer	Alternative Name
Class I		
HERV-Z6969907	Not determined	
HERV-ADP	$tRNA^{Thr}$	ADP-pol
HERV-E	$tRNA^{Glu}$	
HERV-F	$tRNA^{Phe}$	
HERV-F (type b)	$tRNA^{Phe}$	
HERV-FRD	$tRNA^{His}$	
HERV-H	$tRNA^{His}$	RTLV-H
HERV-H49C23	No LTRs	
HERV-I	$tRNA^{Ile}$	RTLV-I
RRHERV-I	$tRNA^{Ile}$	
ERV-9	$tRNA^{Arg}$	
HERV-F (type c)	$tRNA^{Phe}$	
HERV-P	$tRNA^{Pro}$	HuRRS-P
HERV-R	$tRNA^{Arg}$	ERV-R
HERV-R (type b)	$tRNA^{Arg}$	
HERV-T	$tRNA^{Thr}$	HERV-S71
HERV-W	$tRNA^{Trp}$	MSRV
HERV-XA	$tRNA^{Phe}$	
Class II		
HERV-K (HML-1 to HEML-4)	$tRNA^{Lys}$	
HERV-K (HML-5)	$tRNA^{Ile}$	
HERV-K (HML-6)	$tRNA^{Lys}$	
HERV-K (HML-9)	Not determined	
Class III		
HERV-L	$tRNA^{Leu}$	
HERV-S	$tRNA^{Ser}$	
HERV-U2	Not determined	
HERV-U3	Not determined	

Table 1.2 Classification of HERVs. Adapted from Gifford and Tristem 2003.

HERV	Disease associations	Specific action
HERV-F/H	Multiple sclerosis in addition to certain cancers	Expressed in MS patients, particularly in lymphocytes
HERV-W	Multiple sclerosis and schizophrenia	Expressed in white matter lesions in MS patients, association with schizophrenia also reported
HERV-K	Multiple sclerosis	Disease marker

Table 1.3 HERVs and associations in MS and schizophrenia. Taken from Anthony et al. 2011.

Tumor type	HERV transcripts	HERV proteins
Ovarian	*env*, *np9*, *rec*	Env
Melanoma	*pol*, *env*, *gag*, *env, rec*	Pol, Env, Gag, Rec, Np9
Prostate	*gag*, *env*, *np9*	Gag
Lymphoma	*gag*, *env*	Env
Leukemia (CLL, CML, ALL)	*pol*,	Env
Germ cell seminoma	gag, env, np9, rec	Gag, Env, Np9, Rec
Breast	*env, gag, rec*	Env, Rec

Table 1.4 HERV-K (HML-2) activation in solid tumors. In addition, HERV-K viral particles are expressed in all tumor types listed in the table with the exception of ovarian cancer (Wang-Johanning et al. 2007, 2013, 2008; Zhao et al. 2011; Wang-Johanning et al. 2012; Golan et al. 2008; Herbst, Sauter, and Mueller-Lantzscht 1996)

Chapter 2

THE HERV-K ACCESSORY PROTEIN NP9 CONTROLS VIABILITY AND MIGRATION OF TERATOCARCIONOMA CELLS

The work presented in this chapter was submitted to the online journal PLOS One and is currently under revision. All experiments were performed by Susana M. Chan.

Abstract

Human endogenous retroviruses are remnants of ancient germline infections that make up approximately 8% of the modern human genome. The HERV-K (HML-2) family is one of the most recent entrants into the human germline, these viruses appear to be transcriptionally active, and HERV-K viral like particles (VLPs) are found in cell lines from a number of human malignancies. HERV-K VLPs were first found to be produced in teratocarcinoma cell lines, and since then teratocarcinoma has been thought of as the classical model for HERV-Ks, with the NCCIT teratocarcinoma cell line particularly known to produce VLPs. Treatment for teratocarcinoma has progressed since its discovery, with improved prognosis for patients. Since the introduction of platinum based therapy, first year survival has greatly improved even with disseminated disease;

however, it is estimated that 20% to 30% of patients present with metastatic germ cell tumor relapse following initial treatments. Also, the toxicity associated with the use of chemotherapeutic agents used to treat germ cell tumors is still a major concern. In this study, we show that the depletion of the HERV-K accessory protein Np9 increases the sensitivity of NCCIT teratocarcinoma cells to bleomycin and cisplatin. While decreasing the expression of Np9 had only a modest effect on the baseline viability of the cells, the reduced expression of Np9 increased the sensitivity of the teratocarcinoma cells to environmental (serum starvation) and chemical (chemotherapeutic) stresses. Np9 is also essential to the migration of NCCIT teratocarcinoma cells: in a wound closure assay, reduced expression of Np9 resulted in cells migrating into the wound at a slower rate, whereas reintroduction of Np9 resulted in NCCIT cells migrating back into the wound in a manner similar to the control. These findings support the implication that the HERV-K accessory protein Np9 has oncogenic potential.

Introduction

Human endogenous retroviruses (HERVs) account for 8% of the human genome, yet their potential roles in the biology of the cell and in human health or disease remain poorly understood. These ancient viruses were once exogenous viruses that infected germ cells of mammals and other vertebrates numerous times in the course of millions of years, and subsequently integrated their proviral elements into the host genome. These proviruses have then been transmitted over the generations in a Mendelian fashion (Subramanian et al. 2011; Nelson et al. 2003; Jern and Coffin 2008). HERV elements exist in the human genome as retroviral genes (*gag*, *pol*, and *env*) flanked by

two long terminal repeats (LTRs); the LTRs serve as transcriptional promoters (Bannert and Kurth 2004). However, most of the HERV proviral sequences have been rendered nonfunctional due to the accumulation of mutations, insertions, and deletions in crucial retroviral genes (Hughes and Coffin 2004), with the possible exception of the HERV-K (HML-2) subfamily. The HERV-K (HML-2) subfamily is one of the most recent entrants into the human genome, most having only integrated itself between 200,000 and 5 million years ago, and it is the subfamily of endogenous retroviruses that is most conserved and are transcriptionally active, and some have functional open reading frames (ORFs) to code for all of its viral proteins (Barbulescu et al. 1999; Okahara et al. 2004; Tönjes et al. 1996; Sugimoto et al. 2001; Bannert and Kurth 2004; Wang-Johanning et al. 2001).

There are approximately 117 full-length copies and more than 2500 solitary LTRs of HERV-K (HML-2) spanning multiple chromosomes (Subramanian et al. 2011; Babaian and Mager 2016; Wildschutte et al. 2016; Marchi et al. 2014; Contreras-Galindo et al. 2012; Contreras-Galindo et al. 2013; Turner et al. 2001). In addition, our group has discovered that there are at least hundreds of copies of the HERV-K (HML-2) virus found throughout the centromeres of multiple chromosomes, a type-I provirus that we have termed K111 (Contreras-Galindo et al. 2013; Zahn et al. 2015). There are two types of HERV-K (HML-2) viruses: type I is characterized by a 292-bp deletion at the boundary of the *pol* and *env* genes, while type II contains the full sequence for *pol* and *env*. In the type I virus, the 292-bp deletion causes an alternative splicing event that

results in the expression of the accessory protein Np9, while type II expresses the accessory protein Rec (Bannert and Kurth 2004).

The transcription and translation of HERV-K genes and proteins are thought to be tightly repressed under normal physiological conditions, but there is evidence of HERV-K reactivation in different malignancies. For example, even though no infectious HERV particles have been detected to date, HERV-K (HML-2) have been found to produce viral like particles (VLP) in the tumor tissues of patients with breast cancer, leukemia, lymphoma, melanoma, and teratocarcinoma, and in the blood of HIV-infected individuals (Seifarth et al. 1998; Wang-Johanning et al. 2001; Bieda, Hoffmann, and Boller 2001; Büscher et al. 2005; Löwer et al. 1993; Muster et al. 2003; Contreras-Galindo et al. 2012; Contreras-Galindo et al. 2013, 2006; Contreras-Galindo et al. 2008; Contreras-Galindo, Almodóvar-Camacho, et al. 2007; Contreras-Galindo, López, et al. 2007). In addition, the increased transcription and expression of the HERV-K accessory proteins Np9 and Rec are thought to have potentially important roles in cellular functions that may contribute to oncogenesis (Kaufmann et al. 2010; Denne et al. 2007; Galli et al. 2005; Chen et al. 2013; Armbruester et al. 2004).

HERV-K Rec is a 14 kDa protein that is a functional homolog of HIV Rev (Yang et al. 1999; Magin, Löwer, and Löwer 1999). Rec aids in the shuttling of unspliced mRNA out of the nucleus, and has been associated with tumor development similar to testicular carcinoma in nude mice (Galli et al. 2005; Yang et al. 1999). The HERV-K accessory protein Np9 is a 9 kDa protein that shares its first 14 amino acids with HERV-

K Rec, both of which are translated from the HERV-K *env* reading frame (Armbruester et al. 2002). A recent study showed that the *np9* and *rec* transcripts are not restricted to disease states (Schmitt et al. 2015). However, the actual HERV-K Rec and Np9 accessory proteins appear to be expressed mainly in malignant tissues. Rec and Np9 proteins have been detected in primary and metastatic melanoma biopsies and melanoma cell lines but not found in melanocytes (Muster et al. 2003; Büscher et al. 2006). Also, *np9* transcripts have been found in transformed cell lines and tumors such as mammary carcinomas, germ cell tumors, and leukemia blood lymphocytes (Armbruester et al. 2002).

The role that these accessory proteins play in promoting oncogenesis is still not well defined. However, there has been some progress in identifying potential interacting partners and the functions of these accessory proteins in different cellular pathways. HERV-K Np9 and Rec have both been shown to physically and functionally interact with the promyelocytic zinc finger (PLZF) tumor suppressor and inhibit its function as a transcriptional repressor. The PLZF tumor suppressor is a known transcriptional repressor of the c-*myc* proto-oncogene. The co-expression of Np9 or Rec with PLZF removes the transcriptional repression of the c-*myc* promoter by PLZF, resulting in the overexpression of c-Myc and altered expression of c-Myc regulated genes, thus affecting cell proliferation and survival (Denne et al. 2007). HERV-K Np9 has also been shown to interact with the RING-type E3 ubiquitin ligase LNX (ligand of Numb protein X) (Armbruester et al. 2004), and Np9 has been found to play a critical role in different cell signaling pathways by activating β-catenin, ERK, Akt and Notch1 (Chen et al. 2013).

The expression of Np9 is crucial for the survival and growth of myeloid and lymphoblastic leukemia cells: reduced expression of Np9 caused growth inhibition of myeloid and lymphoblastic leukemia cells, whereas overexpression of Np9 promoted the growth of leukemia cells (Chen et al. 2013). Lastly, NOD-SCID mice developed larger tumors at a faster rate when injected subcutaneously with lymphoma cells overexpressing Np9 as compared to mice that received lymphoma cells with a control vector (Chen et al. 2013). Further studies are necessary to examine the role of Np9 in other types of tumors.

In the studies presented here, we investigated the function of Np9 in teratocarcinoma, a classical model for HERV-K and cancer. It was in teratocarcinoma cell lines that investigators first saw the production of VLPs, first termed human teratocarcinoma-derived viruses (HTDV), and it was later determined that HERV-K was responsible for encoding HTDV (Bhardwaj et al. 2015; Löwer et al. 1981; Bieda, Hoffmann, and Boller 2001; Boller et al. 1993). HERV-K (HML-2) mRNA and proteins are also highly expressed in teratocarcinoma (Götzinger et al. 1996; Armbruester et al. 2002). Also, the NCCIT teratocarcinoma cell line has been shown to express K111 (Zahn et al. 2015; Contreras-Galindo et al. 2017).The aim of the present study was to investigate whether the expression of Np9 supports or promotes tumorigenesis. We show that decreasing expression of Np9 with CRISPR/Cas9 decreases the viability of the NCCIT teratocarcinoma cell line when it is subjected to environmental stress (serum starvation) or chemotherapeutic agents (bleomycin and cisplatin) that are used in clinical settings as a part of a cocktail to treat testicular germ cell tumors (TGCT)

(Price and Peters 1992; Meyts et al. 2015; Dasari and Bernard Tchounwou 2014; Malavalli, Karra, and Muniyappa 2013; Williams et al. 1987; Winter and Albers 2011). Further, the reduced expression of Np9 decreased cell migration and invasiveness of teratocarcinoma cells, and re-introduction of Np9 rescued the migration of the NCCIT teratocarcinoma cells. Thus, we show that Np9 is crucial to the viability and mobility of teratocarcinoma cells, and decreasing its expression can potentiate the effectiveness of chemotherapeutic agents used in the clinic.

Results

Np9 controls cellular viability when teratocarcinoma cells are stressed

In view of the potential link between Np9 and teratocarcinoma, we performed loss-of-function studies to investigate the role of Np9 in the viability of the NCCIT teratocarcinoma cell line. We used two independent CRISPR/Cas9 constructs to make permanent cell lines in which Np9 expression had been knocked down. The CRISPR/Cas9 system is an efficient gene editing system that uses guide RNAs specifically designed to target and edit the gene of interest (Wang, La Russa, and Qi 2016); in our study, we used two independent Np9 guide RNAs to knock-down and reduce the number of Np9 copies in NCCIT teratocarcinoma cells, and isolated individual clones. Typically, the CRISPR/Cas9 system is used to mutate or knock-out (KO) a gene of interest (Lieber 2010; Wang, La Russa, and Qi 2016). Given the high number of Np9 gene copies in the human genome and the sequence heterogeneity

from one Np9 locus to another, the use of CRISPR/Cas9 is expected to result in a partial knock-down (KD). For example, the type I virus K111 discovered by our group can be found in hundreds of copies across 15 different centromeres (Contreras-Galindo et al. 2012; Zahn et al. 2015; Contreras-Galindo et al. 2013). Although the K111 proviruses do not seem to be replication competent, they code for many Np9 proteins, including some variants.

In our study, we used two independent Np9 guide RNAs to knock-down and reduce the number of Np9 copies in NCCIT teratocarcinoma cells. As can be seen in Figure 2.1A, knocking down the expression of Np9 was successful. However, the reduced expression of Np9 varied among the different clones that were isolated. In the two knock-down clones presented in this study, the expression of Np9 was reduced approximately by 80% and 20% in the knock-down clones 8 and 9, respectively. Np9 protein expression can only be seen after treatment with epoxomicin to inhibit the proteasome pathway, as Np9 typically has a very short half-life (Armbruester et al. 2004). Even though Np9 is a 9 kDa protein, it has been shown to be represented as a 12.5 kDa signal (Heyne et al. 2015), perhaps due to post-translational modifications. When Np9 was knocked down, we observed that it had a modest effect on the growth of NCCIT cells as represented by the MTT assay, which measures metabolic activity (Figure 2.1B). When we further examined the effect of Np9 knockdown in this teratocarcinoma cell line, consistent with the modest impact on cell viability we found only a small alteration in the cell cycle distribution, more so in Np9 KD clone 8 that causes a greater reduction in Np9 expression (Figure 2.1C). However, many

oncoproteins, as well as other biologically crucial molecules, exert their effects primarily under duress. Therefore, we subjected the knock-down and control cells to serum starvation, and found a marked reduction in cellular metabolism in the two knock-down clones (KD clone 8 and clone 9) as compared to a clone in which only a scramble CRISPR/Cas9 guide RNA was used (Figure 2.1D). Therefore, taken together, we see that the reduction in the levels of Np9 does have a significant effect on the growth and metabolism of this type of cancer cell, but primarily when the cells are put under stress. This prompted us to move on to examine the effect of Np9 knock-down in the setting of chemotherapeutic agents used in the clinic.

Decreased Np9 expression increases sensitivity of teratocarcinoma cells to chemotherapeutic agents

In the clinical setting, testicular teratocarcinomas are frequently treated with combinations that include bleomycin and cisplatin, two highly toxic agents (Williams et al. 1987; Winter and Albers 2011; Dasari and Bernard Tchounwou 2014; Price and Peters 1992; Meyts et al. 2015). As decreasing Np9 expression sensitized NCCIT cells to starvation stress, we next investigated whether cells that had Np9 expression knocked down would be more susceptible to chemical stress induced by bleomycin and cisplatin. Indeed, when Np9 expression was diminished NCCIT cells treated with bleomycin (Figure 2.2A) or cisplatin (Figure 2.2B) for 48 hours showed markedly reduced cell viability. This loss of viability was seen with both Np9 KD clones, as compared to the scramble control.

By appearance, the NCCIT cells in which Np9 was knocked-down appeared to be apoptotic through the loss of adhesion. We therefore stained NCCIT cells (KDs and Scr control) with activated Caspase 3/7 green detection dye and SYTOX AADvanced viability dye to discriminate early and late stage apoptotic cells from live and dead cells. We found that, in spite of the limited effect on overall growth and metabolism as measured by MTT assay (Figure 2.1B), diminished Np9 expression did lead to some increase in apoptosis even in the absence of any stress (Figure 2.3). However, this increase in apoptosis was magnified when bleomycin was applied to the cells (Figure 2.3).

Decreasing Np9 levels severely affects migration of NCCIT cells

The data above demonstrate that the effect of Np9 on the growth, metabolism, and viability of teratocarcinoma cells is particularly visible when the cells are put under stress. We therefore wanted to examine another aspect of oncogenesis that reflects cell migration, and so employed a wound-closure assay. In this assay, a scratch is induced to cells growing in a monolayer and then the time it takes for the "wound" to close is monitored. This is one important test for examining the contribution of a cellular protein to the oncogenic process (Sahai 2005; Hulkower and Herber 2011). As can be seen in images in Figure 2.4A and graphically in Figure 2.4B, knocking down Np9 expression with CRISPR/Cas9 led to a marked slowing in wound closure. At 24 hours (Figure 2.4B), the wound closure was only approximately 25% to 50%, as opposed to

somewhere between 75% and 90% in the control cells. Therefore, a decrease in Np9 expression clearly affects the ability of cells to migrate.

In order to confirm that Np9 is crucial to wound healing, we employed the piggybac system to stably overexpress the Np9-mCherry fusion protein; the mCherry was used as a visual indicator for the stable expression of Np9. Strikingly, when we overexpress Np9 in the NCCIT cells in which Np9 expression has been reduced with CRISPR/Cas9 editing, the wound healing is restored essentially to normal. This is seen in the pictures in Figure 2.5A and is depicted graphically in Figure 2.5B, showing that we were able to restore migratory function of the teratocarcinoma cells by reintroducing Np9. This rescue experiment offers striking evidence that Np9 is important to wound closure in NCCIT cells, and further suggests an important role for this protein in the growth and movement that is necessary for oncogenesis.

Discussion

HERVs have long been implicated in autoimmune disorders and oncogenesis (Balada, Ordi-Ros, and Vilardell-Tarrés 2009; Urnovitz and Murphy 1996; Perron and Lang 2010; Curtin et al. 2012; Mameli et al. 2012; Iramaneerat et al. 2011; Wang-Johanning et al. 2007; Reiche, Pauli, and Ellerbrok 2010; Büscher et al. 2005, 2006; Serafino et al. 2009; Wang-Johanning et al. 2001, 2003, 2008; Goering, Ribarska, and Schulz 2011; Ishida et al. 2008; Contreras-Galindo et al. 2008; Depil et al. 2002; Schiavetti et al. 2002). However, the fact that there are so many copies, over a hundred

copies of the standard HERVs and hundreds to thousands of copies of K111 and K222, picking apart the specific contributions of HERV-K to cancer has been quite difficult (Contreras-Galindo et al. 2013; Zahn et al. 2015). Adding to the complexity of HERV-K biology in the context of human diseases, there is evidence of HERV-K viral particle production in certain cancers and transformed cell lines, such as teratocarcinoma; however, whether these viral particles are actually replicative remains unknown and the subject of debate (Contreras-Galindo et al. 2012; Turner et al. 2001; Löwer et al. 1993; Seifarth et al. 1998; Muster et al. 2003; Büscher et al. 2005; Bieda, Hoffmann, and Boller 2001; Beimforde et al. 2008; Contreras-Galindo et al. 2015; Young and Bieniasz 2007; Hanke, Hohn, and Bannert 2016; Dewannieux et al. 2006; Dube et al. 2014).

In the field of HERV-K biology, the Np9 protein has been of particular interest in view of its potential to function as an oncogene. Indeed, Np9 has been shown to control cellular signal transduction pathways that modulate expression of growth genes such as Notch, Akt, Wnt/ß-catenin, and Myc (Chen et al. 2013; Denne et al. 2007; Armbruester et al. 2004). Further, it has been shown to interact with the promyelocytic leukemia zinc finger tumor suppressor (Denne et al. 2007). In the Notch pathway, Np9 has been shown to interact with Numb, and has been thought to perhaps stimulate Notch signaling via this interaction, although the details have remained quite unclear (Armbruester et al. 2004). Here, we offer strong evidence that reducing the expression of Np9 decreases the cell viability of NCCIT cells, especially when they are challenged by serum starvation or chemotherapeutic agents. The effect is primarily mediated through apoptosis, although the mechanistic effect of combining the cisplatin agent with

Np9 knock-down remains unclear. Perhaps most remarkably, we find using wound healing assays that Np9 is vital for the proliferation/invasion phenotype, further strengthening our observations that Np9 is important for the growth and likely oncogenic properties of teratocarcinoma cells.

Teratocarcinoma is often a treatable disease, but the treatment regimens are often quite toxic. Furthermore, the relapse rate is high in advanced stage disease (Winter and Albers 2011; Price and Peters 1992; Malavalli, Karra, and Muniyappa 2013). Here we show that a reduction in Np9 sensitizes NCCIT cells to the clinically important chemotherapeutic agents bleomycin and cisplatin. Therefore, our data would suggest that targeting Np9 in teratocarcinoma will lead to better outcomes if proper drugs/delivery mechanisms can be obtained. As HERV-K and Np9 have also been implicated in other malignancies (Löwer et al. 1993; Büscher et al. 2006; Bieda, Hoffmann, and Boller 2001; Seifarth et al. 1998; Muster et al. 2003; Contreras-Galindo et al. 2012, 2008; Gonzalez-Hernandez et al. 2012; Contreras-Galindo et al. 2013), it is certainly possible that targeting Np9 may sensitize these other cancers to chemotherapeutic agents. Taken together, Np9 appears to be a key protein propelling teratocarcinoma viability and a potentially interesting target for future therapeutics.

Materials and Methods

Cell lines and cell culture

The teratocarcinoma cell line NCCIT was obtained from ATCC and cultured in RPMI (Thermo Fisher Scientific (TFS), catalog #11875-093), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P-S), at 37°C in a 5% CO_2 cell culture incubator. The medium was changed every 2 to 3 days. Cells were cultured in T-75 Falcon Tissue Culture Treated Flasks (Fisher Scientific, catalog #353112) until 75% confluent and split with TrypLE Express (TFS, catalog #12604013) for 5 min in the 37°C incubator. Cells were re-suspended in RPMI supplemented with 10%FBS and 1% P-S. Cells were counted with Countess Automated Cell Counter (TFS) using Countess™ Cell Counting Chamber Slides (TFS, catalog #C10228).

CRISPR/Cas9 plasmid construct and transfection

The CRISPR/Cas9 guide RNAs were designed using the online CRISPR Design Tool (http://crispr.mit.edu) to specifically target HERV-K Np9. The Np9 guide RNAs (gNp9-1: gcatcatggcccgttctcga; gNp9-2: ggttttgtcgaaaagaaaag) were cloned into the pSpCas9(BB)-2A-Puro (pX459) V2.0 plasmid. The pSpCas9(BB)-2A-Puro (pX459) V2.0 was a gift from Feng Zhang (Addgene, plasmid #62988) (Supplementary Figure 2.1). As a control, a scramble sgRNA (gScr: gagatcgagtgccgcatcac) cloned into the pX459

plasmid was used; the scramble plasmid construct was kindly provided by Dr. Xiaoyan Jia from the Univeristy of Michigan.

NCCIT cells were seeded at a density of 1,000,000 cells per well in a CytoOne 6-well tissue culture plate (USA Scientific, catalog # CC7682-7506) and grown for 24 hours. For transfection of pX459 constructs, we incubated FuGENE® HD transfection reagent (Promega, catalog #E2311) with OPTI-MEM reduced serum medium (TFS, catalog #31985088), and 500 µg of each plasmid was incorporated at a 1:6 ratio (DNA:Transfection reagent) for 30 min at room temperature before being introduced into cells. After a 24 hour transfection with sgRNAs, the medium was changed to RPMI supplemented with 2 µg/mL puromycin (Sigma, catalog #P8833) for selection, and the NCCIT cells were selected for 3 days and then cultured in RPMI for 2 days to recover. After 2 days of recovery, the NCCIT clones were transfected with two additional rounds of CRISPR/Cas9 constructs. As there are hundreds of copies of Np9 scattered around the genome, some with slightly different sequences, CRISPR/Cas9-mediated genome editing results in knock-down, rather than a knock-out cell line.

Immunoblot analysis of Np9 knockdown

NCCIT cells were treated with 0.3 µM epoxomicin (Millipore, catalog #324800) for 24 hours to allow for the stabilization of Np9. NCCIT cells were collected with a cell scraper and lysed in cold RIPA Buffer (Sigma, catalog #R0278) supplemented with cOMPLETE protease inhibitor cocktail (Roche, catalog # 11697498001). Protein lysates

(33 µg protein per sample) were separated in Bio-Rad 4-20% Mini-PROTEAN® TGX gels (Bio-Rad, catalog #4561094). Proteins were transferred to 0.45 µm pore size polyvinylidene difluoride membranes (PVDF) (Millipore, catalog # IPVH00010), incubated with anti-Np9 rat monoclonal antibody 10B1 (1:25) overnight at 4°C, and then incubated with rabbit anti-rat secondary antibody (1:5000) (Invitrogen, catalog #619520) (Heyne et al. 2015). The anti-Np9 rat monoclonal antibody was kindly provided by Dr. Klaus Römer from the University of Saarland Medical Center. Membranes were incubated with SuperSignal femto chemiluminescent substrate (TFS, catalog #34095). As a loading control, the PVDF membrane was incubated with mouse anti-GAPDH (1:10000) (Santa Cruz, catalog #166545), and then incubated with horse anti-mouse secondary antibody (1:10000) (CST, catalog #7076S). We used ImageJ to analyze the intensity of the Np9 signal to determine the efficiency of the CRISPR/Cas9-mediated gene KD.

Starvation and MTT assay

To synchronize and starve the teratocarcinoma cells, the NCCIT cells were seeded at a density of 1000 cells per well in a Falcon 96-well tissue culture plate (Fisher, catalog #353072) and cultured in 100 µL RPMI supplemented with 1% P-S for 24 hours. After 24 hours, the medium was removed and changed to 100 µL full RPMI medium (time 0). At time 0, each well was treated with 10 µL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenytltetrazolium Bromide) (5 mg/mL) for 3 hours, and then 100 µL of solubilization solution (10% SDS in 0.01M HCl) was added to each well to

stop the reaction. The plate was then wrapped in aluminum foil and incubated in a 37°C/5% CO_2 incubator overnight, and was analyzed the following day with a Tecan GENios plate reader. Days 4, 5 and 6 were done in similar manner and optical density (OD) measurements were obtained. The optical density measurements were used to calculate percent cell viability, days 4, 5 and 6 were divided by OD obtained on day 0, resulting number was multiplied by 100 to determine percentage viability.

Bleomycin and cisplatin treatments and MTT assay

NCCIT cells were seeded at a density of 1000 cells per well in Falcon 96-well tissue culture plates (Fisher, catalog #353072) and cultured in full RPMI media for 24 hours. At 24 hours, the media was changed to media supplemented with 5, 15, 30, 60 or 120 µg/mL bleomycin (Cayman Chemical, catalog #13877) or 0.1, 0.5, 1, 15, 15 µg/mL cisplatin (Cayman Chemical, catalog #13119) and the plates were cultured for 48 hours. MTT assay was performed at 0 and 48 hours post-treatment. Calculations were done in a similar manner as previously described.

Cell Cycle Analysis

NCCIT cells were seeded at a density of 220,000 cells per well in a 6-well CytoOne tissue culture plate (USA Scientific, catalog #CC7682-7506), cultured in RPMI, and the media was changed every 24 hours. The cells were trypsonized with TrypLE Express, collected and centrifuged at 700 rpm for 5 mins. The cell pellet was washed

once with 1X phosphate buffered saline (PBS) (TFS, catalog #10010023) and fixed with 70% ice-cold ethanol for 30 mins on ice. The fixed cells were washed with 1X PBS, re-suspended with FxCycle PI/RNase staining solution (Molecular Probes, catalog #F10797), and stained in the dark for 30 mins prior to analysis. The cells were analyzed with a Bio-Rad ZE5 cell analyzer at the University of Michigan Flow Cytometry Core.

Flow cytometry for activated caspase 3/7 apoptosis assay

NCCIT cells were seeded at 150,000 cells per well in a 6-well CytoOne tissue culture plate (USA Scientific, catalog #CC7682-7506) and cultured in full RPMI medium for 24 hours. After 24 hours, the medium was changed to mock treatment (full RPMI media), RPMI medium containing 30 µg/mL bleomycin or 1 µg/mL cisplatin, or cells were placed under serum starvation conditions (serum free media). After 24 hours, the cells were trypsonized with TrypLE Express, collected, washed with 1X PBS (PBS), and stained with CellEvent Caspase 3/7 Green Detection Reagent (TFS, catalog #C10427) for 60 mins; for the last 5 mins the cells were stained with SYTOX® AADvanced Dead Cell Stain (TFS, catalog #C10427). Caspase 3/7 analysis was performed with a Sony SH800 Cell Sorter at the University of Michigan Flow Cytometry Core.

Wound closure assay

Wound closure assay was performed with NCCIT Np9 CRISPR cells or with control NCCIT scramble cells. The tissue culture plates were treated with 0.001% poly-

L-lysine solution (Sigma, catalog # P8920) and stored at 4°C until use. NCCIT cells were seeded at a density of 200,000 cells per well in a 12-well CytoOne tissue culture plate (USA Scientific, catalog #CC77682-7512) and cultured in RPMI for 24 hours. After 24 hours, each well was scratched linearly with a pipette tip, and images were captured at 0 and 24 post-scratch. To determine percent migration, images were measured used to measure the width of the wound through the entire length of the wound within the image using the NIS Elements image software. The percent migration was calculated by dividing the width of the wound at time 24 with time 0 and then multiplying by 100 to obtain percentage of migration.

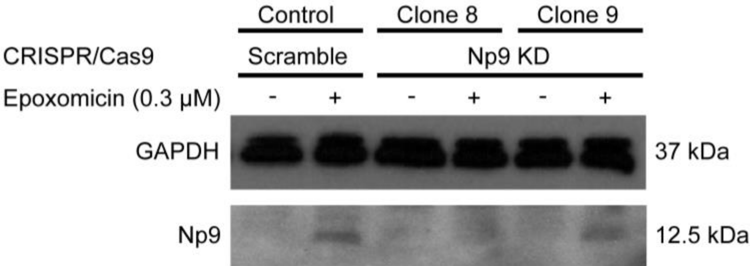
Np9 complementation plasmid construct

Re-introduction of Np9 into NCCIT Np9 KD cells was performed using the piggyBac transposon system. Np9 complementation was performed with the PB-CAG-Np9-mCherry transposon, and the PB-CAG-H2B-mCherry transposon was used as the control. NCCIT cells (scramble, Np9 KD #8 and #9) were seeded at a density of 1,000,000 cells per well in a CytoOne 6-well tissue culture plate. NCCIT scramble control cells and NCCIT Np9 KD cell clones 8 and 9 were co-transfected with PB-CAG-H2B-mCherry transposon plasmid and pCAG-PBase transposase plasmid at a 1:1 ratio as a control. NCCIT Np9 KD clones 8 and 9 were co-transfected with PB-CAG-Np9-mCherry transposon and pCAG-PBase transposase plasmid at a 1:1 ratio. Twenty-four hours post transfection, the cells were selected for mCherry using a Sony SH800 cell sorter, and allowed to grow for a week in full media. The cells were selected a second

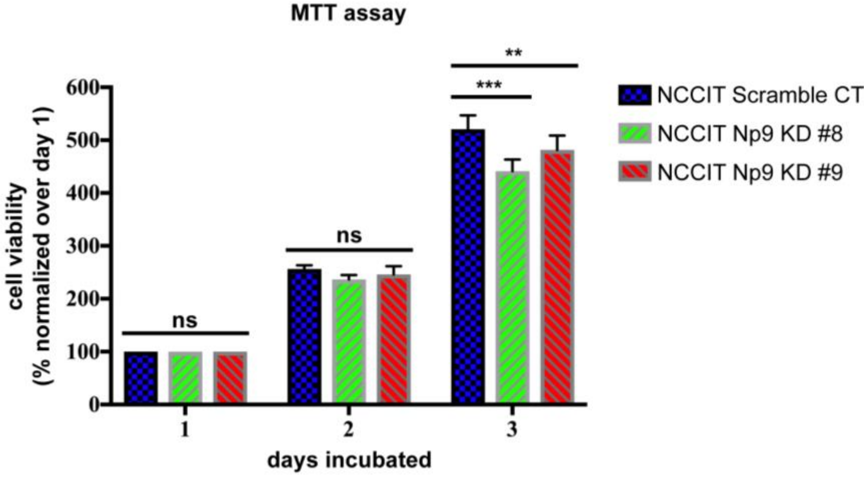
time for mCherry a week after the first selection to ensure that the mCherry expressing constructs were stably integrated. These cells were used for a wound closure assay done in a similar manner as previously described.

Figures

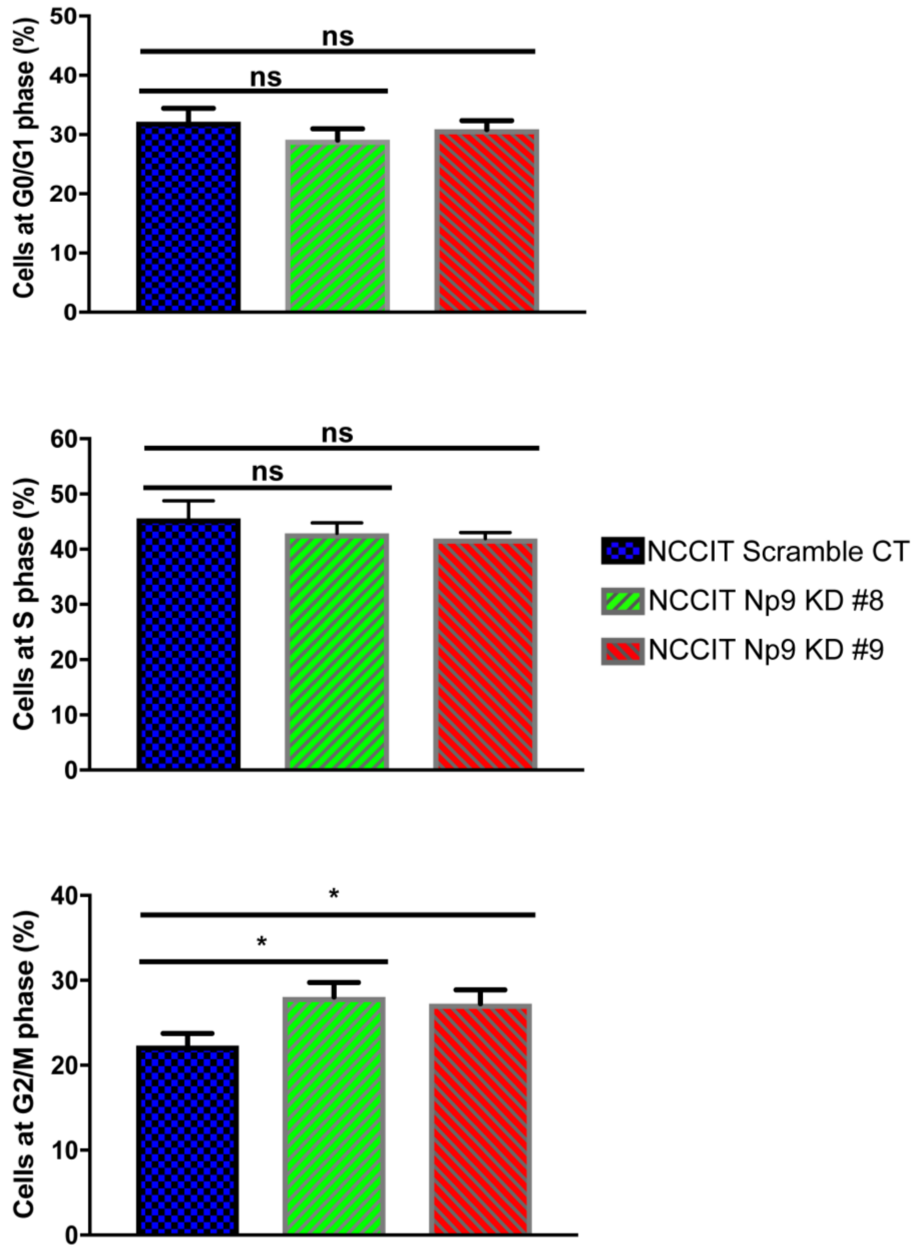
A)



B)



C)



D)

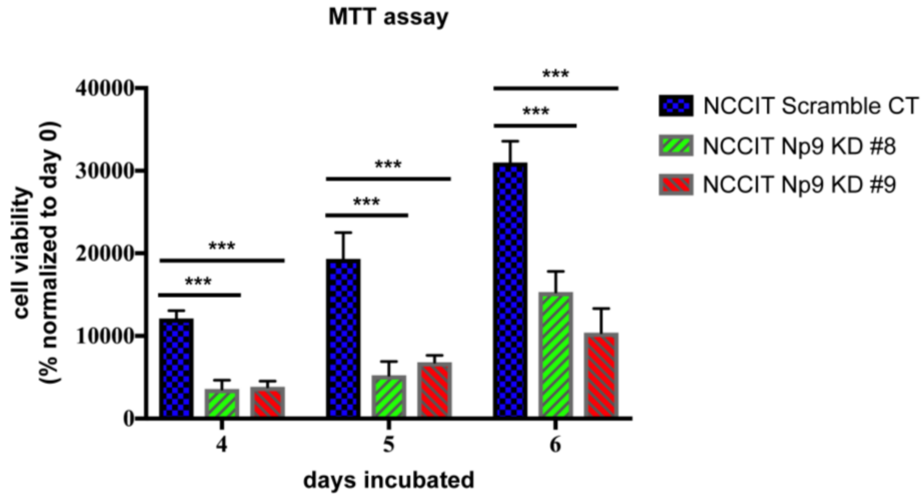
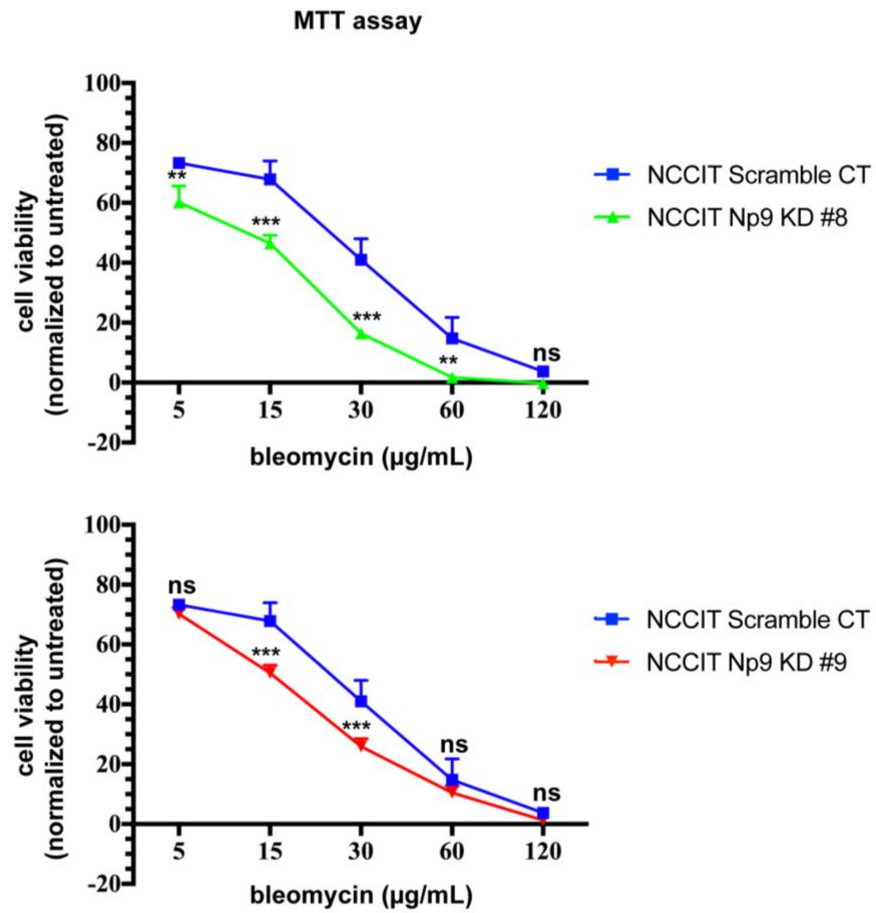


Figure 2.1 Reduced expression of Np9 in NCCIT teratocarcinoma cells increases sensitivity to environmental stress. (A) NCCIT cells were either transfected with a scramble CRISPR/Cas9 system or Np9 CRISPR/Cas9 guide RNAs. After three rounds of transfection with CRISPR/Cas9 Np9 guides, Clones 8 and 9 were selected, and the level of Np9 was shown to be reduced by 80% and 20%, respectively; Np9 densitometry was analyzed with ImageJ. (B) NCCIT scramble control and Np9 KD cells were cultured in full media and viability was measured with MTT assay (n=6 for each cell line). Reduced expression of Np9 had a limited effect on the viability of the teratocarcinoma cells. (C) NCCIT scramble control and Np9 KD cells (n=4 for each cell line) were cultured in full media for 48 hours, and media was changed every 24 hours. Cell cycle analysis was performed with the Bio-Rad ZE5 cell analyzer. Reduced expression of Np9 had a very modest effect on the distribution of the cell cycle in teratocarcinoma cells. (D) NCCIT scramble control and Np9 KD cells were cultured in serum free media (environmental stress) for 24 h, and after 24 h media was changed to full media, and viability was measured with MTT assay (n=8 for each cell line). The reduced expression of Np9 increased sensitivity to environmental stress and resulted in fewer viable cells. T bars denote the standard deviations of the means; P values were determined using student t-test analysis. ns not significant, $^*P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$**.** The Np9 CRISPR/Cas9 guide RNAs were designed and provided by our collaborators, Dr. Orly Reiner and Dr. Tamar Sapir. The scramble CRISPR/Cas9 guide RNA was kindly provided by Dr. Xiaoyan Jia. Dr. JoAnn Sekiguchi provided reagents necessary for cell cycle analysis.

A)



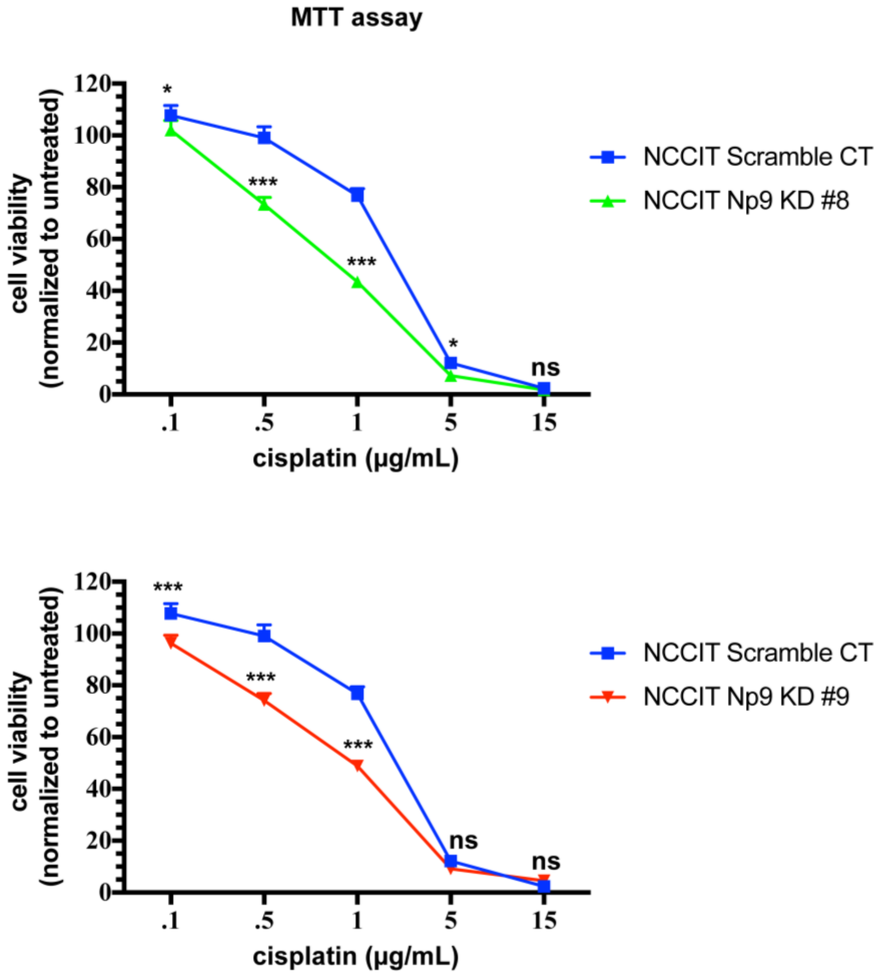
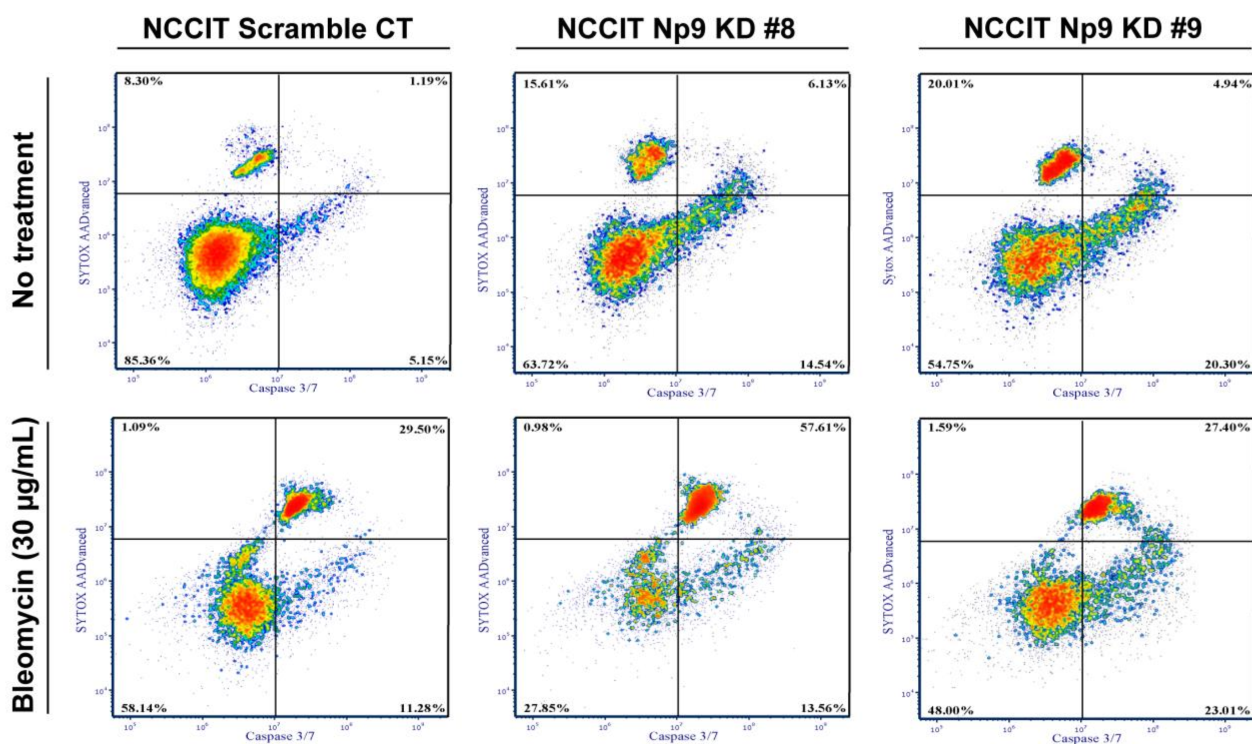


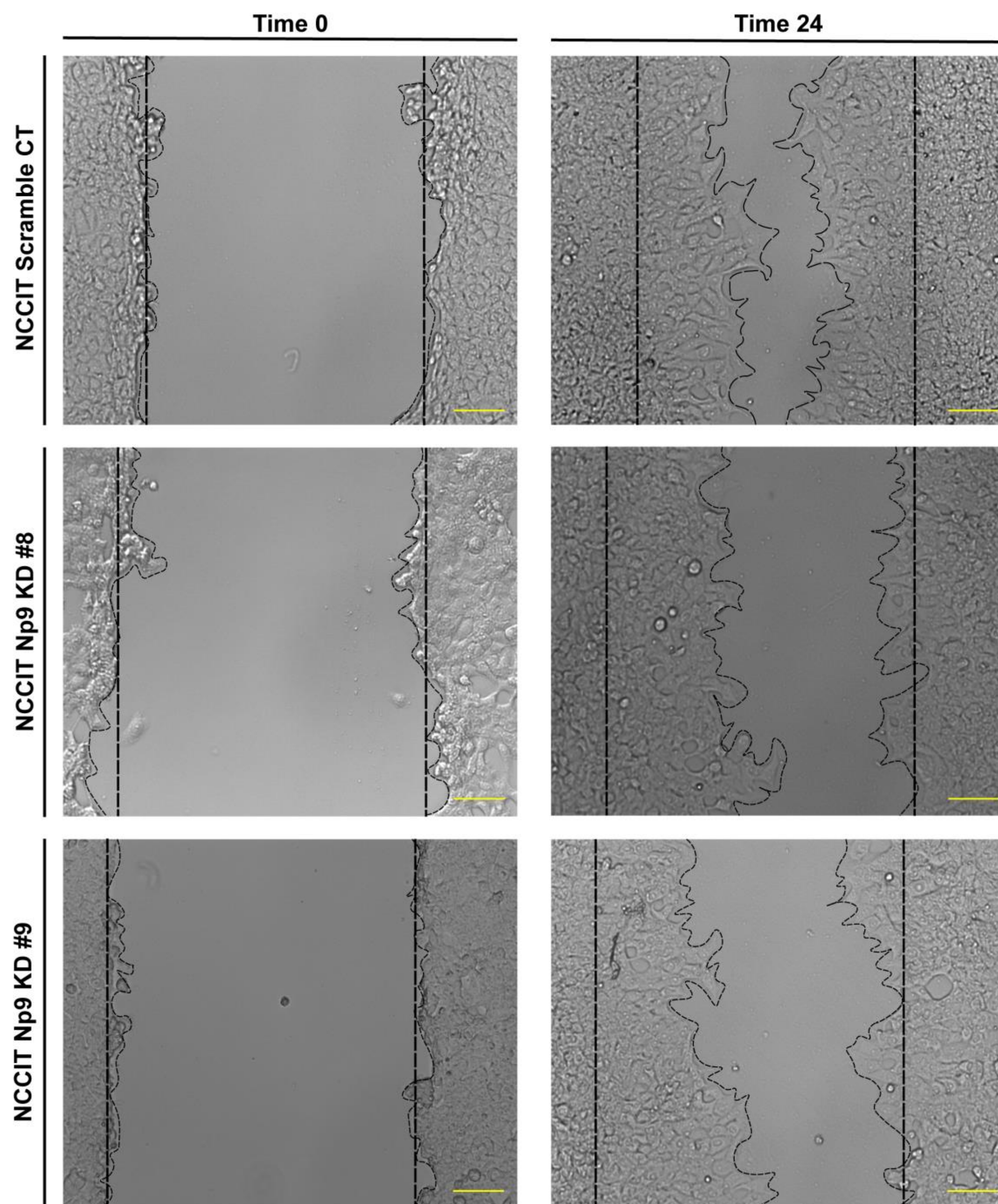
Figure 2.2 Reduced expression of Np9 in NCCIT teratocarcinoma cells increases sensitivity to chemical stress. NCCIT scramble control and Np9 KD cells were cultured in regular media for 24 h (n=4 for each cell line). The following day, the media was changed to mock treatment (full media) or media supplemented with (A) bleomycin (30 μg/mL) or (B) cisplatin (1 μg/mL) and cultured for 48 h; at 48 h viability was measured with MTT assay. Reduced expression of Np9 resulted in increased sensitivity to bleomycin or cisplatin treatment with decreased viability of NCCIT cells. T bars denote the standard deviations of the means; P values were determined using student t-test analysis. ns not significant, *$P<0.05$, **$P<0.001$, ***$P<0.0001$



Apoptotic cells (%)		
Cell line	No treatment	Bleomycin (30 µg/mL)
NCCIT Scramble CT	6.3	40.8
NCCIT Np9 KD #8	20.7	71.2
NCCIT Np9 KD #9	25.2	50.4

Figure 2.3 Reduced expression of Np9 in NCCIT teratocarcinoma cells increases sensitivity to chemical stress, resulting in apoptotic cells. NCCIT scramble control and Np9 KD cells were cultured in full media. The following day media was changed to either mock treatment (full media) or treatment (30 µg/mL bleomycin). After 24 h of treatment, the cells were stained with SYTOX AADvanced and Caspase 3/7 and apoptosis analysis was performed with a Sony SH800 Cell Sorter. Np9 KD clones 8 and 9 were naturally more apoptotic than scramble control cells: 20 and 25% compared to 6%, respectively. The reduction of Np9 expression in NCCIT cells increased sensitivity to bleomycin, with increased apoptosis in Np9 KD clones 8 and 9 compared to scramble control cells: 71% and 50% compared to 40%, respectively. The percent of apoptotic cells represented in the table was calculated from the bottom right and upper right quadrants, with the bottom right quadrant being early stage apoptotic cells and the upper right quadrant being late stage apoptotic cells.

A)



B)

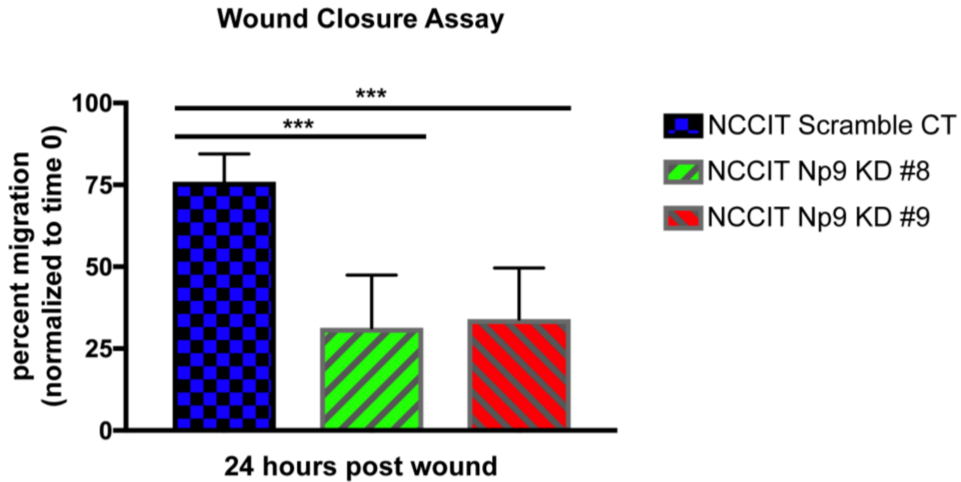
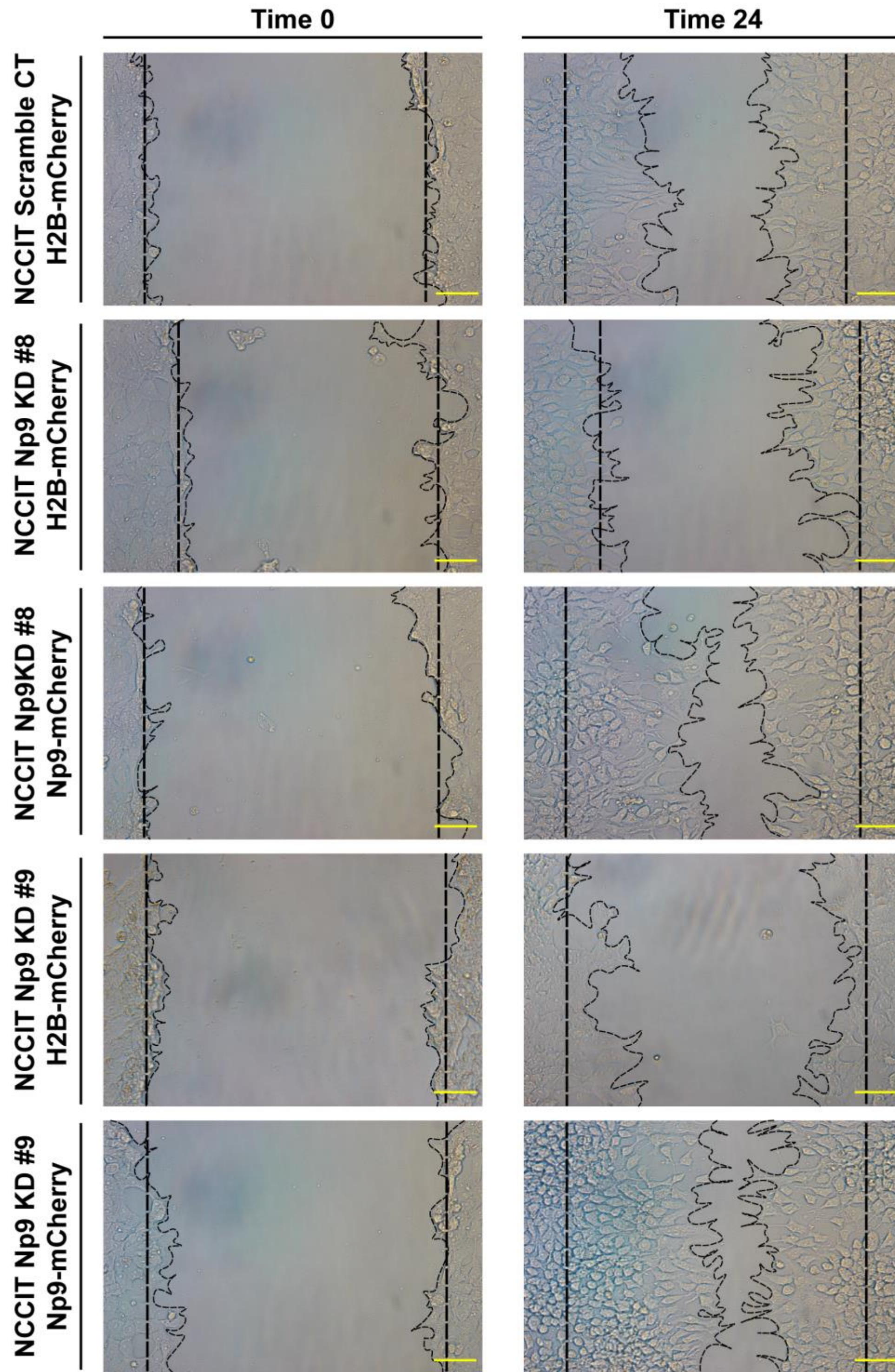


Figure 2.4 Np9 is essential for cell migration and wound closure. (A) NCCIT scramble control and NCCIT Np9 KD clones 8 and 9 were used for the migration assay. A total of 200,000 cells for each cell type were transferred into a 12-well plate. The following day, a scratch was created down the middle, and pictures were taken at time 0 and at 24 hours. The images are representative of 3 independent experiments done in duplicate. Scales bars, 100 µm. (B) The wound was measured at time 0 and at 24 hours. The percent of migration was plotted over time 0. The measurements are representative of 3 independent experiments done in duplicate. The width of the wounds was measured in each image and totaled for scramble CT (n=26), KD #8 (n=27), and KD #9 (n=28). The reduction in the expression of Np9 in NCCIT cells resulted in reduced cell migration post-wound as compared to scramble control cells. T bars denote the standard deviations of the means; P values were determined using student t-test analysis. ***$P<0.0001$

A)



B)

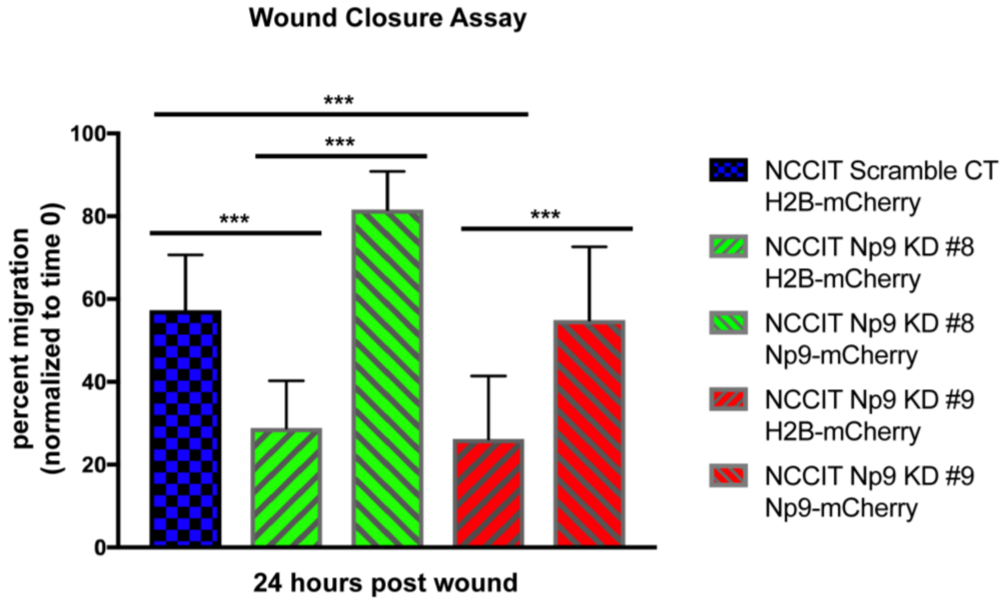
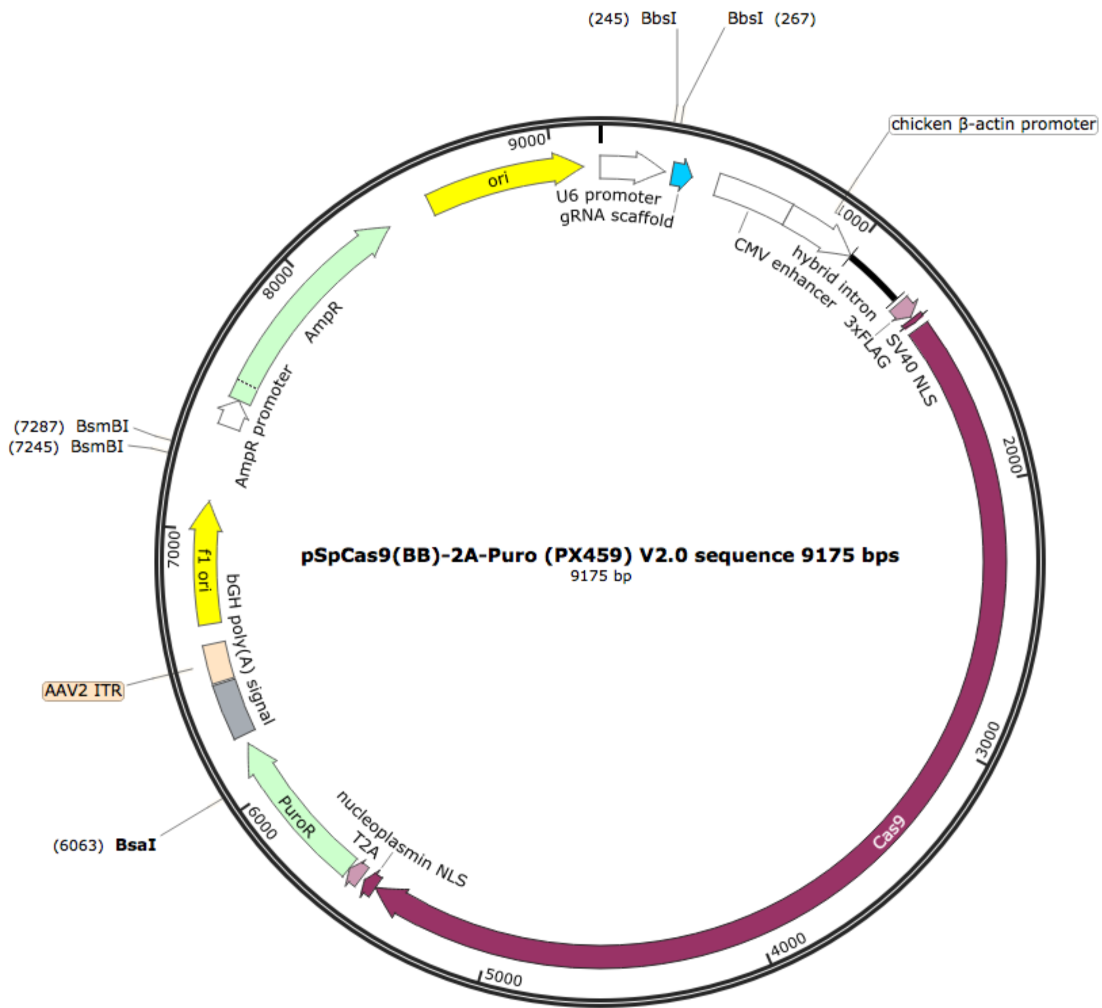
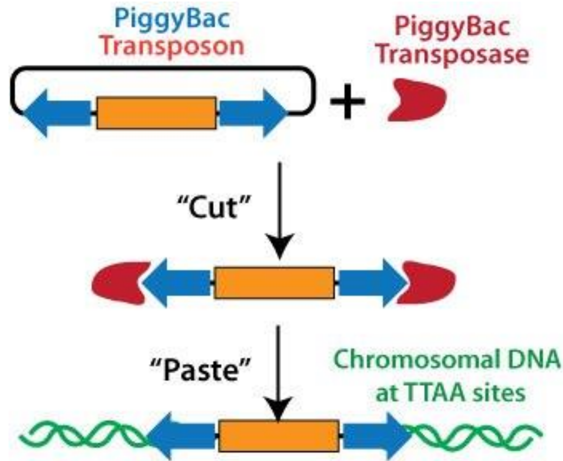


Figure 2.5 Re-introduction of Np9 into teratocarcinoma cells rescues the migration phenotype. (A) NCCIT scramble, Np9 KD clones 8 and 9 with H2B-mCherry control clones and NCCIT Np9 KD clones 8 and 9 with Np9-mCherry clones were used for the migration assay. A total of 200,000 cells for each cell type were transferred into a 12-well plate. The following day, a scratch was created down the middle, and pictures were taken at time 0 and at 24 hours. The images are representative of 2 independent experiments done in duplicate. Scales bars, 100 μm. (B) The wound was measured at time 0 and at 24 hours. The percent of migration was plotted over time 0. The measurements are representative of 2 independent experiments done in duplicate. The width of the wound was measured for each sample: scramble CT-H2B-mCherry (n=83), Np9 KD #8 H2B-mCherry (n=135), Np9 KD #8 Np9-mCherry (n=156), Np9 KD #9 H2B-mCherry (n=160), and Np9 KD #9 Np9-mCherry (n=145). The re-introduction of Np9 into Np9 KD cells rescued the migratory phenotype of the NCCIT cells almost to the level of the scramble control, and in the case of Np9 KD clone 8 complemented with Np9-mCherry, the re-introduction of Np9 resulted in faster migration compared to the scramble control. T bars denote the standard deviations of the means; P values were determined using student t-test analysis. ns not significant, ****P*<0.0001. The mCherry constructs were made by Drs. Tamar Sapir and Orly Reiner at the Weizmann Institute.

Supplementary Figures



Supplementary Figure 2.1 A map of pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid. BsbI recognition sites were used to replace the cassette with the cDNA sequence for the Np9 guide RNAs. Image taken from Addgene.com.



Supplementary Figure 2.2 An illustration of the PiggyBac Transposon system. The PiggyBac (PB) transposon is a moveable genetic element that transposes between the vector and chromosome through a cut and paste mechanism. The PB transposase (Red) recognizes the transposon specific inverted terminal repeats (Blue) located at both ends of the vectors that flank the transgene (Orange), cuts the PB transposon, and integrates the contents into TTAA chromosomal sites. The PB system has been shown to have the highest transposition activity in mammalian cells. Image adapted from Biocat.com.

A) ATGAACCCATCGGAGATGCAAAGAAAAGGGCCTCCGCGGAGATGTCTGCAGGT
GTACCCAACAGCTCCGAAGAGACAGCGACCATCGAGAACGGGCCATGATGAC
GATGGCGGTTTTGTCGAAAAGAAAAGGGGGAAATGTGGGGAAAAGCAAGAGA
GATCAGATTGTTACTGTGTCTGTGTAGAAAGAAGTAGACATAGGAGACTCCATT
TTGTTATGTGC

B) MNPSEMQRKGPPRRCLQVYPTAPKRQRPSRTGHDDDGGFVEKKRGKCGEKQER
SDCYCVCVERSRHRRLHFVMC

Supplementary Figure 2.3 HERV-K 101 Np9. (A) The HERV-K 101 *np9* nucleotide sequence was the reference sequence used in the design of the Np9 CRISPR guide RNAs, and the *np9* gene was cloned into the PB-CAG-mCherry vector for the complementation experiments. (B) The HERV-K 101 Np9 amino acid sequence.

Chapter 3

DISCUSSION

Prior to the sequencing of the human genome, it was estimated that our genome consists of a hundred thousand or more genes, but that was proven to be an over-estimation. The human genome consists of only twenty to twenty-five thousand genes that make up 1.1% of the DNA sequence, with the remaining 98.9% originally being falsely labeled “junk” DNA (Lander et al. 2001; Collins et al. 2004; Chial 2008). The large bulk of genetic material was considered “junk” because it had no known biological function. However, we now know that some of the “junk” sequences can serve as regulatory elements that are integral to biological function (Maston, Evans, and Green 2006; Plank and Dean 2014). In addition, transposable elements make up about 42% of the genome (Lander et al. 2001). These transposable elements consist of endogenous retroviruses characterized by the presence of long terminal repeats, and short interspersed nuclear elements (SINES) and long interspersed nuclear elements (LINES) that do not have LTRs; LINES can encode reverse transcriptase. HERVs are LTR-positive retroelements that make up 8% of the human genome (Lander et al. 2001; Bannert and Kurth 2004; Bannert and Kurth 2006; Weiss 2006; Subramanian et al. 2011).

Interestingly, the existence of HERVs in the human genome is the result of ancient infections by exogenous retroviruses and subsequent integration of retroviral elements into germ cells; these proviral sequences have been inherited in the Mendelian fashion (Nelson et al. 2003; Jern and Coffin 2008; Subramanian et al. 2011). HERVs are distributed across the genome and the distribution has been suggested to be non-random; it has been reported that they migrated to regions of chromosomes with high levels of heterochromatin, thus increasing their chances to be retained in the host genome (Kurdyukov et al. 2001). These proviral elements have accumulated insertions, mutations, and deletions in the open reading frames of pertinent viral genes, which rendered them incapable of replication (Hughes and Coffin 2004). While most HERVs are defective, a limited number have retained the potential to produce viral products such as viral particles, viral transcripts, and viral proteins (Löwer et al. 1993; Tönjes et al. 1996; Seifarth et al. 1998; Johnston et al. 2001; Sugimoto et al. 2001; Bieda, Hoffmann, and Boller 2001; Wang-Johanning et al. 2001; Yi et al. 2001; Ruda et al. 2004).

Evidence thus far suggests that the expression of HERV products can either be beneficial in biological processes, a causative factor in human malignancies, or an innocent bystander in disease states. Most HERVs entered primates 60 to 30 million years ago, and have been rendered inactive due to the accumulation of mutations (Bannert and Kurth 2004). However, the most transcriptionally active family and one of the most recent entrants into the human genome is the HERV-K (HML-2) subfamily

(Tönjes et al. 1996; Seifarth et al. 1998; Barbulescu et al. 1999; Okahara et al. 2004; Johnston et al. 2001; Sugimoto et al. 2001; Wang-Johanning et al. 2001; Yi et al. 2001; Ruda et al. 2004). The abundance of the HERV-K (HML-2) subfamily in modern-day humans is a result of replication, re-infection, and recombination during human evolution (Belshaw et al. 2004). Even though to date there is no evidence of an infectious and replicative HERV-K (HML-2) virus in modern humans, this subfamily is considered to be "active" because the proviruses can produce viral like particles, express viral transcripts, and code for viral proteins (Löwer et al. 1993; Seifarth et al. 1998; Bieda, Hoffmann, and Boller 2001; Muster et al. 2003; Büscher et al. 2005). Futher, HERV-K (HML-2) can be passaged from one cell to another (Contreras-Galindo et al. 2015). Therefore, it comes as no surprise that there has been a great deal of interest in HERVs in general, and HERV-K in particular, and their potential function in human biology and disease.

HERV expression beneficial for development of the placenta

Considering that HERVs make up a sizeable portion of the human genome, it has been a topic of discussion whether these ancient germ line infections were retained because they performed, or perform, useful biological functions. Indeed, it has been found that the envelope protein from the HERV-W, HERV-FRD, and HERV-R families has been linked to the development of human placenta; these proteins are termed syncytin-1, syncytin-2, and ERV-3 Env, respectively (Bolze, Mommert, and Mallet 2017). Some of the critical aspects of placenta development include fusion, proliferation,

angiogenesis, immune tolerance, and tissue survival. These envelope glycoproteins have been shown to have fusogenic and immunosuppressive properties, thus supporting essential functions of placenta development (Malassiné et al. 2007). Syncytins have been shown to have similar roles in the placental development of mice (Gong et al. 2007).

HERV expression linked to neurological and autoimmune diseases

HERVs have been linked to autoimmune diseases such as multiple sclerosis (MS) and sporadic amyotrophic lateral sclerosis; the role of HERV in MS was described previously. Sporadic amyotrophic lateral sclerosis is a progressive neurodegenerative disease, and is usually fatal, except in the case of some ALS patients with HIV infection; the anti-retroviral therapy used to treat HIV infection has sometimes been shown to reverse ALS-like syndromes (Bowen et al. 2016; Alfahad and Nath 2013; Orsini et al. 2012; Calza et al. 2004; Casado et al. 1997; Galassi et al. 1998; Verma, Ziegler, and Kepes 1990). Reverse transcriptase activity has been detected in the blood and brain tissue of ALS patients, prompting the search for the exogenous retroviruses responsible (MacGowan et al. 2007; McCormick et al. 2008; Steele et al. 2005; Viola et al. 1975). However, the search for exogenous retroviruses that can be linked to ALS has not been successful. Interestingly, HERV-K was found to be expressed in the neurons of ALS patients but not in the neurons of healthy individuals, and the expression of HERV-K envelope protein was shown to cause the retraction and beading of neurites (Douville et al. 2011; Li et al. 2015). The expression of HERV-K Env caused a decrease in cell

numbers, thus suggesting that the envelope protein could contribute to neurotoxicity and neuronal death (Li et al. 2015). Furthermore, the expression of HERV-K Env in the embryonic mouse brain resulted in the degeneration of motor neurons (Li et al. 2015). Thus, mounting evidence suggests that HERV-K expression might play a role in the pathophysiology of ALS, although this remains to be clarified.

HERVs are also believed to be pathogenic in rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). RA and SLE are very complex diseases, and the induction of disease requires genetic susceptibility, environmental factors, and inopportune circumstances (Tugnet et al. 2013). It is suggested that HERVs induce RA and SLE through immune dysregulation and molecular mimicry. The theory of molecular mimicry is that a foreign protein that shares sequence homology to self-peptides can result in self-reactive cells, such as B or T cells (Tugnet et al. 2013). HERV-K has been detected in the plasma of RA patients, with higher titers observed in patients with active disease; RA patients exhibited elevated levels of HERV-K *gag* expression as compared to healthy controls (Freimanis et al. 2010). It is thought that HERV expression can generate an immune response and induce disease through molecular mimicry, providing a continuous source of antigen for the self-reactive immune cells. HERV antigens are similar to exogenous viral antigens that may cross-react with self-antigens, and therefore could elicit pathological antibodies (Tugnet et al. 2013). As an example, the gag protein of HTLV-related endogenous sequence-1 (HRES-1) has sequence homology and cross-reactivity with the U1-small nuclear ribonucleoprotein (U1-snRNP); the U1-snRNP is an autoantigen, and a target of

autoreactive B and T cells in rheumatic diseases such as SLE (Perl et al. 1995). In a study, it was shown that 50% of SLE patients had anti-HRES-1 antibodies that bound to the U1-snRNP autoantigen, while antibodies were only detected in 3.6% of healthy individuals, suggesting that the expression of the HERV protein triggered an autoimmune response directed against U1-snRNP (Perl et al. 1995). Normally, self-reactive B and T cells are eliminated to ensure that the immune system does not attack itself. However, in the case of SLE patients, dysregulation of apoptosis allows for the self-reactive cells to bypass deletion (Emlen, Niebur, and Kadera 1994). Taken together, it is possible that HERVs induce immune reactivity in rheumatoid diseases.

HERV expression and cancer

As noted previously, HERV-K has been implicated in different human malignancies such as breast cancer, prostate cancer, ovarian cancer, melanoma, germ cell tumors, leukemia, and lymphomas (Seifarth et al. 1998; Wang-Johanning et al. 2001; Bieda, Hoffmann, and Boller 2001; Büscher et al. 2005; Löwer et al. 1993; Muster et al. 2003; Contreras-Galindo et al. 2012; Contreras-Galindo et al. 2013, 2006; Contreras-Galindo et al. 2008; Contreras-Galindo, Almodóvar-Camacho, et al. 2007; Contreras-Galindo, López, et al. 2007). High level of HERV-K transcripts can be seen in disease states but generally not in healthy individuals, and the expression of HERV-K proteins appears to be particularly restricted to malignancies (Schmitt et al. 2015). The HERV-K accessory proteins Np9 and Rec have been suggested to be oncoproteins (Chen et al. 2013; V. Armbruester et al. 2004; Kaufmann et al. 2010; Denne et al. 2007;

Galli et al. 2005; Gross et al. 2011). Most HERVs are silenced because most have taken residence in areas of the genome that are hyper-methylated. However, global hypo-methylation has been seen in some cancers and, therefore, the activation of HERV-Ks result from this process (Mullins and Linnebacher 2012). It is important to note that the activation or expression of HERV-K and HERV-K products has been seen in all the human malignancies noted above.

Interestingly, the HERV-K Env protein has been shown to be over-expressed in breast cancer cells (BC cells) and tissues (Zhao et al. 2011). In a recent study, investigators knocked-down the expression of HERV-K Env in breast cancer cells, which hindered BC cell proliferation, migration, and invasion; knocking-down HERV-K Env prevented BC cells from forming tumors, thus preventing metastasis (Zhou et al. 2016). The downregulation of HERV-K blocked the expression of Ras, p-RSK, and p-ERK, which are tumor associated genes, and the re-introduction of HERV-K Env in HERV-K Env knock-down BC cells resulted in the restoration of the Ras/Raf/MEK/ERK signaling pathway (Zhou et al. 2016). These findings led the investigators to conclude that the activation of the HERV-K Env protein is essential for the tumorigenesis and metastasis of breast cancer cells (Zhou et al. 2016).

The expression of HERV-K Env, Rec and Np9 proteins has been detected in melanomas and melanoma cells but not in melanocytes (Büscher et al. 2005, 2006), and the activation of HERV-K has been implicated in the malignant transformation of melanoma cells (Serafino et al. 2009). Furthermore, UV irradiation of normal human

epidermal melanocytes induced the expression of HERV-K *rec* and *np9* transcripts; the *rec* and *np9* transcript levels remained unchanged in UV irradiated melanoma cells, suggesting that Rec and Np9 could play a functional role in melanoma formation (Reiche, Pauli, and Ellerbrok 2010).

HERV-K 111

In recent years, during our study of the activation of HERV-K by the HIV TAT accessory protein, we discovered HERV-K (HML-2) RNA sequences in HIV-1 patients that were not present in the recently annotated human genome assembly. We termed the newly-discovered HERV-K (HML-2) type 1 virus K111. The K111 virus inserted itself into the genome of the hominid lineage prior to the split between chimpanzees and humans. This virus exists in chimpanzees as a single copy (none in gorillas or lower primates), but can be found in multiple copies in the modern day human, and copies of this virus are spread across the centromeres of 15 chromosomes. During the evolution of humans, the virus expanded in copy numbers through a process resembling homologous recombination. The expansion of the K111 viruses suggests that recombination took place between the centromeres of various chromosomes during human evolution. From our investigation, we saw a significant variation in the sequence of K111 proviruses in each centromere. To date, it is still not clear how many copies of K111 exists in the human genome, but it appears that there are between 100 to one thousand copies. However, most of the viral genes of K111 are mutated and cannot produce viable viral products, with one exception: the K111 virus contains an intact

open reading frame for the Np9 protein and variants of Np9. For this reason, we were intrigued to investigate the reason for retaining the coding sequence for the accessory protein. Could the HERV-K accessory protein Np9 play a role in development or promote oncogenesis, or does it exist as a simple bystander in health and disease?

The implication of Np9 in cancer and its disruptive influence on cell signaling pathways

The activation and inactivation of cell signaling pathways are highly modulated; any perturbations in the cell signaling pathways can be detrimental, and lead to uncontrolled cell proliferation and tumorigenesis. In recent years, some light has been shed on the possible functions of Np9 and Rec in human biology in terms of interacting partners, and their role in different cell signaling pathways.

As noted previously, the physical interaction between Rec and Np9 with the promyelocytic zinc finger protein results in the de-repression of c-*myc*, and improper regulation of c-*myc* can result in over-proliferative cells and promote oncogenesis (Denne et al. 2007). Also, Np9 has been shown to interact with LNX (ligand of numb protein X), an E3 ubiquitin ligase that targets Numb in the Notch signaling pathway (Armbruester et al. 2004). Further, Np9 can activate ß-catenin, ERK, Akt, and Notch1, and promote the growth of human leukemia stem/progenitor cells (Chen et al. 2013). The interaction between Np9 and repressive elements, such as PLZF and LNX/Numb, alters the normal biological function of the repressive elements, and results in the

activation of signaling pathways (Denne et al. 2007; Armbruester et al. 2004). Furthermore, Np9 has been shown to activate pro-proliferative cell signaling pathways, such as Wnt/ß-catenin and Notch1 (Armbruester et al. 2004; Chen et al. 2013). Therefore, we set out to explore the role of Np9 in other malignancies.

The role of Np9 in teratocarcinoma cell viability

To further examine the role of Np9 in cancer, we compared the expression level of *np9* transcripts in different human cancer cell lines that were thought to be associated with HERV-K (Figure 3.1). Certain of these cell lines have also been shown to produce HERV-K (HML-2) VLPs. Specifically, the cell lines that we studied were derived from human breast cancer, prostate cancer, germ cell tumors (ovarian and testicular), and melanoma. Even though np9 transcripts were found in all the cancer cell lines tested, they were more highly expressed in some of the breast cancer cell lines, melanoma cell lines, and germ cell tumor cell lines. The expression of *np9* transcripts varied among germ cell tumor cell lines, with a higher expression level in testicular derived germ cell tumor cell lines than in ovarian derived germ cell tumor cell lines.

Teratocarcinoma is a testicular germ cell tumor that has been shown to be more prevalent in young men, which is concerning considering the chances of relapse and the toxicity of treatment regimens. The treatment regimens used for this cancer can be quite effective even in disseminated disease. However, relapse can occur in individuals with late stage disease a year after concluding treatment. The treatment regimen

usually consists of bleomycin and cisplatin, both of which are highly toxic chemotherapeutic agents. Considering that teratocarcinoma occurs in young men, long-term remission and fertility are also major concerns and development of less toxic and more effective treatment regimens is necessary. We therefore considered whether the expression of Np9 in testicular germ cell tumor cells was the result of the disease, was simply a disease marker, or whether Np9 expression promotes tumorigenesis.

We hypothesized that Np9 promoted tumorigenesis in NCCIT teratocarcinoma cells, a cell line that express viral like particles containing K111. However, the reduction of Np9 in NCCIT teratocarcinoma cells using CRISPR/Cas9 mediated genome editing resulted in only modest alterations to the viability of the cells, which was quite puzzling. It is possible that full depletion of Np9 is necessary to see very significant hindrance to the viability of NCCIT cells. However, tools necessary to deplete the genome of all Np9 and its variants are not available. Also, it is possible that the function of Np9 in NCCIT teratocarcinoma cells is not one of promoting proliferation but that of survival. When we subjected the teratocarcinoma cells to environmental stress in conjunction with reduced Np9 expression, there was marked reduction in cell viability. The striking reduction in viability in the Np9 KD cells compelled us to question the effect of chemotherapeutic agents on the KD cells. We reasoned that if Np9 played a role in the survival of NCCIT cells, then the reduction of Np9 would increase teratocarcinoma cell sensitivity to bleomycin and cisplatin. We indeed found that the Np9 KD cells were more susceptible to bleomycin or cisplatin treatment, and the response to the chemotherapeutic agents was dose-dependent. The reduction of Np9 worked synergistically with the

chemotherapeutic agents, thus reducing cell viability. Further, treating NCCIT cells with bleomycin resulted in the increased activation of caspase dependent apoptotic cell death in Np9 KD cells; the mechanism of increased cell death is still unclear in terms of cisplatin treatment.

The studies up to that point were focused the role of Np9 in teratocarcinoma cell viability. We decided to look at another crucial hallmark of cancer, which is the migration of cancer cells. The migration of cancer cells is an important aspect of tumor progression; metastasis is a result of cancer cells migrating from a centralized region to adjacent regions, thus increasing the level of difficulty in the clearance of circulating cancer cells even after primary tumor removal. Impressively, reduced expression of Np9 greatly hindered the migration ability of the NCCIT cells, and the re-introduction of Np9 in KD cells rescued the migration phenotype.

If further studies *in vivo* and *in vitro* continue to demonstrate that Np9 expression plays a pivotal role in the invasiveness of the cancer cells it would prompt a search for an agent that would inactivate Np9 and be used as part of treatment regimens. However, it is important to stress the difficulty in targeting HERVs in the human genome. Considering that 8% of the human genome consists of HERVs, the first hurdle that must be overcome is the complexity of specifically targeting HERVs or HERV products. Adding to the complexity is the possibility that insufficient reduction of Np9 will lead to therapeutic failure, whereas too much reduction might harm Np9 cellular function as discussed in the Appendix.

Identifying Np9’s role in different cell signaling pathways in teratocarcinoma cells

Testicular germ cell tumor incidence has increased in the last decade and it is the most common solid tumor in young males (Cooper et al. 2008). However, the molecular mechanisms of teratocarcinoma formation are still not well understood. Aberrant activation of cell signaling pathways is a common event in human tumor progression. Investigators attempted to determine if the aberrant cell signaling processes give rise to the formation of teratocarcinoma. For example, a study led by Jing Hao explored the mechanism and function of the Wnt/ß-catenin signaling pathway in the pathogenesis of teratocarcinoma (Zhang et al. 2012). Researchers treated P19 mouse teratocarcinoma cells with all trans-retinoic acid and a GSK3ß inhibitor to induce P19 cell differentiation and activate the Wnt/ß-catenin signaling pathway, respectively. In their study, investigators measured P19 cell proliferation by looking at gene expression using quantitative RT-PCR, protein expression using Western blotting, and cell proliferation using BrDU incorporation. Their results showed that GSK3ß inhibitor treatment of P19 teratocarcinoma cells results in the activation of the Wnt/ß-catenin signaling pathway, as represented by the nuclear translocation of ß-catenin, the upregulated the expression of c-*myc* and pluripotent-related genes such as *oct4*, *sox2*, and *nanog*. Further, GSK3ß inhibition blocked cell differentiation normally induced by all-trans retinoic acid. In summary, researchers found that the activation of Wnt/ß-catenin cell signaling pathway can promote cell proliferation of P19 teratocarcinoma cells, and the upregulation of c-*myc* inhibited differentiation (Zhang et al. 2012).

During our studies on the effect of Np9 knockdown in NCCIT teratocarcinoma cells, we wanted to determine whether Np9 played a role in cell signaling pathways involved in teratocarcinoma. Considering that the Wnt/ß-catenin signaling pathway might play a role in teratocarcinoma progression, we investigated whether knocking down Np9 in NCCIT teratocarcinoma cells affected the expression of Wnt related proteins (Figure 3.2). In the absence of Wnt-signal (off-state), ß-catenin is phosphorylated by CK1 and the APC/Axin/Gs3kß complex leads to its ubiquitination and subsequent proteasomal degradation. In the presence of Wnt-signal (on-state), ß-catenin is stabilized and translocated to the nucleus where it binds to LEF/TCF transcription factors, displaces co-repressors, and ultimately recruits additional co-activators to Wnt target genes.

When we knocked down the expression of Np9 in NCCIT teratocarcinoma cells, we did not see a difference in the expression of ß-catenin (active form) (Figure 3.2); therefore, we concluded that the reduced expression of Np9 has no effect on the Wnt/ß-catenin signaling pathway. Recent studies have shown that Np9 play a role in the MAPK/Erk, c-myc/AKT, and the Notch1 signaling pathways in human leukemia cells (Chen et al. 2013). We determined that the reduced expression of Np9 in NCCIT cells has no effect on the expression of phosphorylated c-myc or phosphorylated Akt (Figure 3.2).

We then considered the role of Np9 in other signaling pathways. To begin, we looked at the effect of knocking down Np9 in NCCIT cells on the MAPK/Erk signaling

pathway, which is involved in cell growth and differentiation. To our surprise, reduced expression of Np9 in NCCIT cells resulted in the downregulation of phosphorylated Erk1/2 (Figure 3.3A), but it had no effect on Mek1/2 or phosphorylated Mek1/2, which are upstream of Erk1/2 (Figure 3.3B), suggesting Np9 expression plays a role in the regulation of Erk1/2. The MAPK/Erk signaling pathway controls transcription and cell cycle progression through the activation and regulation of MAPK/Erk-related proteins such as p90RSK (transcription) and cdc25c (cell cycle progression).

Next, we wanted to determine if knocking down Np9 in NCCIT teratocarcinoma cells altered the expression of MAPK/Erk related proteins that regulate transcription and cell cycle progression. We saw that the reduced expression of Np9 in NCCIT cells downregulated the expression of cdc25c, but the Np9 downregulation had no effect on p90RSK (Figure 3.3B). Therefore, we conclude the reduced expression of Np9 results in the downregulation of the MAPK/Erk pathway and cdc25c, and can potentially disrupt cell cycle progression.

Lastly, we wanted to determine whether the reduced expression of Np9 in NCCIT teratocarcinoma cells alter the Notch1 cell signaling pathway. As mentioned previously, the Np9 protein interacts with the Notch repressive element LNX/Numb. Therefore, we sought to answer whether the reduction in Np9 expression alters the activation of the Notch1 pathway. As shown in Figure 3.4, the reduction of Np9 in NCCIT teratocarcinoma cells resulted in the upregulation of phosphorylated Numb, which is the inactive form that would be subjected to proteasomal degradation. Interestingly, we

witnessed a downregulation of MAML-1, a Notch transcriptional co-activator, which indicated a downregulation of the Notch pathway. Our attempt at elucidating the mechanistic role of Np9 in different signal transduction pathways illustrated that Np9 may play a role in the MAPK/Erk and in the Notch pathway in teratocarcinoma cells. However, the preliminary studies presented here on the role of Np9 in signal transduction pathways were not always replicable, and therefore, further studies would be necessary to fully to validate the findings.

Conclusion

Evidence thus far suggests that the role of HERVs in human biology, disease and malignancy is incredibly complex. HERVs makeup a sizeable portion of the human genome and the HERV-K (HML-2) subfamily is one of the most recent entrants into the human genome; this subfamily has remained transcriptionally active, and can produce viral products. Since our discovery of the HERV-K type 1 virus termed K111 in the centromeric region of human chromosomes, we have determined that there are at least 100 to one thousand copies spread across 15 different human chromosomes. The K111 virus is highly mutated in almost all viral genes, rendering it incapable of producing a replicative virus. However, it retains the open reading frame for the accessory protein Np9. We speculated that the coding sequence for Np9 was retained in the modern day human because it performed a role in human biology.

In our study of Np9 in teratocarcinoma cells, we have discovered that Np9 played a different role in tumorigenesis than originally expected. We initially hypothesized that the role of Np9 in tumorigenesis was that of promoting cell proliferation. However, the Np9 accessory protein actually promotes tumorigenesis by reducing the sensitivity of teratocarcinoma cells to environmental and chemical stresses, thus promoting cell viability. Further, Np9 is essential for the migration of teratocarcinoma cells, and migration of cancer cells is crucial for metastatic progression. Therefore, there is mounting evidence that the HERV-K Np9 accessory protein is an oncoprotein. Also, our preliminary studies on the role of Np9 in signal transduction pathways illustrate that the function of Np9 is complex. A continuation of the study on identifying potential interacting partners of Np9 could provide a clue as to the role of Np9 in teratocarcinoma.

Conversely, preliminary studies done in collaboration with Dr. Orly Reiner and Dr. Tamar Sapir suggest that the expression of Np9 is beneficial for brain development (See Appendix). Taken together, our data suggest that the role of Np9 in human biology is can be either beneficial or detrimental, and will require further studies to understand the complexity of its role.

Figures

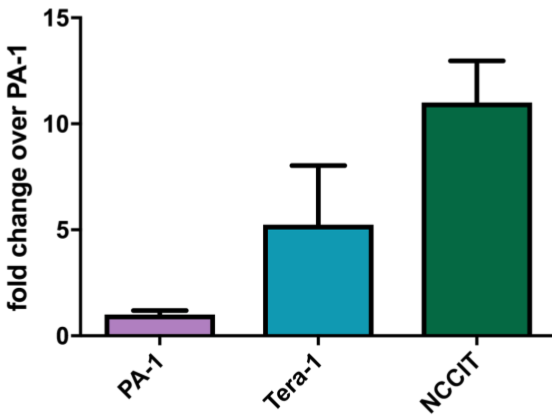


Figure 3.1 Quantitative RT-PCR of *np9* mRNA in different teratocarcinoma cells. RNA was extracted from three teratocarcinoma cell lines: Pa-1, Tera-1, and NCCIT. qRT-PCR was performed to quantitate the expression of *np9* mRNA in the three teratocarcinoma cells; the expression of *np9* was normalized to GAPDH, a housekeeping gene. The expression of *np9* mRNA in Tera-1 and NCCIT was compared to that of Pa-1, as Pa-1 is a an ovarian derived teratocarcinoma cell line known to not produce VLPs, and is known to express little to no HERV-Ks. The expression of *np9* mRNA in the three teratocarcinoma cell lines varied, with NCCIT being the teratocarcinoma cell line to have the highest expression of *np9*. Data were obtained by Susana M. Chan.

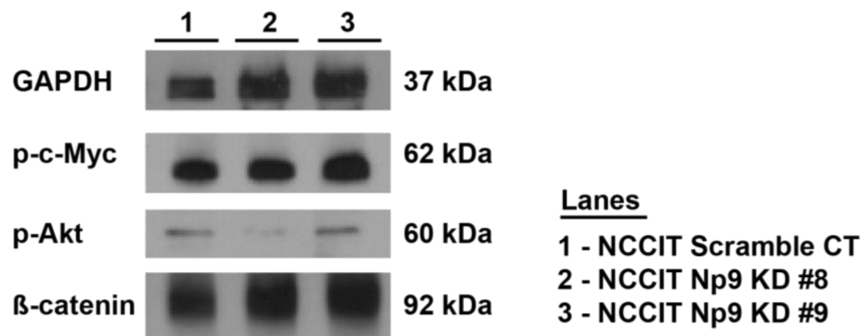
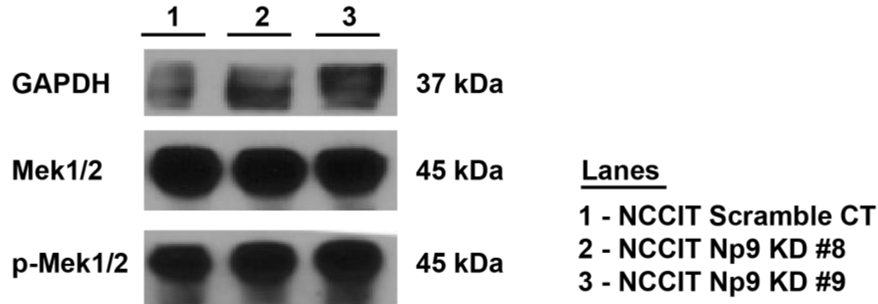


Figure 3.2 The reduced expression of Np9 in teratocarcinoma had no effect on the c-myc and Wnt/ß-catenin signaling pathways. Knocking down the expression of Np9 in NCCIT teratocarcinoma cells did not affect the Wnt/ ß-catenin signaling pathway, nor did it alter the expression of phosphorylated c-Myc or phosphorylated Akt. Data were obtained by Susana M. Chan.

A)



B)

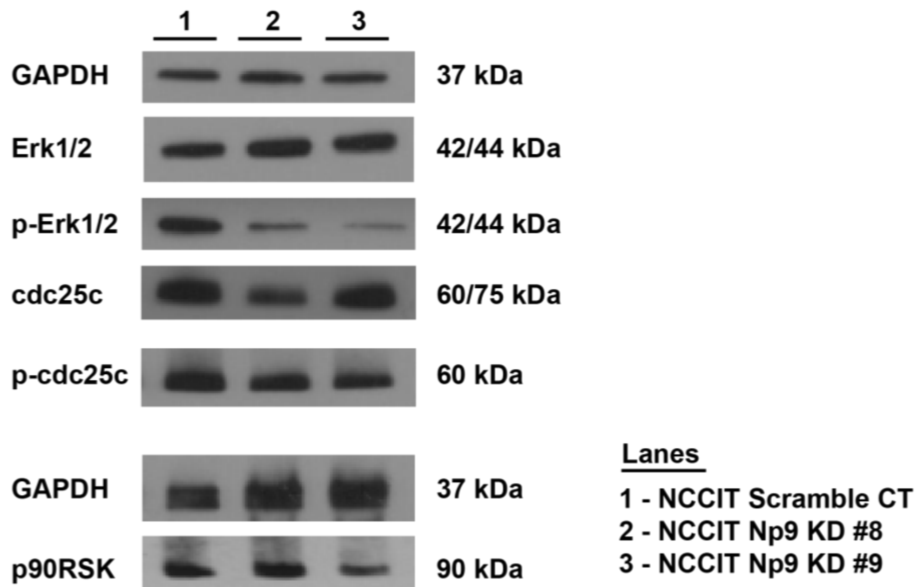


Figure 3.3 The reduced expression of Np9 in teratocarcinoma cells downregulated the MAPK/Erk signaling pathway. (A) Knocking down the expression of Np9 in NCCIT cells had no effect on Mek1/2 and p-Mek1/2, a MAPK element upstream of Erk1/2. (B) Knocking down the expression of Np9 resulted in the downregulation of p-Erk1/2 and cdc25c, a downstream element of Erk1/2 that plays a role in cell cycle progression. The reduced expression of Np9 had no effect on p90RSK, a downstream element of Erk1/2 responsible for transcription. Data were obtained by Susana M. Chan.

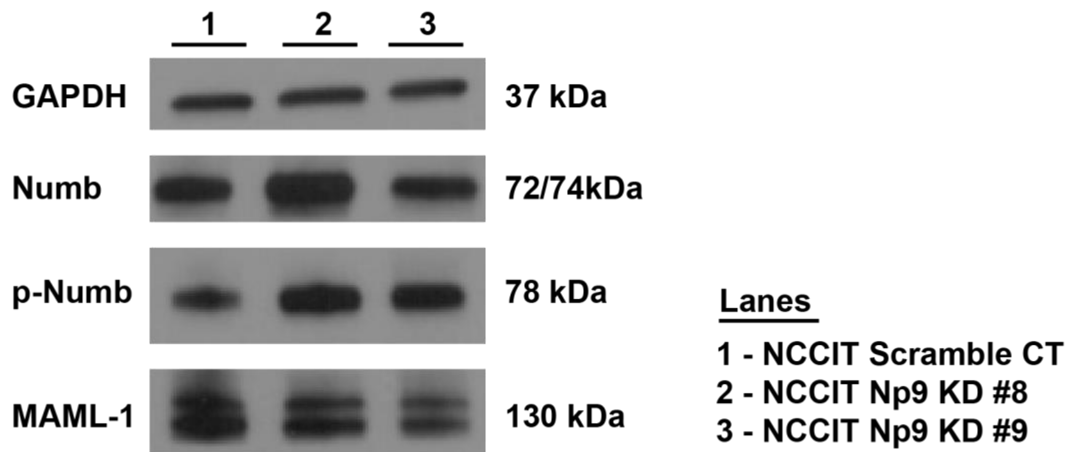


Figure 3.4 The reduced expression of Np9 resulted in the altered regulation of Numb, a Notch repressive element. Knocking down the expression of Np9 in teratocarcinoma cells resulted in the increased phosphorylation of the Numb protein, normally a Notch repressive element. In the phosphorylated state, Numb is subjected to proteasomal degradation. However, there seemed to be no increase in Notch activation indicated by the downregulation of MAML-1, a Notch co-activator. Data were obtained by Susana M. Chan.

Appendices

The role of Np9 in brain development

My thesis has focused on the role of Np9 in human disease by looking at the function of Np9 in human teratocarcinoma cells. However, we also wondered whether, analogous to syncytin, Np9 might play a role in normal human development. We hypothesized that Np9 might be important in the development of the human brain, as K111 is so abundant, expressed only in human, and produces only Np9. Dr. Orly Reiner and Dr. Tamar Sapir at the Weizmann Institute of Science in Israel have led a collaboration with us on the role of Np9 in brain development and neurological diseases.

Brain development throughout the course of human life is an exceptionally complex and time-sensitive process. During the embryonic and fetal stages of early life, the brain must undergo high, but modulated, levels of neuronal proliferation, differentiation, migration, synaptogenesis, and apoptosis. Subsequently, the brain continues to mature through adolescence and into adulthood in a manner and pacing distinct from that of its prenatal development. The precise control of these cell fate determinations over the course of an individual's life is vital for the successful development of the human brain. Multiple signaling pathways have been identified to be involved in these cell fate determinations, and we and our collaborators focused on two

of the pathways thought to be under control of Np9: Wnt/ß-catenin and Notch (Lui, Hansen, and Kriegstein 2011). Alterations or imprecise control of these same pathways that are important for successful brain development have also been associated with neurological diseases such as Alzheimer's disease and schizophrenia (Al-Harthi 2012).

During embryogenesis, Wnt expression promotes neural stem cell proliferation through the translocation of ß-catenin into the nucleus to act as a transcription factor (Lui, Hansen, and Kriegstein 2011). Later in central nervous system (CNS) development, Wnt signaling is important for inducing neuronal and astroglia differentiation and suppressing oligodendrocyte differentiation (Lui, Hansen, and Kriegstein 2011). The Notch signaling pathway plays a key role in the brain, activating the HES family genes, the BLBP gene, and both n- and c-Myc (Lui, Hansen, and Kriegstein 2011). As a result, during CNS development Notch signaling plays an active role in stem cell self-renewal, inhibiting neuronal commitment, promoting astrocyte-oriented fates, and affecting both brain morphogenesis and neuronal migration (Lui, Hansen, and Kriegstein 2011). Additionally, the Notch pathway is highly active in ventricular radial glia cell and outer radial glia cells (Lui, Hansen, and Kriegstein 2011). Importantly, the results of both Wnt/ß-catenin and Notch signaling are dependent on the context of other signaling cascades, and thus can result in apparently opposing outcomes at various times in brain development.

Considering the vastly increased presence of HERV-K *np9* gene in humans as compared to primates, the complete absence in other mammals, and the interaction of

Np9 in pro-tumorigenic pathways that overlap with those known to be important in brain development, Np9 struck us as an intriguing candidate to be involved in driving the emergence of the modern-day human brain. We examined the expression of *np9* transcripts in post-mortem brain samples from human adults. For the study, RNA was extracted from five brain regions (cerebellum, amygdala, hippocampus, nucleus accumbens, and dorsolateral prefrontal cortex) of four deceased adult patients, and the expression of *np9* mRNA was determined by qRT-PCR. The results of these experiments are shown in Figure A.1, in which the expression of *np9* mRNA was normalized to *gapdh* and plotted relative to that of Tera-1 teratocarcinoma cell lines, previously shown to express high levels of both *np9* mRNA and protein. Strikingly, all brain regions tested expressed equivalent or greater *np9* mRNA than did Tera-1 cells.

We and our collaborators hypothesized that expression of Np9 into the mouse embryonic brain would affect signaling in the pathways previously mentioned, and thus alter the developing mouse brain through changes in the number of progenitor cells and perhaps the ratio between different types of progenitors residing in the ventricular zone (VZ) and sub-ventricular zone (sVZ). Subsequently, Np9 expression might then also alter cell fate determination, neuronal migration, positioning in the cortex, neuronal morphology and/or neuronal connectivity. For our study, Np9 was expressed in the developing embryonic mouse brain at E13, when the relative proportion of cycling cells is high. Np9 was introduced by *in utero* electroporation of a Np9 mammalian expression plasmid tagged with a fluorescent protein; the fluorescent protein allowed for the monitoring of Np9 expression and determined the success of the electroporation. The

preliminary experiment was conducted in combination with a Notch reporter system, and showed that Np9 increased Notch activity, and altered cellular localization within the mouse brain after only 24 hours, with retention of cells in the apical region of the VZ (Figure A.2). Currently, our collaborators are knocking-down Np9 in human brain organoids using CRISPR/Cas9 and studying the effect on cellular migration and on the Wnt/ ß-catenin and Notch cell signaling pathways. Thus, while expression of Np9 can drive malignancy, the HERV-K protein may also play a role in healthy human brain development.

Figures

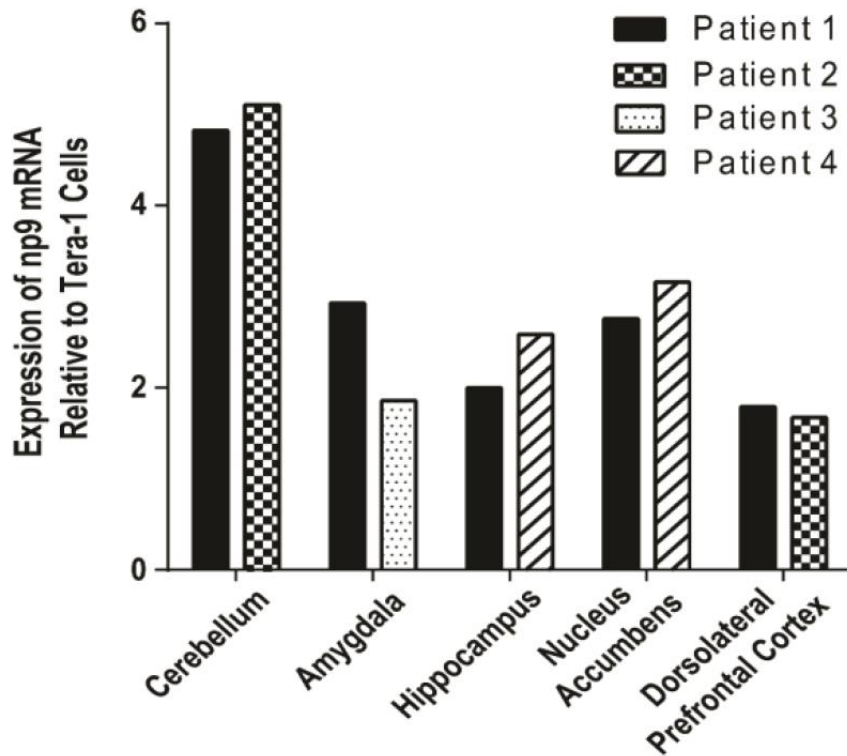


Figure A.1 Relative Np9 expression in post-mortem adult human brain samples. RNA was extracted from the indicated brain regions of four deceased human adults and the expression of HERV-K *np9* mRNA analyzed compared to that of the highly expressing Tera-1 teratocarcinoma cell line (equal to 1) by quantitative real time RT-PCR. Amplification of *gapdh* mRNA was used for normalization, and the relative expression graphed was calculated using the $\Delta\Delta$cT method. Data were obtained by Dr. Derek Dube.

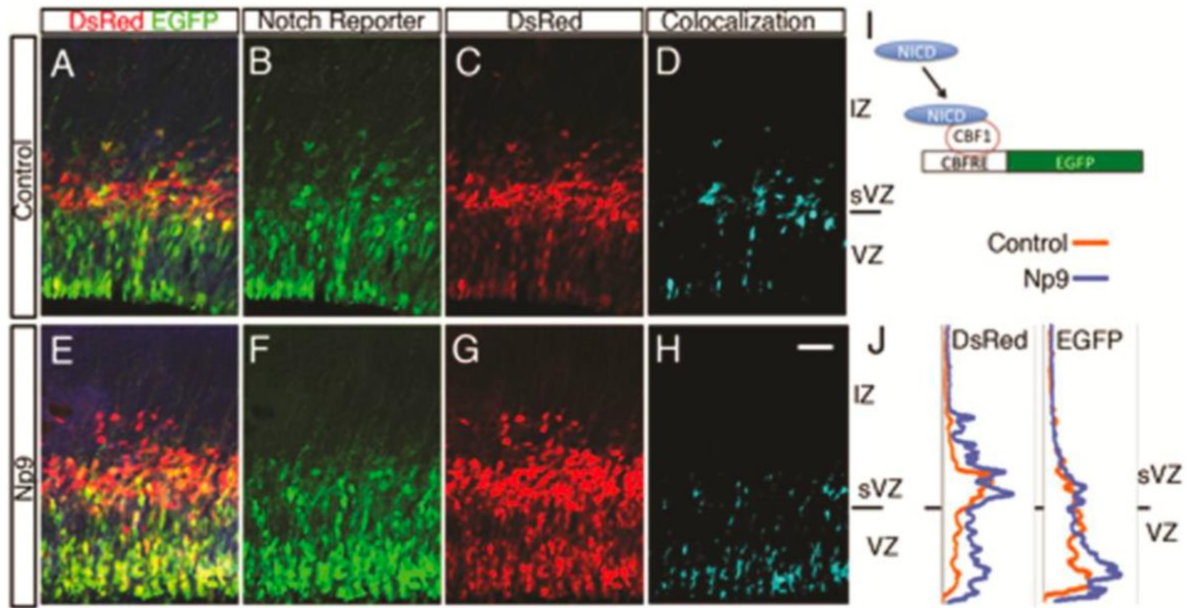


Figure A.2 Forced expression of Np9 affects Notch activity in the developing cortex. CBFRE-EGFP was in utero electroporated to E13 mouse embryos together with DsRed expression plasmid (A-D) or with DsRed and Np9 (E-H). The brains were collected one day later, sectioned and stained with anti-GFP. (I) EGFP signal represents NICD-CBF1 binding to CBF responsive element and is a readout for Notch activation. The Notch activity in the control brains is localized in the VZ, with a peak expression in the nuclei at the apical surface and a sharp drop of activity in sVZ and intermediate zone (IZ) (A, B J). A small portion of double labeled cells (DsRed+ EGFP+) is seen in the basal aspeact of the VZ and, to a lesser level, in the apical side of the VZ (D). Over-expression of Np9 (E-H), caused labeled cells to be retained in the apical regions of the VZ (G). These cells generally exhibit high Notch activity (H). A histogram (J) of signal levels along the VZ/sVZ and the IZ demonstrates the differences between control (orange) and Np9 over-expressing brains (blue). Scale is 50 microns. Data obtained by Drs. Orly Reiner and Tamar Sapir at the Weizmann Institute as part of an ongoing collaboration with Susana M. Chan and Dr. David Markovitz.

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