Fakultät für Medizin

Analyzing the role of human endogenous retrovirus, HERV-K(HML-2), on cortical neuron differentiation

Vidya Padmanabhan Nair

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades einer **Doktorin der Naturwissenschaften (Dr. rer. nat.)** genehmigten Dissertation.

Vorsitz: Prof. Dr. Thomas Misgeld

Prüfer innen der Dissertation:

1. Prof. Dr. Ulrike Protzer

2. Prof. Dr. Wolfgang Wurst

3. Prof. Dr. Benjamin Schusser

Die Dissertation wurde am 23.02.2021 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 07.12.2021 angenommen.

Table of Contents

Abstract

The Human Genome Project (HGP) has revealed that essentially more than 45% of the human genome consists of mobile genetic elements, most of which comes under the class of retrotransposons. Out of these, more than 8% of the genomic make up encompass sequences of infectious retroviruses known as human endogenous retroviruses (HERVs) which are presumed to be integrated 2-40 m.y.a during primate evolution and amplified throughout the evolutionary timescale. In this thesis, I generated a CRISPR activation and CRISPR inhibition system in human embryonic stem cells to actively regulate the most recently integrated member of the HERV family, HERV-K(HML-2) and attempted to delve deeper into the mechanistic detailing of how these elements influence the neuronal differentiation and brain development. To this end, the HERV-K(HML-2) overexpressing stable H9-dCas9-VP64 and HERV-K(HML-2) repressed stable H9-dCas9-KRAB embryonic stem cell lines were established for active transcriptional regulation of HERV-K(HML-2) LTRs. Steady monitoring of the differentiation of HERV-K(HML-2) activated cells into cortical neurons showed considerable decrease in the overall MAP2 expression levels as well as decreased functionality of neurons, suggesting the potential role of these elements in impairment of cortical neuronal development and subsequent roles in manifestation of several neuron-related disorders. Interestingly, the differentiation into dopaminergic neuronal lineage or repression of HERV-K(HML-2) showed no signs of an inefficient differentiation pattern, suggesting a cortical specific outcome. A whole genome RNA sequencing of HERV-K(HML-2) activated cortical neurons showed enrichment in neuron-related genes, suggesting a clear correlation of HERV-K(HML-2) activation and neuron-related gene expression. We observed that activation of HERV-K(HML-2) transcription resulted in the synchronous upregulation of Neurotrophic Tyrosine Receptor Kinase 3 (NTRK3) expression along with other genes associated with neuronal differentiation and development. CRISPRa of NTRK3 and differentiation into cortical neurons resulted in a phenotype similar to that of HERV-K(HML-2) activation observed earlier. Furthermore, analysis of forebrain organoids generated from HERV-K(HML-2) activated cells showed a difference in overall size and organisation of deep brain layers and expression of cell type specific markers. Taken together, these results conclusively demonstrate the active participation of HERV-K(HML-2) elements in revamping and regulating the progression of cortical neuronal differentiation and remodelling the homeostasis of human brain development which could potentially translate into several neuron related dysfunctions.

Zusammenfassung

Das Human Genome Project (HGP) hat gezeigt, dass im Wesentlichen mehr als 45 % des menschlichen Genoms aus mobilen genetischen Elementen besteht, von denen der größte Teil in die Klasse der Retrotransposons fällt. Davon umfassen mehr als 8% des Genoms Sequenzen infektiöser Retroviren, die als humane endogene Retroviren (HERVs) bekannt sind und von denen man annimmt, dass sie während der Evolution der Primaten 2-40 Mio. Jahren integriert und über die evolutionäre Zeitskala hinweg amplifiziert wurden. In dieser Arbeit generierten wir ein CRISPR-Aktivierungs- und CRISPR-Inhibitionssystem in humanen embryonalen Stammzellen, um das jüngste integrierte Mitglied der HERV-Familie, HERV-K(HML-2), aktiv zu regulieren, und versuchten, die mechanistischen Details zu ergründen, wie diese Elemente die neuronale Differenzierung und Gehirnentwicklung beeinflussen. Zu diesem Zweck wurden die HERV-K(HML-2) überexprimierenden stabilen H9-dCas9-VP64 und HERV-K(HML-2) unterdrückenden stabilen H9-dCas9-KRAB embryonalen Stammzelllinien für die aktive transkriptionelle Regulation der HERV-K(HML-2) LTRs etabliert. Die ständige Überwachung der Differenzierung von HERV-K(HML-2) aktivierten Zellen in kortikale Neuronen zeigte eine erhebliche Abnahme der gesamten MAP2-Expressionsniveaus sowie eine verringerte Funktionalität der Neuronen, was auf die mögliche Rolle dieser Elemente bei der Beeinträchtigung der kortikalen neuronalen Entwicklung und die anschließende Rolle bei der Manifestation verschiedener neuronenbezogener Störungen hindeutet. Interessanterweise zeigte die Differenzierung in die dopaminerge neuronale Linie keine Anzeichen eines ineffizienten Differenzierungsmusters, was auf ein kortikalspezifisches Ergebnis hindeutet. Eine Ganzgenom-RNA-Sequenzierung von HERV-K(HML-2) aktivierten kortikalen Neuronen zeigte eine Anreicherung in neuronenbezogenen Genen, was auf eine klare Korrelation von HERV-K(HML-2)-Aktivierung und neuronenbezogener Genexpression hinweist. Wir beobachteten, dass die Aktivierung der HERV-K(HML-2)-Transkription zu einer synchronen Hochregulierung der Expression der Neurotrophen Tyrosin-Rezeptor-Kinase 3 (NTRK3) zusammen mit anderen Genen führte, die mit der neuronalen Differenzierung und Entwicklung assoziiert sind. Die CRISPRa von NTRK3 und die Differenzierung in kortikale Neuronen führte zu einem ähnlichen Phänotyp wie die zuvor beobachtete Aktivierung von HERV-K(HML-2). Darüber hinaus zeigte die Analyse von Vorderhirn-Organoiden, die aus HERV-K(HML-2) aktivierten Zellen generiert wurden, einen Unterschied im Gesamtwachstum und in der Organisation der tiefen Hirnschichten sowie in der Expression von zelltypspezifischen Markern. Zusammengenommen zeigen diese Ergebnisse schlüssig die

aktive Beteiligung von HERV-K(HML-2)-Elementen an der Erneuerung und Regulierung des Fortschreitens der kortikalen neuronalen Differenzierung und der Umgestaltung der Homöostase der menschlichen Gehirnentwicklung, was sich potenziell in verschiedenen neuronenbezogenen Dysfunktionen niederschlagen könnte.

Abbreviations

AA	Ascorbic Acid
AD	Alzheimer's Disease
AKAP12	A-Kinase Anchoring Protein -12
ALS	Amyotrophic Lateral Sclerosis
APS	Ammonium Per Sulphate
ATP	Adenosine Tri Phosphate
BDNF	Brain-Derived Neurotrophic Factor
Bp	Base Pair
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CAS9	CRISPR Associated Protein 9
CHRDL1	Chordin Like 1
CLSTN2	Calsyntenin 2
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
CSF	Cerebrospinal fluid
CTIP2	B cell lymphoma 11B
DEPC	Diethyl PyroCarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxyribonucleotide TriPhosphate
E Coli	Escherichia Coli
EDTA	Ethylene Diamine Tetra Aceticacid
EMX2	Empty Spiracles homeobox 2
EPHA4	Ephrin Type A Receptor 4
FASN	Fatty Acid Synthase
FCS	Fetal Calf Serum
FOXA2	Forkhead box protein A2
FOXG1	Forkhead box protein G1
GDNF	Glial Cell Derived Neurotrophic Factor

GFP	Green Fluorescent Protein
HERV	Human Endogenous Retroviruses
hESc	Human Embryonic Stem Cells
hiPSc	Human Induced Pluripotent Stem Cells
HIV	Human Immunodeficiency Virus
HTLV	Human T-Lymphotropic Virus
IFA	Immunofluorescence assay
IkB	Inhibitory Kappa-B
ISG56	Interferon Stimulated Gene 56
KAP1	KRAB associated protein 1
kb	Kilobases
kDa	Kilo Dalton
KI67	Marker of Proliferation 67
KRAB	Kruppel Associated Box domain
L1CAM	L1 cell adhesion molecule
LDN	Low Dose Naltrexone
LMXA1	LIM homeobox transcription factor 1
LTR	Long Terminal Repeats
MAP2	Microtubule Associated protein 2
mRNA	Messenger RNA
MS	Multiple Sclerosis
MXA	Myxovirus Interferon Induced Regulatory Protein
NF-kB	Nuclear Factor Kappa- B
NTRK3	Neurotrophic Tyrosine Kinase Receptor 3
NURR 1	Nuclear Receptor Related Protein 1
OTX1	Orthodenticle homeobox 1
PAX6	Paired Box protein 6
PCR	Polymerase Chain Reaction
PFA	Poly Formaldehyde
PO3F2	POU Class homeobox 2
PTPRT	Protein Tyrosine Phosphatase Receptor Type T

qRT PCR	Quantitative RealTime PCR
RELN	Reelin
ROBO1	Roundabout homolog 1
RPS6KA1	Ribosomal S6 Kinase 1
RPS6KA2	Ribosomal S6 Kinase 2
SATB2	Special AT rich sequence binding protein 2
SCD	Schizophrenia Disorder
SEMA3A	Semaphorin 3A
sgRNA	Single Guide RNA
SOX2	SRY box transcription factor 2
TAQ	Thermophilus Aquaticus
TBR1	T- box brain transcription factor 1
TGF B	Transforming Growth Factor Beta
TH	Tyrosine Hydroxylase
TRIM28	Tripartite Motif containing 28
TRIS	Hydroxymethyl amino methane
TRK	Tyrosine Kinase
VP64	Herpes Simplex virus Viral Protein 64
WB	Western Blotting

1. Introduction

1.1. Retroviridae

Retroviridae comprises a large and diverse family of positive sense, single stranded (ss) RNA viruses, the Retroviruses (Coffin; 1996). Retroviral particles or virions are enveloped, and all retroviruses are characterized by their peculiar mechanism of replication, the reverse transcription of the RNA genome into ds-DNA during their life cycle. The reverse transcribed DNA is permanently integrated into the host genome as the provirus (Coffin; 1996, Belshaw et al; 2004).

The Retroviridae family is classified into two subfamilies Orthoretrovirinae and Spumaretrovirinae, based on the infectivity, genetic and morphological properties (Coffin; 1996, Murphy et al; 1995). The subfamily of Orthoretrovirinae holds six genera of retroviruses, Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus and Lentivirus. The Spumaretrovirinae subfamily includes a single genus, Spumavirus.

1.1.1 Genomic organization of a retrovirus

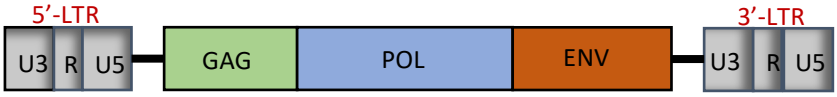


Figure 1.1: Schematic showing the organization of a retroviral provirus integration. *The coding regions, GAG, POL and ENV are represented as boxes flanked by the 5'and 3'LTRs that regulate transcription(Figure modified from Stoye; 2012)*

The genomic organization of retroviruses consists of three major coding domains, gag, pol and env, sandwiched between 5' and 3' long terminal repeats or LTRs, produced during reverse transcription, as depicted in **Figure 1.1**. Both LTRs are essential for retroviral transcription as the 5' LTR functions as a promoter necessary for transcriptional initiation.The GAG region codes for Matrix, capsid and nucleocapsid proteins, while information for envelope proteins is coded by the ENV region. The enzymes, integrase and reverse transcriptase, necessary for reverse transcription and provirus formation, are expressed from the POL coding region. In addition to the three major coding regions which are present in all retroviruses, many viruses also harbors additional open reading frames, which code for accessory proteins (Coffin; 1996, Belshaw et al; 2004).

Many retroviruses are associated with serious infections in birds and mammals, including humans. Members of the Orthoretrovirinae are known to infect vertebrates and causes severe

lifelong cases of immunodeficiency and cancers. Human immunodeficiency (HI) virus HIV-1 and HIV-2 of the genus Lentivirus are causative agents of acquired immunodeficiency syndrome (AIDS), in humans. The murine leukemia viruses (MLVs) cause cancer in mouse hosts. (Greene and Peterlin; 2002, Popovic et al; 1984)

1.1.1 Life cycle of a retrovirus

One of the most extensively researched and studied retrovirus is the lentivirus, HIV. The life cycle of a retrovirus **(Figure 1.2)** can be clearly understood from the different pathways followed by the HIV. The major steps in the life cycle of a retrovirus are:

1) Attachment of virus to host cell
2) Entry of Virus into cell
3) Reverse transcription of the RNA genome into ds-DNA via an RNA-intermediate
4) Integration of the provirus into the host genome.
5) Viral gene expression
6) Virion assembly
7) Budding of viral particles
8) Maturation of Viral particles

As a first step towards replication, the virus attaches itself to the host cell via specific host receptor/ co-receptor complexes and facilitates the fusion of the viral envelope to the host cell membrane. The viral genome is released into the cytoplasm and the reverse transcription of the genome is initiated by the enzyme reverse transcriptase. Thus, viral ss-RNA is transcribed into ds-proviral DNA using a host tRNA (transfer RNA) as primer, which binds to the primer binding site (PBS) at the 5'end of the viral RNA (Rous; 1911). The proviral DNA together with the host and viral proteins establish the pre-integration complex (PIC). The PIC mediates the integration of provirus into the host genome inside the nucleus. 5' LTR serves as a promotor and enhancer for transcription initiation for viral gene expression upon integration. Viral proteins assemble at the plasma membrane of the host cell and different cellular factors facilitates the budding of viral proteins. The polyproteins are cleaved using an enzyme protease during maturation, leaving behind functional viral proteins for release (Karn and Stoltzfus; 2012, Stoye; 2012).

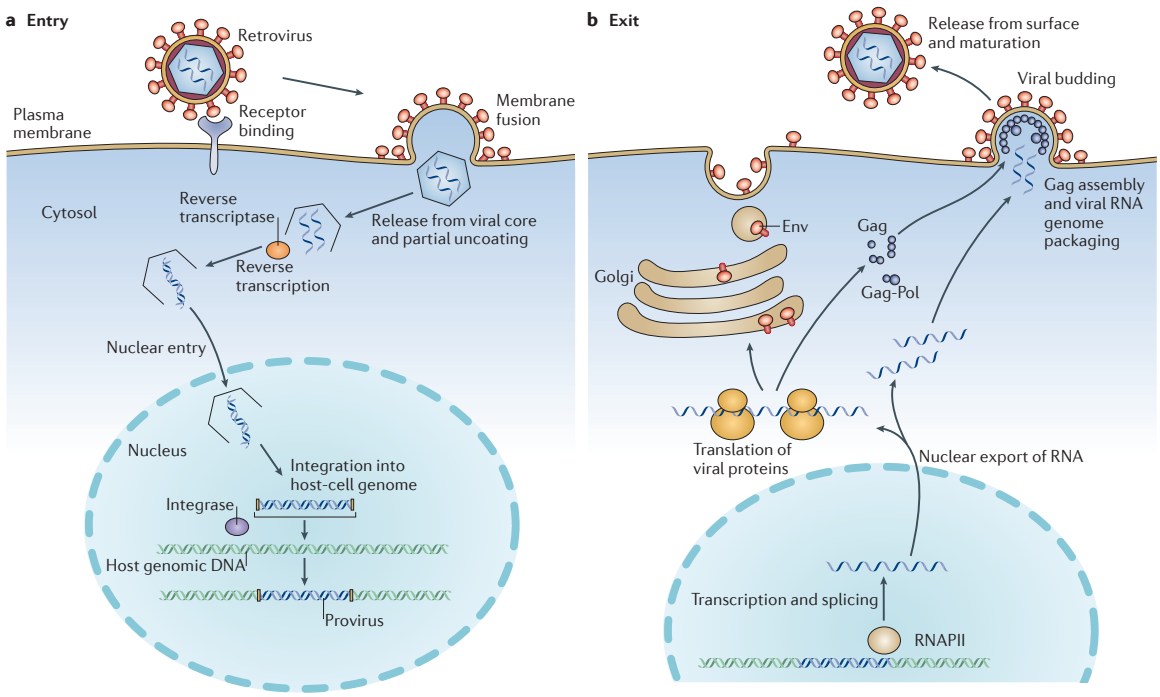


Figure 1.2: Replication of HIV-1. *Schematic figure showing the different stages of replication of a retrovirus (Figure taken from Stoye; 2012)*

1.2. Transposons

Until later half of 20th century, scientific community considered and identified genes as stable entities which was arranged in a linear fashion like 'beads in a string' (Morgen T H; 1922). A paradigm shifting discovery in Zea mays genome made by Barbara McClintock in the late 1940s established the groundbreaking idea that genes could be mobile and these elements, called as 'jumping genes' could impart reversible alteration in expression levels of other genes (McClintock; 1950). Decades later, these elements were recognized as Transposable elements or Transposons.

Human Genome Project (HGP) identified that almost 50% of our genomic make up is comprised of distinct pieces of DNA which are capable of moving within and in between genomes (Lander et al; 2001). These genomic elements, then termed as 'junk DNA' are now recognized as crucial players in the cellular gene expression and regulation. The extend of contribution of TEs to the diversity of the human genome becomes all the more relevant since only 2% of the human genome accounts for protein coding genes (Gregory; 2005). Transposable elements can be broadly classified into two major classes: - DNA transposons and Retrotransposons, as shown in **Figure 1.3**. DNA transposons constitute approximately 3% of the human genome. They are characterized by their ability to snip themselves out of the

genome and integrate into new genomic sites by a ‘cut and paste’ mechanism using the enzyme integrase. These elements have lost their ability to move around the genome and the last transposition competent integrant was active 37 million years ago in the primate evolutionary scale (Feschotte and Pritham; 2007, Pace and Feschotte; 2007, Richardson et al; 2015). The second major class of transposable elements is the retrotransposons. Retrotransposons constitute about 97% of the global transposon milieu of our genome. The genetic mobility or transposition is facilitated by a ‘copy-paste mechanism’ of the DNA. Following the transcription of DNA to RNA, these retrotransposons duplicate through the RNA intermediate using the enzyme reverse transcriptase and reverse transcribe to RNA and inserts into new genomic locations (Richardson et al; 2015) Retrotransposons are further subdivided into two groups based on the presence or absence of the long terminal repetitive sequences at the 5’ and 3’ends, Non-LTR retrotransposons and LTR retrotransposons. Non-LTR retrotransposons, the only group of transposable elements known to be currently active in humans, collectively comprises about one-third of the human genome (Cordaux and Batzer; 2009). This class includes Long Interspaced Nuclear Elements (LINEs) and Short Interspaced Nuclear Elements (SINEs). LINEs or L1 elements make approximately 17% of the human genome with more than 500,000 copies present as a result of over 150 million years of continuous transposition and mobilization. L1 elements are by far the most successful transposable element by mass. A typical L1 element has a genomic length of approximately 6kb harboring a 5’ UTR, an internal RNA polymerase II promoter, two open reading frames, an ORF1 coding for an RNA binding protein and an ORF2 coding for proteins with endonuclease and reverse transcriptase like functions, and a 3’ UTR with a poly adenylation tail. Owing to deletions, truncations, and rearrangements, only about 100 copies of L1 elements are currently functional. SINE elements are non-autonomous and are dependent on LINE-encoded proteins for retro transposition, making it a ‘parasite’s parasite’. There are two types of SINE elements, Alu elements and SVA elements. There are over one million copies of Alu elements scattered in the human genome as a result of mobilization and transposition of over 65 million years, making it the most successful transposon in terms of copy number. In each one out of 20 births, specific Alu elements make a new genomic insertion. A canonical Alu element spans about 300bp in length. An SVA element is made up of a Short interspaced nuclear element, a Variable number tandem repeat element and an Alu element. The genome of an SVA element is approximately 2kb in length and like the Alu elements, it depends on the L1 transposition machinery for mobilization. SVA elements are active throughout 25 million years of hominid evolution. Owing to their incessant activity and amplification over millions of years, non-LTR

retrotransposons have had a colossal impact on the structural and functional evolution of primate genomes. (Cordaux and Batzer; 2009, Richardson et al; 2015, Goke and Ng; 2016)

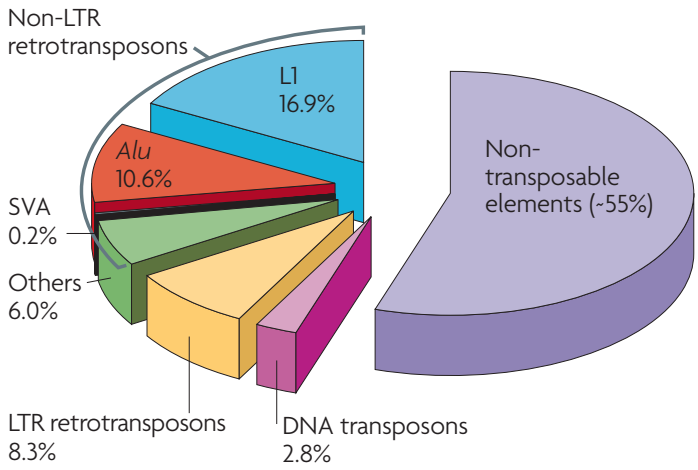


Figure 1.3: Distribution of Transposable elements in human genome - *Schematic figure showing the distribution of transposable elements in the human genome (Figure taken from Cordaux and Batzer; 2009)*

1.3. Human Endogenous Retroviruses (HERVs)

The second major class of Retrotransposons are the LTR retrotransposons, which majorly includes a group of repetitive viral sequences called the Endogenous Retroviruses. These elements are remnants or footprints of ancestral exogenous retroviral infections of germline cells. Over the course of millions of years of hominoid evolution, these viral elements got integrated into the DNA and were amplified through retro transposition, constituting what we call the human endovirome (Stoye; 2012, Ecco; 2020). Retroviral integration usually occurs within somatic cells. However, occasional integration events of germ line cells result in vertical transfer of retroviral sequences from parent to offspring. The well-studied drafts of human genome sequencing shows that around 8-9% of our entire genetic makeup is composed of these ancient retroviral sequences. (Lander et al; 2001, Griffiths; 2001, Li et al; 2001). The genomic organization of the provirus of an endogenous retrovirus is similar to that of exogenous retroviruses with viral gag, pol and env genes sandwiched between a 5' and 3' long terminal repeats. Endogenous retroviruses are capable of replication but rendered inactive by recombinational deletion between two repeat regions termed long terminal repeats (LTRs) located at the 5′ and 3′ ends or by random mutation during DNA replication of the host genome. During this period of insertion, replication and inactivation, the viral copy number may increase via retrotransposition and mobilization within the genome. Essentially, these elements are silent and are replication defective due to the accumulation of several deleterious mutations,

frameshifts and deletions, particularly in the env region. There are several 'solitary LTRs' scattered in our genome which are the result of these deletions and subsequent homologous recombination. These LTRs still hold the potential to influence cellular gene expression. There are more than 600,000 integrants of HERVs identified, out of which around 500,000 are solo LTRs (Goke and Ng; 2016, Griffiths; 2001, Benachenhou et al; 2009).

1.3.1. Classification of HERVs

Human Endogenous Retroviruses are broadly classified into three major classes, Class I, Class II and Class III, based on the sequence similarity to the infectious exogenous retroviruses (Kauffman; 2005). HERV classes can be further defined into individual subfamilies, which makes it a convenient feature to distinguish different groups of HERVs from one another **(Figure 1.4)**. They are arranged into 22 independent acquired families, based on the tRNA amino acid core of the primer binding site in the transcriptional start site (Römer et al; 2017). Thus, members of the HERV-K family contain a PBS with a sequence similar to a region of $tRNA^{Lys}$, whereas the HERV-E by $tRNA^{Glu}$ and HERV-H family is primed by $tRNA^{His}$. Of these, 17 families belong to the class I HERVs, 3 to the class II HERVs and 2 to the class III HERVs. The Class I endogenous retroviruses show sequence similarity to gammaretroviruses like Mouse Mammary Viruses (MMV), eg. HERV-W, HERV-H. The Class II exhibit sequence homology to betaretroviruses like Mouse mammary tumor viruses (MMTV), eg. HERV-K. The Class III endogenous retroviruses are similar to spumaretroviruses in sequence, eg- HERV-L, HERV-S (Goke and Ng; 2016, Griffiths; 2001) Albeit the classification of a large majority of HERV groups into different classes, this system of classification fails for many other HERV groups, owing to the fact that many HERVs could be primed by same tRNA but not necessarily fall into the same phylogeny with respect to the sequence homology of the pol genes of these groups. (Griffiths; 2001).

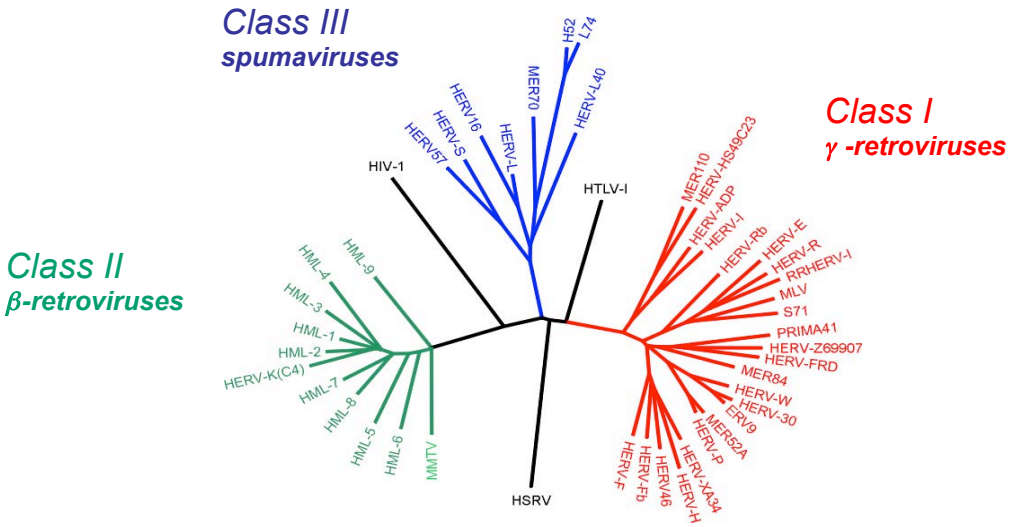


Figure 1.4:Classification of endogenous retroviruses *– Schematics showing different classes and subfamilies of human endogenous retroviruses (Figure taken from Mager and Medstrand; 2003)*

HERVs demonstrate variability at many taxonomic levels. From the detailed studies of transmission, integration and expansion of several exogenous retroviruses at genome level, it can be shown that HERVs exhibit closer taxonomic relation to animal retroviruses like MLV and MMTV rather than human exogenous retroviruses such as HIV or HTLV-1. A DNA microarray- based approach revealed that in comparison to the Endogenous Retroviral content of humans, New World Monkeys (NWMs) project a reduced and Old-World Monkeys (OWMs) display a more restricted copy number of most ERV classes (Stengel et al; 2006). A pol-specific microarray revealed eight families of gammaretroviruses, nine families of betaretroviruses, and five subgroups of HERV-L elements detected in OWMs. This suggests that, following the demarcation of New World Monkeys lineage from Old World Monkeys and great apes, a major invasion and expansion of pol containing endogenous retroviral elements must have occurred (Stengel et al; 2006, Greenwood et al; 2005) Thus, at the DNA level, both de novo integration and vertical transmission of existing insertions by retro transposition and reinfection occurred among different species. It has to be added that despite the transmission and expansion of ERVs at DNA level in OWMs, a major share of retroviral elements integrated before the OWM and great ape lineages contributed to divergent evolution and this explains their similar genomic distribution pattern among catarrhines (Greenwood et al; 2005, Mager and Freeman; 1995).

1.3.2. Human Endogenous Retrovirus-K (HML-2)

Deep Sequence analysis of the endogenous retroviral elements in the human genome reveals that there are three endogenous retroviral elements with a complete genome organization of gag pol and env genes. Interestingly, all three of these full-length open reading frames belong to the HERV-K(HML-2) family of human endogenous retroviruses, although presented with significant mutations in the reverse transcriptase regions (Griffiths; 2001, Stengel et al; 2006). Evolutionary history suggests HERV-K subfamily of human endogenous retroviruses as the most recently integrated member of the human genome around 2.5 million years ago (Sverdlov; 1998, Hughes and Coffin; 2004). HERV-K family of endogenous retroviruses are subdivided into 11 subgroups (HML-1-HML-11), (Chen et al; 2019) with the most-studied one being HERV-K(HML-2). HERV-K(HML-2) has two major types of proviruses, type I and type II (Subramanian et al; 2011, Hohn, Hanke, and Bannert ; 2013, Bannert and Kurth; 2004). Type I is characterized by the deletion of a 292-bp fragment at the pol/env boundary encoding two variant proteins, Np9 and Rec, respectively (Tavakolian, Goudarzi, and Faghihloo; 2019, Denne et al; 2007, Huang et al; 2013) which is absent in type-II. The type II provirus produces the regulatory protein Rec by a singly spliced transcript, while the type I provirus produces Np9 through a doubly spliced transcript in the pol/env boundary region. In humans, HERV-K(HML-2) insertional polymorphisms are common and proposed to be still occurring, suggesting recent retro transposition and reinfection events (Hohn, Hanke, and Bannert; 2013, Belshaw et al; 2005, Macfarlane and Badge; 2015). Because of this activity, HERV-K(HML-2) is one of the best studied HERV-subtypes.

1.3.3. Human Endogenous Retrovirus-H

HERV-H is one of the most successful endogenous retrovirus which has been domesticated by the human genome and demonstrated to be actively transcribed during early developmental stages of embryogenesis. (Römer et al; 2017). The HERV-H family is primed by a histidine t-RNA and the transcription of these elements is promoted by the LTRs. It has been shown that HERV-H is bound by transcription factors such as Nanog, OCT-4 and LBP9 which are markers of pluripotency and that HERV-H acts as a long noncoding RNA which maintains the naïve state of embryonic stem cells and loss of HERV-H transcriptional activity marks the commencement of cell differentiation. (Santoni, Guerra, and Luban; 2012, Robbez-Masson and Rowe; 2015, Gemmell, Hein, and Katzourakis; 2019). HERV-H loci has been reported to act as regulatory elements with a stage and region-specific expression. It was also shown that

modified cells changed their morphology to fibroblast like structures and changes in transcription factor profiles upon interference with HERV-H transcript levels (Lu et al; 2014). It has also been demonstrated that there is a directional selection of highly transcribed HERV-H elements by the genome due to its extremely high divergence (Gemmell, Hein, and Katzourakis; 2019, Göke et al; 2015). Thus HERV-H is a classic example of a retroviral sequence to be functionally co-opted by the host genome.

1.4 Epigenetic regulation of Human Endogenous Retroviruses

Endogenous Retroviruses are predominantly silent in our genome owing to the tight epigenetic control of these sequences. Establishment of heterochromatin contributes to the low transcription rates of HERVs in most cell types. Recognition of the ERV sequence by DNA binding factors, mostly by the large family of KRAB zinc finger (KRAB-ZnF) proteins marks the repression of endogenous retroviruses. The recruitment of chromatin modifying enzymes is via the Krüppel associated box (KRAB) domain binding to the corepressor TRIM28/KAP1. (Friedman et al; 1996). It has been suggested that KRAB-ZnF proteins have coevolved with Endogenous Retroviral elements to maintain the internal homeostasis of the transcriptome. (Emerson and Thomas; 2009, Thomas and Schneider; 2011). TRIM28 recruits the histone methyltransferase SETDB1/ESET to establish the chromatin modification, which facilitates the action of the repressive chromatin mark H3K9me3 (Schultz et al; 2002, Frietze, O'Geen et al; 2010). Additionally, TRIM 28 is bound by the NuRD histone deacetylase complex, containing Histone Deacetylase 1 (HDAC1) (McDonel et al; 2009), and the heterochromatin protein 1 (HP-1)(Sripathy et al; 2006) are directed to the loci of repression. Studies have shown that TRIM28 is not only necessary for the establishment of heterochromatin at ERV loci but is also involved in the stage and region-specific activation of ERVs and genes located in the vicinity of the activated ERVs during development in mice and human neural progenitor cells (Rowe et al. 2013, Brattås et al; 2017). However, it has been demonstrated, in the human as well as the murine system, that not all ERVs are upregulated after TRIM28 knockdown in embryos and stem cells (Brattås et al; 2017). In early embryos silenced ERV sequences are characterized by the presence of H3K9me3 and H4K20me3, while differentiated cells lacks this repressive mark (Mikkelsen et al; 2007). TRIM28 pathway is mainly necessary to initiate ERV regulation during early development and that in later stages other factors keep these elements in a repressed state.

The list of potential repressive factors continues with histones(Elsässer et al; 2015), histone chaperones, sumoylation factors and chromatin modifiers(Yang et al; 2015). Studies on mouse

embryonic stem cells demonstrated an important role for histone methylation (including H3K9me3 and H4K20me3) in silencing active ERVs during embryogenesis (Maksakova, Mager, and Reiss; 2008, Mikkelsen et al; 2007, Martens et al; 2005). Loss of DNA methylation leads to heavy activation of ERV elements in differentiated cells (Hutnick et al; 2009, Hutnick, Huang et al; 2010), stressing the importance of this epigenetic mechanism for ERV silencing in more developed cells.

1.5 Activation of Human Endogenous Retroviruses

Substantial evidence of endogenous retroviral activation in the genome exists, which shows that HERVs can be upregulated by environmental factors, such as external chemical substances, physical agents, host epigenetic regulators, cellular factors and exogenous viral infections.

Chemical agents such as phytohemagglutinin (PHA) and phorbol-12-myristate-13-acetate (PMA) have been reported to induce expression of different HERV families in different cell types. HERV-H transcription was activated in normal peripheral T-cells treated with with PHA alone or in combination with phorbol-12-myristate-13-acetate (PMA) (Balestrieri et al; 2019). PMA treatment in primary macrophages and monocytoid cells showed increased transcription of HERV-W, HERV-K, and HERV-H (Johnston et al; 2001, Liu et al; 2016). Moreover, physical agents, such as UVB irradiation and X-rays, have also been shown to act as inducers of endogenous retroviral activity (Hohenadl et al; 1999, Vincendeau et al; 2015) Cytokines also play vital roles in the expression of HERVs. Treatment of cytokines such as tumor necrosis factor-α, interleukin (IL)-1α, and IL-1β upregulated the expression of HERV-R (Katsumata et al;1999)

Role of exogenous viruses in the activation of endogenous elements has been reported in several studies. Infections with Epstein-Barr virus (EBV) has been shown to upregulate the transcription of env gene of HERV-K18 and HERV-W (Mameli et al; 2012, Sutkowski et al; 2001). EBV associated superantigen activity (SAg) was shown to be disrupted by HERV-K 18 env specific antiserum suggesting a therapeutic potential of HERV repression in EBV infections (Sutkowski et al; 2001).

Herpes virus antigens have been shown to increase the reverse transcriptase activity of HERVs. Combined action of Herpes virus antigens and activated HERV-H elements demonstrated an elevated cellular immune response in multiple sclerosis patients (Brudek et al; 2007, Chen, Foroozesh, and Qin; 2019, Brudek et al; 2004). Herpes simplex virus-1 (HSV-1) infection was shown to induce transcription of HERV-K, through HSV-1 immediate-early protein ICP0

(Kwun et al; 2002). Increased expression of HERVs has also been shown to be induced by retroviruses such as HIV-1 in vitro. Analysis of HIV patient samples also revealed increased transcript levels of HERV-K HML2 (Garrison et al; 2007, Tandon et al; 2011, SenGupta et al; 2011, Jones et al; 2012). Chronic Hepatitis C infections can cause Sjögren's syndrome, which is a result of increased HERV expression levels (Moyes et al; 2005, Dantec et al; 2012) Chronic hepatitis C virus infection results in hyper reactivity to an autoantigen, GOR (Michel et al; 1992). This protein shows sequence homology to Hepatitis C nucleocapsid and HTLV-1 gag. Thus, GOR could be speculated to be an autoantigen of retroviral origin in liver due to Hepatitis C infection. (Quiroga et al; 2007) Tax protein of HTLV-1 virus has been reported to activate HERV LTRs (Toufaily et al; 2011, Perzova et al; 2013). HERV transcription may be activated not only by viruses but also by other infectious agents such as the protozoan parasite Toxoplasma gondii. Neuroepithelial cells infected with Toxoplasma gondii showed increased expression levels of HERVs (Frank et al; 2006, Sher, Denkers, and Gazzinelli; 1995).

1.6 Human Endogenous Retroviruses in cellular regulation

Human Endogenous Retroviruses have been domesticated in human genome through millions of years of insertion, amplification and conservation throughout the evolutionary progression. (Nelson et al; 2003). This has enabled the retroviral sequences and the host genome to evolve different mechanisms of survival, coexistence and at times, complete control. Endogenization of retroviruses have made the host genome to evolve and develop a complex array of transcription factors to control and coordinate the retroviral sequences to their benefit. Most of the retroviral elements have lost their ability of transposition and have gained novel functions by preserving the retrotransposon sequence and gaining of functionality in a different context. Transcriptional functionality of retrotransposons can be observed throughout the genome where endogenous elements are internalized as enhancers and regulatory elements by influencing the genes located in the proximity without changing or activating the retrotransposon identity. These elements can also act as promoter sequences for initiating the transcription of a retroviral sequence which in turn provides isoforms for genes and imparts cell type specificity for coding competent genes. Endogenous elements can also transcribe on their own and generate long noncoding RNAs which can influence the spatial and temporal expression of critical gene networks, as depicted in **Figure 1.5** (Goke and Ng; 2015).

1.6.1. Endogenous retroviruses as regulatory elements

Cellular Pluripotency is one of the most important characteristic features of embryonic stem cells. (Romito and Cobellis; 2016). Studies have shown that at least one-fourth of the transcription factor binding sites of pluripotency associated genes, OCT-4 and Nanog have originated from endogenous transposable elements (Hammachi et al; 2012, Hutchins and Pei; 2015, Kunarso et al; 2010). These primate specific sequences act as regulatory elements by virtue of their ability to integrate novel genes into existing networks. (Chuong et al; 2017). Retrotransposon- derived enhancer activity has been observed in pregnancy related organs such as Placenta and endometrium. A special class of endogenous retroviruses, RLTR13D5 present in murine trophoblastic stem cells harbor several enhancers that are active during placentation (Chuong et al; 2013). These elements contain transcription factor binding sites for ElF5, Cdx2, Eomes which are involved in trophoblast regulatory network. (Frendo et al; 2003, Chuong; 2018). Similar pattern of endogenous retroviral regulatory element assembly was also reported in human endometrial tissues (Lynch et al; 2011). A cryptic promoter of HERV-E is reported to be associated with the transcription of salivary amylase (Ting et al; 1992). The regulatory region of the GABA receptor B1 gene (GABBR1) harbors a HERV-W element. (Hegyi; 2013). Studies have also revealed promoter potential of varying strength in HERV-K LTRS (Buzdin et al; 2006). Transcription of HERV is usually initiated by a TATA box motif along with other vital promoter elements. However, studies demonstrate a TATA less promoter activation and transcription of the HERV-K LTR by the transcription factors Sp1 and Sp3 (Fuchs et al; 2011). HERVs can even serve as the only promoters for specific genes, as the BAAT gene expressed in the liver solely from a retroviral LTR (Carlton et al; 2003). A HERV-K(HML-2) element acts as an enhancer for the schizophrenia-associated gene PRODH (Suntsova et al; 2013).

1.6.2. Human Endogenous Retroviruses in Protein coding genes

Studies have shown the active involvement of human endogenous retroviruses in gene expression and functional translation of host mRNAs. Syncytin-I is a protein which plays a major role in placenta formation during early embryonic development of mammals. It has been associated with the membrane fusion process during the formation of syncytiotrophoblast, the syncitial cell layer which is involved in regulating maternal-fetal exchanges in the placenta, and to the suppression of the maternal immune response against the fetus, thus establishing fetal tolerance by the mother (Mi et al; 2000, Koch and Platt; 2007) Syncytin-I is one of the classic examples of acquisition and domestication of retroviral genes by the host genome. It is shown to be an env-derived gene of a human endogenous retrovirus (HERV) of the HERV-W

family inserted in human Chr.7q21 (Dunlap et al; 2006, Grandi and Tramontano; 2017). Gag and pol sequences of the provirus harbored several inactivating mutations, but the env region maintained an open reading frame which codes for a 538 amino acid long polypeptide which was involved in the receptor-mediated fusogenic activity (Blond et al; 2000, Kwun et al; 2002, Sverdlov; 1998, Caceres and Thomas; 2006). A molecular evolution study of the HERV-W provirus in several ape species and humans showed a conserved expression profile and functional role of Syncytin-I (Mallet et al; 2004).

1.6.3. Human Endogenous Retroviruses as Long Noncoding RNAs (lncRNAs)

Regulation of non-coding RNAs involves significant interference from Human Endogenous Retroviruses. lncRNAs are known to influence cellular transcription, chromatin state, splicing patterns, and are observed to be of significant importance in brain development (Qureshi and Mehler; 2012). HERVs are associated with a large number of long non-coding RNAs (lncRNAs), which overlaps with transcriptional start sites (Kelley and Rinn; 2012) of several crucial gene sequences. lncRNAs derived from endogenous retroviruses are reported to be crucial in the pathology of mental disorders such as Schizophrenia (Slokar and Hasler; 2016). HERV LTRs also act as promoters which drive the expression of long non-coding regulatory RNAs in the maintenance of pluripotency in stem cells. This can be explained in the context of Human Endogenous Retrovirus-H. Presence of HERV-H RNA has even been suggested as a marker for pluripotency in human embryonic stem cells (ESCs) (Ohnuki et al; 2014). Chimeric transcripts of several HERV families are stage-specifically expressed during early development (Göke, Lu et al; 2015) human central nervous system development (Brattås et al; 2017). HERVs also play a major role in eukaryotic mRNA polyadenylation by providing new polyadenylation signals when transcribed along with existing genes. Because of this fact, amongst others, there is a strong negative selection on sense orientation of HERVs and of integration in gene introns (van de Lagemaat, Medstrand, and Mager; 2006). Multiple mRNAs utilize HERK-K LTRs sequences as polyadenylation signals (Baust et al; 2000) such as HHLA2 as well as HHLA3. Furthermore, HERVs can regulate gene expression through RNA interference. It has been shown that there are HERV-K inserts in the introns of the genes SLC4A8 and IFT172 which serve as promoters for transcripts that are complementary to exons of the genes resulting in the downregulation of the corresponding mRNAs (Gogvadze et al; 2009).

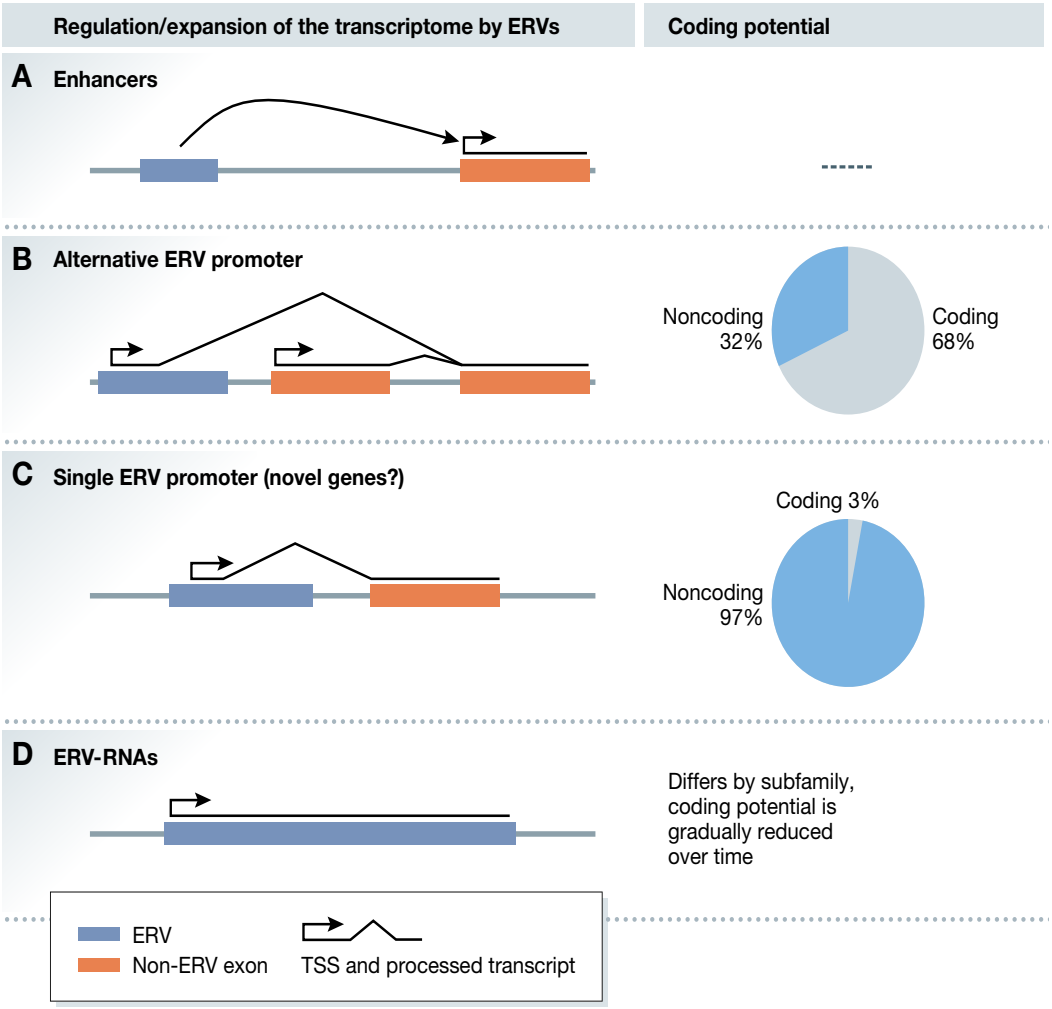


Figure 1. 5 Cellular Regulation by endogenous retroviruses*. Schematic showing the different regulatory roles of endogenous elements (Figure taken from Goke and Ng; 2016)*

1.7 Human Endogenous Retroviruses in diseases

HERVs are subjected to tight epigenetic control and most of them are heavily silenced except when are they functionally indispensable to the host genome (Hurst and Magiorkinis; 2017). HERVs can contribute to acquiring inherited diseases, by induction of gene instability and variability, recombination, insertional gene disruption, autoimmunity, superantigenic stimulation, production of immunosuppressive factors, cancer activation/inactivation of growth controlling genes, etc. Several clinical situations have been attributed to one or more HERVs, as particularly with respect to some neurological diseases, as discussed below.

1.7.1. Human Endogenous Retroviruses in autoimmune disorders

Autoimmune diseases are a complicated condition arising from an abnormal response from self to a functioning entity of one's own body (Gutierrez-Arcelus, Rich, and Raychaudhuri; 2016). These are complex disorders in which genetic susceptibility confers a disease onset, but it is

neither sufficient nor scalar for the disease progression. Retroviruses have been incessantly discussed as etiological factors of autoimmune diseases and antibodies to viral regions of retroviruses, including Human Endogenous Retroviruses, have been reported in patients with autoimmune disease (Rucheton et al; 1985, Li et al; 1996, Nelson et al; 1999), but the role of retroviruses in these diseases remains unclear. Several reports point towards the active role of endogenous retroviruses in autoimmune disease manifestations. For example, it has been demonstrated that HERV-K family harbors binding sites for many pro-inflammatory factors (Manghera and Douville; 2013), related to rheumatoid arthritis (Freimanis et al; 2010). Peripheral blood and synovial fluid in RA patients showed higher levels of HERV-K10 expression in (Ejtehadi et al; 2006, Perl et al; 2010) comparison with healthy controls.

Aberrant activity of both HERV-E and HERV-K elements has been demonstrated in the T-lymphocytes of systemic lupus erythematosus (SLE) patients (Wu et al; 2015, Shi et al; 2014). Presence of HERV-W proteins were detected in patient samples with osteoarthritis (Bendiksen et al; 2014). HERV-Fc1 expression was shown to be elevated in patients with active multiple sclerosis (Bendiksen et al; 2014) whereas expression levels of HERV-W, HERV-K and HERV-E was increased in psoriatic lesions (Lättekivi et al; 2018). Recent studies also suggest increased expression levels of HERV-H and HERV-W pol genes in Type -1 Diabetes patients when compared to healthy controls (Tovo et al; 2020, Levet et al; 2019). These studies and observations demonstrate active involvement of human endogenous retroviral elements in immune system related disorders and a potential pathogenic and therapeutic implications of these acquired sequences.

1.7.2. Human Endogenous Retroviruses in Cancer

One of the major implicated roles of human endogenous retroviruses is its causative role in cancer progression. Several research studies demonstrate the expression of HERVs in cancer tissues, but its causative role in cancer development remains controversial. Several genomic factors such as insertional mutagenesis, immune modulation, putative oncogenes and environmental factors like UV radiation have been proposed as causes of HERV activation and protein expression in cancer tissues(Grandi and Tramontano; 2018).

The most recently integrated member of the HERV families is the HERV-K family of endogenous retroviruses. The HERV-K (HML-2) proviruses has retained open reading frames for most of the viral proteins. HERV-K family has been associated with the development of several human cancers such as breast cancer, lung cancer, prostate cancer, hepatocellular carcinoma (HCC), melanomas, germ cell tumor, leukemia and, lymphoma (Cegolon et al; 2013,

Mayer et al; 1999, Downey et al; 2015, Wang-Johanning et al; 2003). The neoplastic transformation of cells mediated by HERVs can be via direct expression of viral RNA, or indirectly via cellular gene regulation and expression of tumor-associated genes. Viral products of HERV-K, HERV-H, HERV-R and HERV-T has been detected in blood and tissue samples of patients with lung and breast cancers. HERV trans activation in these patients were higher than healthy controls. This was further confirmed from the experiments conducted with HERV-K specific antibodies and shRNAs which resulted in inhibition of cancer growth both in vitro and in vivo. Furthermore, both Np9 and Rec has been shown to have oncogenic potential and are present in variety of tumors and cancer cell types. These proteins exhibit physical and functional interaction with the promyelocytic leukemia zinc finger (PLZF) tumor suppressor to regulate cancer cell proliferation and survival by transforming and modifying the expression of the c-Myc proto-oncogene (Bannert et al; 2018, Lin et al; 2013). As mentioned before, the HERV-W gene product Syncytin-I is essential for normal placental development. Aberrant cell fusion by extraplacental expression of Syncytin has been demonstrated in the context of cancer cells and metastasis (Bjerregaard et al; 2006).

1.7.3. Human Endogenous Retroviruses in neurological disorders

Endogenous retroviruses are pathogenic in some respects. However, an explicit cause-effect relationship of pathogenesis has not been established yet. Role for HERVs in neurological and neuropsychiatric disorders such as multiple sclerosis (MS) and schizophrenia (SCZ) has been proposed and is discussed widely.

1.7.3.1. Human Endogenous Retroviruses in MS and SCZ

Multiple sclerosis or MS is a demyelinating disease of the central nervous system characterized by fatigue, and disturbed sensory, motor, and cognitive functions (Dobson and Giovannoni; 2019). The MS pathological process includes disruption of the blood- brain barrier, inflammation, demyelination and axonal degeneration (Trapp and Nave; 2008). The course of MS pathogenesis varies in clinical symptoms and progression, ranging from benign MS, over relapsing–remitting forms to progressive MS (Berger and Reindl; 2007). MS epidemiology involves both genetic and environmental components with genetic susceptibility. Viruses, such as human herpesvirusesand HERVs are often suggested as triggers or etiologic factors in MS (Fotheringham; 2005, Christensen; 2007). Specific HERV families with varied aspects of activation and pathogenicity have been reported for MS pathogenicity. Reports have shown that envelope genes and gene products of HERV-W and HERV-H/F are involved in the disease pathogenesis. Antivirals generated against HERVs has proven to be efficient therapeutic

agents. Interestingly, reports suggest a higher prevalence of MS outcome in women. This observation could be linked to the presence of a HERV-W copy on the X chromosome that has an almost intact ORF for the env protein (Arru et al; 2007, Garcia-Montojo et al; 2014). Administration of HERV-W env protein to mice models shows MS specific phenotypes, suggesting the contribution of HERV-W in the development of MS (Perron et al; 2013). Syncytin-1, a HERV-W envelope-encoded glycosylated protein exhibits increased glial expression within MS lesions. Overexpression of Syncytin-1 in glia cells induces endoplasmic reticulum stress which leads to neuroinflammation and the induction of free radicals, thereby degenerating the proximate cells. The neutral amino acid transporter, ASCT1 which is expressed on glial cells, is the receptor of Syncytin-I. It has been reported to be suppressed in white matter of MS patients. Antioxidants has been shown to improve the neuropathogenic effects caused by the activity of Syncytin-I, raising the possibility of using these agents as therapeutics for neuroinflammatory diseases (Antony et al; 2011, Antony et al; 2007, Sakai et al; 2003).

Schizophrenia is a mental disorder characterized by the progressive impairment of cognitive functions such as thought processing, memory, functioning etc. Although no clear etiology of schizophrenia has been identified so far, many factors such as alterations in neurotransmission, decreased synaptic plasticity and hippocampal volumes have been identified as the physiological conditions that might contribute to the development of the disease. In addition to these factors, several psychological and environmental interactions also play a major role in the onset and progression of this disorder (Heinrichs and Zakzanis; 1998).

Studies on the HERV transcript levels in patient blood samples, postmortem brain samples and cerebrospinal fluids (CSF) demonstrated consistent evidence of the role of endogenous retroviruses in Schizophrenia. An elevation in HERV-W, ERV9 and HERV-K 10 transcription was shown to be associated with the progression of the disease. Inflammatory responses from HERV-W results in an increase of IL-6, TNF-α, and IL-1β which has been linked to pathogenesis of schizophrenia (Yao et al; 2008). Glutamate transmission in brain has been reported to be dysfunctional in schizophrenic patients. This can be linked to the aberrant expression of N-methyl-D- Aspartate receptor and hASCT1 and hASCT2 which are the cellular receptors of HERV-W in brain (Kantrowitz and Javitt; 2012, Johansson et al; 2020).

1.7.3.2. Human Endogenous Retroviruses in ALS

Amyotropic Lateral Sclerosis is a severe case of neurodegenerative disorder characterized by the progressive loss of motor-neurons which leads to muscle weakness, motor dysfunctions

and respiratory failure. The fatality rate tends to be around 90% within first 5 years of onset. The etiology of the disease remains largely unknown, but research points out to complex, multifactorial, interplay of multiple genes, environmental and age-related factors and other random events (Rowland and Schneider; 2001, Hardiman et al; 2017). Probable causative agents include diet, athleticism, heavy metals, neurotoxins, viruses, etc (Zufiría et al; 2016). Multiple mutations SOD-1 and TDP-43 have also been reported in cases of ALS (Jeon et al; 2019, Neumann et al; 2006, Pokrishevsky et al; 2012). Endogenous retroviral activation has been reported in several cases of ALS. The env protein of the HERV-K is attributed to be involved in the neurodegenerative phenotype in ALS. A study conducted in transgenic mouse model expressing env of HERV-K developed ALS-like phenotype with severe motor dysfunctions, retraction and beading of neurites in the brain. It was also accompanied by loss of motor cortex volume, decreased synaptic activity etc. It was also shown that HERV-K expression was regulated via TAR DNA binding protein-43 in the LTR of the virus (Li et al; 2015, Mayer et al; 2018, Douville et al; 2011).

It can be stated that for a large array of neurologic disorders such as MS, SCZ, ALS, and HIV-associated dementia (HAD), the involvement of endogenous retroviruses, especially HERVs is conclusive (Guo et al; 2018, Dolei; 2018, Sankowski et al; 2019). Activation of these elements is essentially a prerequisite for pathogenesis and causality of these disorders as most HERV sequences remain quiescent and inactive in non-pathological conditions. Thus, the importance of regulatory pathways, epigenetics status and moreover the mechanistic detailing of how HERV elements achieve this neurodegenerative phenotype is imperative.

1.8 CRISPR-based transcriptional regulation of endogenous retroviruses

The CRISPR/Cas9 (clustered regularly interspaced palindromic repeats/CRISPR-associated) technique is a revolutionary breakthrough of modern science derived from the adaptive immune system of bacteria. It is characterized by its ability to site specifically cleave or edit DNA sequences. (Horvath and Barrangou; 2010, Deltcheva et al; 2011). The method is based on an endonuclease Cas9 and a CRISPR RNA duplex comprised of a CRISPR RNA (crRNA) and a target specific trans-activating CRISPR RNA (tracrRNA). A single guide RNA constitutes a chimeric RNA duplex of about 20 nucleotides. The canonical base pairing of the sgRNA and the target sequence facilitates the recruitment of Cas9. However, it is imperative that the sgRNA binding site is near a protospacer adjacent motif (PAM), a 2-6bp long DNA sequence(Gasiunas, Barrangou et al; 2012). In most cases, Streptococcus pyogenes-derived Cas9 is used with a PAM sequence of NGG. Cas9 proteins from other bacterial species are

recruited to DNA sequences with other PAM motifs in proximity. Nevertheless, there is a NGG sequences approximately every 8bp in the human genome, making sgRNA target sites identifiable for almost every locus. (Jinek et al; 2012, Gasiunas et al; 2012, Cong et al; 2013, Hou et al; 2013).

Two catalytic nuclease domains, termed RuvC-like and HNH domain, cleave the DNA post the recruitment of Cas9 protein to the target site. A blunt-end double strand break which is imperfectly repaired by the DNA repair machinery, causes frameshift mutations and thus disrupt the gene expression. The nuclease-deactivated Cas9 or dead Cas9 (dCas9) protein is commonly fused to effector domains that have regulatory functions and can highly specifically and stably control transcriptional repression or activation of specific endogenous genes (Cheng et al; 2013, Gilbert et al; 2014, Gilbert et al; 2013). These variants of the CRISPR/Cas9 method have been termed CRISPRactivation (CRISPRa) and CRISPRinterference (CRISPRi), correspondingly.

In CRISPRa, CRISPR-mediated gene activation, the dCas9 protein is fused to transcriptional activator proteins or protein domains, to facilitate gene expression **(Figure 1.6).** VP64, a tetrameric complex of the herpes simplex virus transacting activator protein VP16, is one example for such a protein, that is used ubiquitously. Efficient activation has been shown for both reporter genes and endogenous genes when multiple sgRNAs targeting different sequences in the same promoters were expressed together to recruit multiple activators (Cheng et al; 2013, Maeder et al; 2013).

Most commonly used for the transcriptional inhibition is a dCas9-KRAB fusion protein. The KRAB domain is present in many zinc finger protein- based transcription factors and is known as one of the strongest transcriptional repressors (Margolin et al; 1994). Repression levels of up to 90-99% can be achieved with the most effective sgRNAs (Gilbert et al; 2014, Margolin et al; 1994, Maeder et al; 2013, Kearns et al; 2014).

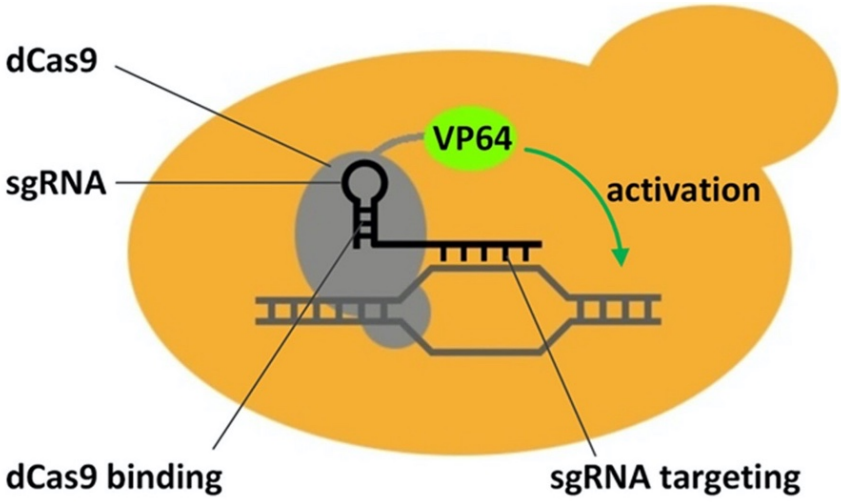


Figure 1. 6: CRISPR- based regulation of transcription *- Schematic showing the CRISPR activation (CRISPRa) mechanism for transcriptional activation of target loci (Figure taken from Pengsong et al; 2018)*

Recent attempts have been made to manipulate the transcriptional activity of endogenous elements using CRISPR technology. By virtue of gRNA multiplexing and combination of CRISPR activation and interference to Chimeric Assembly of gRNA Oligonucleotides, commonly known as CARGO (Gu et al; 2018), a novel method of gRNA multiplexing and delivery, activation and repression of HERV-K(HML-2) LTR5Hs has been achieved (Fuentes et al; 2018). Additionally, CRISPRa has also been actively used to activate and augment the expression of endogenous genes (Wang et al; 2019). Thus, CRISPRa and CRISPRi provides remarkable cutting-edge technological edge to regulate the silent elements of our genome.

2. Materials and Methods

2.1 Materials

Table 2. 1: Antibodies

Primary Antibody	Provider	Reference No
Mouse Anti-Cas9	Abcam	ab191468
Mouse Anti-GAPDH	EMD Millipore Corp	CB1001-500UG
Mouse Anti-MAP2	Sigma-Aldrich	M-1406
Mouse Anti-SYNAPSIN-I	Santa Cruz Biotech	sc-376623
Mouse Anti-SOX2	Santa Cruz Biotech	sc-398254
Rat Anti-CTIP2	Abcam	ab18465
Mouse Anti-OCT-4	Santa Cruz Biotech	sc-5279
Rabbit Anti-NANOG	Abcam	ab80892
Rabbit Anti- PAX6	Biolegend	901301
Rabbit Anti-TBR1	Abcam	ab31940
Goat Anti- FOXA2/HNF-3β	R&D biosystems	AF2400
Rabbit Anti-TBR2	EMD Millipore Corp	AB2283
Rabbit Anti-SATB2	Sigma-Aldrich	HPA001042
Mouse Anti-Vimentin	Dako	M0725
Mouse Anti- Human HERV-K Env	Austral Biologicals	HERM-1811-5
Mouse Anti- Human HERV-K-Gag	Austral Biologicals	HERM-1841-5
AlexaFluor Goat Anti-Mouse 488	Thermo Fischer Scientific	R37120
AlexaFluor Goat Anti-Rabbit 488	Thermo Fischer Scientific	A32731
AlexaFluor Goat Anti-Rat 488	Abcam	ab150157
AlexaFluor Donkey Anti-Goat 488	Abcam	ab150129
AlexaFluor Goat Anti-Mouse 594	Abcam	ab150116
AlexaFluor Goat Anti-Rabbit 594	Thermo Fischer Scientific	A11012
AlexaFluor Goat Anti-Rat 568	Thermo Fischer Scientific	A-11077
AlexaFluor Goat Anti-Rabbit 647	Thermo Fischer Scientific	A-27040

Table 2. 2 : Media

Media	Provider

DMEM	Gibco Life Technologies
Essential 8 Flex	Gibco Life Technologies
Essential 8	Gibco Life Technologies
Essential 6	Gibco Life Technologies
Neurobasal Media	Gibco Life Technologies
Opti-MEM	Gibco Life Technologies
DMEM-F12	Gibco Life Technologies

Table 2. 3: Growth Factors

Growth Factor	Provider	Reference No
LDN 193189 (LDN)	Stemgent	04-0074
SB 431542 (SB)	Tocris	1614
XAV-939 (XAV)	Tocris	3748
Y-27632 dihydrochloride (ROCKi)	Tocris	1254
CHIR 99021 (CHIR)	Tocris	4423
DAPT	Tocris	2634
Recombinant Sonic Hedgehog (Shh)	R&D biosystems	464-SH-200
Recombinant Brain-derived Neurotrophic factor (BDNF)	R&D biosystems	248-BD-025
Recombinant Glial-derived Neurotrophic factor (GDNF)	PeproTech	450-10
Ascorbic acid (AA)	Sigma-Aldrich	A4034-100G
db-cAMP	Sigma-Aldrich	D0627-100MG
TGFß 3	R&D Systems	243-B3

Table 2. 4: Reagents

Reagents	Provider
FBS	Gibco Life Technologies
L-glutamine	ThermoFischer Scientific
Sodium pyruvate	ThermoFischer Scientific
Penicillin/Streptomycin	ThermoFischer Scientific

0.05% Trypsin-EDTA	ThermoFischer Scientific
Knockout Serum (KSR)	ThermoFischer Scientific
Stem cell Pro Accutase	ThermoFischer Scientific
ReLeSR	StemCell Technologies
B27 without Vitamin A	ThermoFischer Scientific
N2 supplement B	StemCell Technologies
N2 Supplement	ThermoFischer Scientific
Progesterone	Sigma-Aldrich,Darmstadt, Germany
Mouse Laminin	ThermoFischer Scientific
Fibronectin	ThermoFischer Scientific
Vitronectin	ThermoFischer Scientific
Poly Orinithine	ThermoFischer Scientific
Matrigel	Corning
Geltrex	ThermoFischer Scientific

Table 2. 5: Buffers

Buffer	Constitution
Oligo Annealing Buffer	1M Tris-HCl, pH-8.0+ 5M NaCl+ 500mM EDTA, pH-8.0
Protein Lysis buffer	100mM Tris-HCl (pH-8.0) + 150mM NaCl+ 1% Glycerol+ 1% NP-40+ 0.5% Deoxycholate+ 0.1% SDS+ 0.1% Triton-X 100
Lamellie Buffer	277.8 mM Tris-HCl, pH 6.8 + 44.4% (v/v) glycerol+ 4.4% SDS+ 0.02% bromophenol blue+ 150mM DTT
10x Electrophoretic Buffer	0.25M TRIS+ 1.92M Glycine+ 0.1% SDS
Towbin Transfer Buffer	25mM Tris + 192 mM Glycine, pH 8.3+ 20% Methanol (vol/vol)
TAE buffer	2M Tris-Acetate, 100mM Na2-EDTA, 10mM Tris-HCl
Phosphate Buffered Saline (PBS)	140mM NaCl+ 5.4mM KCl+ 9.7 mM Na2HPO4. 2H2O+ 2mM KH2PO4, pH-7.4

Permeabilisation Buffer for IFA	0.3%Triton X-100 in PBS
Blocking buffer for IFA	0.15%Triton X-100 in PBS+10% FBS +5% BSA
PBS-T	1x PBS+ 1% Tween-20

Table 2. 6 : Chemicals

Chemical	Provider
2-Log DNA ladder	New England Biolabs (NEB), Frankfurt, Germany
30% Acrylamide-Bis Acrylamide solution	Bio-Rad, Munich, Germany
Absolute Ethanol 200-proof Molecular Biology Grade	
Luria Bertani LB Agar	Carl Roth, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich,Darmstadt, Germany
Ammonium Per Sulphate (APS)	Carl Roth, Karlsruhe, Germany
Agarose NEEO Ultra Quality	Carl Roth, Karlsruhe, Germany
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Darmstadt, Germany
ß-Mercaptoethanol	Thermo Scientific, Dreieich, Germany
Chloroform 99.8%	Acros organics Fisher scientific, Geel, Belgium
Isopropanol	Carl Roth, Karlsruhe, Germany
Puromycin	Sigma-Aldrich, Darmstadt, Germany
Blasticidin	Sigma-Aldrich, Darmstadt, Germany
Hygromycin	Sigma-Aldrich, Darmstadt, Germany
Doxycycline	Sigma-Aldrich, Darmstadt, Germany
Geneticin G418	Thermo Fischer scientific, Dreieich, Germany
dNTPs	Thermo Fischer scientific, Dreieich, Germany
Nuclease Free water	NEB
Developer	Agfa, Mortsel, Germany
Fixer	Agfa, Mortsel, Germany

DAPI	Thermo Fischer scientific, Dreieich, Germany
Hoerst	Thermo Fischer scientific, Dreieich, Germany
Triton X-100	Sigma-Aldrich, Darmstadt, Germany
Tween-20	Sigma-Aldrich, Darmstadt, Germany
PBS	Carl Roth, Karlsruhe, Germany
PFA	Carl Roth, Karlsruhe, Germany
DMSO	Sigma-Aldrich, Darmstadt, Germany
EDTA-Molecular Biology Grade	Sigma-Aldrich, Darmstadt, Germany
Tris-HCl, pH-7.4	Carl Roth, Karlsruhe, Germany
Tris-HCl, pH-6.8	Carl Roth, Karlsruhe, Germany
TEMED	Sigma-Aldrich, Darmstadt, Germany
Ethidium bromide	Sigma-Aldrich, Darmstadt, Germany
Page Ruler Protein Marker	Thermo Fischer scientific, Dreieich, Germany
Bromophenol 6X gel running dye	New England BioLabs, Frankfurt, Germany
Xylene Cyanol 6X gel running dye	New England BioLabs, Frankfurt, Germany
Polybrene	Sigma-Aldrich, Darmstadt, Germany
Skimmed Milk powder	Sigma-Aldrich, Darmstadt, Germany
LB growth Media	Carl Roth, Karlsruhe, Germany
SOC media	NEB
SDS	Carl Roth, Karlsruhe, Germany
HCl	Carl Roth, Karlsruhe, Germany
Sucrose	Sigma-Aldrich, Darmstadt, Germany
NaCl	Sigma-Aldrich, Darmstadt, Germany
D-glucose	Sigma-Aldrich, Darmstadt, Germany
Immumount	Thermo Fischer Scientific
NEG blue	Thermo Fischer Scientific
OCT solution	VWR chemicals
TRIZOL	Gibco life Technologies, Dreieich, Germany

Sodium bicarbonate	Sigma-Aldrich, Darmstadt, Germany
DTT	Sigma-Aldrich, Darmstadt, Germany
Glycine	Sigma-Aldrich, Darmstadt, Germany
Methanol	Carl Roth, Karlsruhe, Germany
NaOH	Sigma-Aldrich, Darmstadt, Germany

Table 2. 7: Enzymes

Enyzme	Provider
T4 DNA ligase	ThermoFischer Scientific
Go Taq polymerase	Promega
BfuA1	NEB
EcoR1-HF	NEB
Kpn1-HF	NEB
BamH1-HF	NEB
Superscript Reverse Transcriptase	Invitrogen Life Sciences
RNAse-H	NEB
RNAse-OUT	Invitrogen Life Sciences
DNAse	Qiagen

Table 2. 8: Bacteria

Bacteria	Provider
DH5 alpha	Thermofischer Scientific
XL 10 Gold	Agilent Technologies

Table 2. 9:Recombinant DNA

Recombinant DNA	Identifier	Source
pLKO.1-Puro U6 sgRNA BfuA1 stuffer	Addgene plasmid #50920	A gift from Rene Maehr & Scot Wolfe
pHAGE EF1α dCas9-VP64	Addgene plasmid #50918	A gift from Rene Maehr & Scot Wolfe
pHAGE EF1α dCas9-KRAB	Addgene plasmid #50919	A gift from Rene Maehr & Scot Wolfe

pHAGE TRE-dCas9-VP64	Addgene plasmid #50916	A gift from Rene Maehr & Scot Wolfe
pSPAX.2	Addgene plasmid #12260	A gift from Didier Trono
pMD2.G	Addgene plasmid #12259	A gift from Didier Trono
pLKO.1-U6 HERV-K(HML-2) G3	This study	
pLKO.1-U6 HERV-K(HML-2) G10	This study	
pLKO.1-U6 CHRDL1 G1	This study	
pLKO.1-U6 CHRDL1 G7	This study	
pLKO.1-U6 CLSTN2 G3	This study	
pLKO.1-U6 CLSTN2 G23	This study	
pLKO.1-U6 EPHA4 G2	This study	
pLKO.1-U6 EPHA4 G11	This study	
pLKO.1-U6 NTRK3 G1	This study	
pLKO.1-U6 NTRK3 G17	This study	

Table 2. 10:Oligonucleotides

Oligonucleotide	Upper strand	Lower strand
HERV-K(HML2) G3	AAATGGATTAAGGGCGGTGC	GCACCGCCCTTAATCCATTTC
HERV-K(HML2) G10	ATCCTCCATATGCTGAACGC	GCGTTCAGCATATGGAGGATC
CHRDL1 G1	GCCGGCTTCTCGGGCCAAGT	ACTTGGCCCGAGAAGCCGGC
CHRDL1 G7	GCTCCCTCGTGGTGTGAGGG	CCCTCACACCACGAGGGAGC
CLSTN2 G3	GAGAGTCGGAGTGGAGGCGC	GCGCCTCCACTCCGACTCTC
CLSTN2 G23	GTGACGTCACGGGCCGTCCC	GGGACGGCCCGTGACGTCAC
EPHA4 G2	GATCCCCCACGTTACCTCGA	TCGAGGTAACGTGGGGGATC
EPHA4 G11	GACTGGCGGGCTCACGTCAC	GTGACGTGAGCCCGCCAGTC
NTRK3 G1	GATAACCCGTGCGTTTCGTA	TACGAAACGCACGGGTTATC
NTRK3 G17	GCATTTGAGATTGCGAGGGT	ACCCTCGCAATCTCAAATGC

Table 2. 11:Primers

Primers	Sequence forward (5'-3')	Sequence reverse (5'-3')

RNA Polymerase II	GCACCACGTCCAATGACAT	GTCGGCTGCTTCCATAA
dCas9	TCGGATCTGCTACCTGCAGGAG ATCTTTAG	CAGCCTTATCAGTACTGTCTACCAG CTTCT
HERV-K(HML-2) Pol	GGCCATCAGAGTCTAAACCACG	CTGACTTTCTGGGGGTGGCCG
HERV-K(HML-2) Env	CTGAGGCAATTGCAGGAGTT	GCTGTCTCTTCGGAGCTGTT
HERV-K(HML-2) Gag	AGCAGGTCAGGTGCCTGTAACA TT	TGGTGCCGTAGGATTAAGTCTCCT
MAP2	TTCCTCCATTCTCCCTCCTC	TCTGCGAATTGGCTCTGAC
SYNAPSIN-I	GGAAGGGATCACATCATTGAG G	TGTTTGTCTTCATCCTGGT
SOX-2	CCATGCAGGTTGACACCGTTG	TCGGCAGACTGATTCAAATAATACA G
Ki67	TGACCCTGATGAGAAAGCTCAA	CCCTGAGCAACACTGTCTTTT
PAX6	GTGTCCAACGGATGTGTGAG	CTAGCCAGGTTGCGAAGAAC
FOXG1	AGGAGGGCGAGAAGAAGAAC	TCACGAAGCACTTGTTGAGG
OTX1	GCCTCCCCTTCCAGTCTTTC	GGGCAGAAACACGCCAGTTA
EMX1	TGACGGTTCCAGTCCGAAGT	CCAAGGACAGGTGAGCATCC
SOX1	TACAGCCCCATCTCCAACTC	GCTCCGACTTCACCAGAGAG
EMX2	TCCAAGGGAACGACACTAGC	TCTTCTCAAAGGCGTGTTCC
LMX1	CAGCCTCAGACTCAGGTAAAA GTG	TGAATGCTCGCCTCTGTTGA
TH	GCACCTTCGCGCAGTTCT	CCCGAACTCCACCGTGAA
NurrI	GGCTGAAGCCATGCCTTGT	GTGAGGTCCATGCTAAACTTGACA
FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
OCT4	GGGCTCTCCCATGCATTCAAAC	CACCTTCCCTCCAACCAGTTGC
NANOG	TGGGATTTACAGGCGTGAGCCA C	AAGCAAAGCCTCCCAATCCCAAAC
CHRDL1	CCTGGAACCTTATGGGTTGGT	AACATTTGGACATCTG ACTCGG
CLSTN2	GCACCGGGAGGCGAG	TGTGCTTATTGACTTTAGCCGC
EPHA4	GGAAGGCGTGGTCACTAAAT	TCTGCCATCATTTTTCCTGA
NTRK3	TCCGTCAGGGACACAACTG	GCACACTCCATAGAACTTGACA

Table 2. 12:Kits

Kit	Provider
Rapid DNA Ligation Kit	Roche, Mannheim, Germany
Expand High Fidelity PCR kit	Roche, Mannheim, Germany
GoTaq PCR amplification kit	Promega, Madison, USA
NucleoSpin PCR purification Kit	Mackery-Nagel, Dueren, Germany
NucleoSpin DNA Gel Extraction kit	Mackery-Nagel, Dueren, Germany
NucleoSpin Plasmid DNA isolation Kit	Mackery-Nagel, Dueren, Germany
NucleoBond Xtra Midi Kit	Mackery-Nagel, Dueren, Germany
QIA-amp DNA mini–Genomic DNA isolation kit	Qiagen, Hilden,Germany
RNeasy RNA isolation mini kit	Qiagen, Hilden,Germany
RNAse-free DNase digestion kit	Qiagen, Hilden,Germany
RQ1 RNAse-free DNAse digestion Kit	Promega, Madison, USA
RNA Nano 600 Bioanalyser Kit	Agilent technologies, California, USA
Superscript II First strand cDNA synthesis kit	Invitrogen

Table 2. 13:Softwares and Algorithms

Software	Licenser
Optimised CRISPR design tool	Zhang Lab, MIT
CRISPR-ERA	Lei Stanley Qi Lab, Xiaowo Wang Lab, Stanford
Image J	NIH
Graph Pad Prism 8	GraphPad
Adobe Photoshop	Adobe Inc.
Adobe Illustrator	Adobe Inc.
SnapGene viewer	Insightful Science
LC480 SW 1.5.1	Roche
Zeiss AX10- Zen Pro 2.3 Blue	Zeiss
MS-Office	Microsoft Corp.

Table 2. 14:Instruments

Equipment	Manufacturer
1.0 mm glass plates	Bio-Rad, Munich

10-well combs	Bio-Rad, Munich
-20°C premium NoFrost freezer	Liebherr, Kirchdorf
4°C premium NoFrost fridge	Liebherr, Kirchdorf
-80°C freezer 900 Forma	Thermo Fisher Scientific, Dreieich
Agarose gel chamber model B1A	Peqlab, Erlangen
Agarose gel chamber model B2	Peqlab, Erlangen
Agarose gel comb 22 well, 1.0 mm	Peqlab, Erlangen
Agarose gel comb 8 well, 1.5 mm	Peqlab, Erlangen
Assistent® improved Neubauer chamber	Karl Hecht, Sondheim
Biometra TB2 Thermoblock	Analytic Jena, Jena
Casting stands with 2 casting frames	Bio-Rad, Munich
Cell buffer dam	Bio-Rad, Munich
ChemiDoc Molecular Imager	BioRad, Munich
Combimag REO magnetic stirrer	IKA, Staufen im Breisgau
Eppendorf Research Plus Pipette, 0.1-2.5 µL	Eppendorf, Hamburg
Eppendorf Research Plus Pipette, 0.5-10 µL	Eppendorf, Hamburg
Eppendorf Research Plus Pipette, 100-1000 µL	Eppendorf, Hamburg
Eppendorf Research Plus Pipette, 10-100 µL	Eppendorf, Hamburg
EVOS FL Cell Imaging System	Thermo Fisher Scientific, Dreieich
Gel holder cassettes	Bio-Rad, Munich
Heidolph reax 2000-vortex mixer	Heidolph instruments, Schwabach
Heraeus Fresco 17 centrifuge	Thermo Fisher Scientific, Dreieich
Herasafe biological safety cabinet	Thermo Fisher Scientific, Dreieich
Hercell 150i CO_2 incubator	Thermo Fisher Scientific, Dreieich
Hettich ROTANTA 460/460R centrifuge	Sigma-Alrdich, Darmstadt
Integra Vacusafe	Integra, Biebertal
Jouan CR3i centrifuge	DJB Labcare, Buckinghamshire, UK
LABOCAP caps with handle, silver, 15/16 mm	Roth, Karlsruhe
LightCycler® 480 System	Roche Diagnostics, Mannheim
Mini Trans-Blot® Cell system	Bio-Rad, Munich
Mini-PROTEAN Tetra Cell Tank, lid with power cables and electrode assembly	Bio-Rad, Munich

Mr. Frosty freezing container	Thermo Fisher Scientific, Dreieich
Nanodrop 2000c spectrophotometer	Peqlab, Erlangen
NewClassic MS analytical scale	Mettler-Toledo, Columbus, USA
PIPETBOY acu 2	Integra, Biebertal
PowerPac™ HC	Bio-Rad, Munich
Privileg 9029 GD microwave	Bauknecht, Stuttgart
Sartorius portable scale	Waagenbau, Munich
SPROUT™ Mini-Centrifuge	Heathrow Scientific, Vernon Hills, USA
Test Tube Rotating Shaker 3015	GFL, Burgwedel
Thermostat water bath model 2761	Eppendorf, Hamburg
Universal Hood II Gel Doc	Bio-Rad, Munich

Table 2. 15:Consumable Items

Consumables	**Manufacturer**
12 mL syringe	Becton-Dickinson, Heidelberg
Amicon Ultra-15, PLHK Ultracel-PL Membran, 100 kDa ultracentrifugation falcon tubes	Merck, Darmstadt
CL-XPosure™ film	Thermo Fisher Scientific, Dreieich
Greiner cell scraper	Sigma-Aldrich, Darmstadt
Greiner CELLSTAR® serological pipette, volume 5 mL	Sigma-Aldrich, Darmstadt
Greiner CELLSTAR® serological pipette, volume 10 mL	Sigma-Aldrich, Darmstadt
Greiner CELLSTAR® serological pipette, volume 25 mL	Sigma-Aldrich, Darmstadt
Greiner CELLSTAR® serological pipette, volume 50 mL	Sigma-Aldrich, Darmstadt
Cell culture flasks, 75 cm^2	Sarstedt, Nümbrecht
Cell culture flasks, 175 cm^2	Sarstedt, Nümbrecht
Conical falcon tubes, 15 ml	Sarstedt, Nümbrecht
Conical falcon tubes, 50 ml	Sarstedt, Nümbrecht

FrameStar® 96 Well Plate with qPCR adhesive seal	4titude, Berlin
Falcon® Petri dishes, 100x20 mm	Corning, Durham, USA
Greiner cell culture plates, 6 Well	Sigma-Aldrich, Darmstadt
Parafilm	Bemis, Oshkosh, USA
Kimtech purple nitrile gloves	Kimberly Clark, Irving, USA
TipOne filter tips, 10 µL	USA Scientific, Ocala, USA
TipOne filter tips, 100 µL	USA Scientific, Ocala, USA
TipOne filter tips, 1000 µL	USA Scientific, Ocala, USA
Whatman paper	Sigma-Aldrich, Darmstadt
NITRIL® NEXTGEN® gloves	Meditrade, Kiefersfelden
1.0 mL Eppendorf tubes	Sarstedt, Nümbrecht
PCR tubes 0.2 mL	Biozym Scientific, Oldendorf
2.0 mL Eppendorf tubes	Sarstedt, Nümbrecht
1.5 mL Eppendorf tubes	Sarstedt, Nümbrecht
Nunc® CryoTubes® cryogenic vials, 1.8 mL	Sigma-Aldrich, Darmstadt
EMD Millipore™ Steritop™ Sterile Vacuum Bottle-Top Filters 0.22µM	Thermo Fisher Scientific, Dreieich
Millex HV 0.45 µm filter	Merck, Darmstadt
Nitrocellulose blotting membrane, 0.45µM	GE Healtcare, Munich
12 mL syringe	Becton-Dickinson, Heidelberg
Amicon Ultra-15, PLHK Ultracel-PL Membran, 100 kDa ultracentrifugation falcon tubes	Merck, Darmstadt
CL-XPosure™ film	Thermo Fisher Scientific, Dreieich
Greiner cell scraper	Sigma-Aldrich, Darmstadt
Greiner CELLSTAR® serological pipette, volume 5 mL	Sigma-Aldrich, Darmstadt
Greiner CELLSTAR® serological pipette, volume 10 mL	Sigma-Aldrich, Darmstadt

2.1.1 Cell lines

H9 EF1 dCas9-VP64

The pHAGE-EF1 dCas9-VP64 fusion construct which constitutively express dCas9 from an EF1 alpha promoter was lentivirally transduced to H9 human embryonic stem cell line, selected with puromycin and validated for dCas9 expression using several methods.

H9 EF1 dCas9-KRAB

The pHAGE-EF1 dCas9-KRAB fusion construct which constitutively express dCas9 from an EF1 alpha promoter was lentivirally transduced to H9 human embryonic stem cell line, selected with puromycin and validated for dCas9 expression using several methods.

H9 TRE dCas9-VP64

The pHAGE-TRE dCas9-VP64 fusion construct which inducibly express dCas9 upon doxycycline treatment from a Tetracycline Response Element (TRE) was lentivirally transduced to H9 human embryonic stem cell line, selected with Geneticin and validated for dCas9 expression using several methods.

H9 EF1 dCas9-VP64 HERV-K(HML-2)

The pLKO.1 U6 puro HERV-K(HML-2) gRNA 3 and pLKO.1 U6 puro HERV-K(HML-2) gRNA 10 constructs which express the HERV-K(HML-2) gRNAs from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-VP64 human embryonic stem cell line, selected with puromycin and validated for HERV-K(HML-2) transcription activation using several methods.

H9 EF1 dCas9-KRAB HERV-K(HML-2)

The pLKO.1 U6 puro HERV-K(HML-2) gRNA 3 and pLKO.1 U6 puro HERV-K(HML-2) gRNA 10 constructs which express the HERV-K(HML-2) gRNAs from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-KRAB human embryonic stem cell line, selected with antibiotics and validated for HERV-K(HML-2) transcription repression using several methods.

H9 EF1 dCas9-VP64 control

The pLKO.1 U6 puro control gRNA construct which express the CAG control gRNA from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-VP64 human embryonic stem cell line, selected with antibiotics and validated using several methods.

H9 EF1dCas9-KRAB control

The pLKO.1 U6 puro control gRNA construct which express the CAG control gRNA from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-KRAB human embryonic stem cell line, selected with antibiotics and validated using several methods.

H9 EF1 dCas9-VP64 CHRDL1

The pLKO.1 U6 CHRDL1 gRNA constructs which express the CHRDL1 gRNAs from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-VP64 human embryonic stem cell line, selected with antibiotics and validated for CHRDL1 transcription activation using several methods.

H9 EF1 dCas9-VP64 CLSTN2

The pLKO.1 U6 CLSTN2 gRNA constructs which express the CLSTN2 gRNAs from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-VP64 human embryonic stem cell line, selected with antibiotics and validated for CLSTN2 transcription activation using several methods.

H9 EF1 dCas9-VP64 EPHA4

The pLKO.1 U6 EPHA4 gRNA constructs which express the EPHA4 gRNAs from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-VP64 human embryonic stem cell line, selected with antibiotics and validated for EPHA4transcription activation using several methods.

H9 EF1 dCas9-VP64 NTRK3

The pLKO.1 U6 NTRK3 gRNA constructs which express the NTRK3 gRNAs from a human U6 promoter was lentivirally transduced to H9 EF1 dCas9-VP64 human embryonic stem cell line, selected with antibiotics and validated for NTRK3 transcription activation using several methods**.**

H9 TRE dCas9-VP64 HERV-K(HML-2)

The pLKO.1 U6 puro HERV-K(HML-2) gRNA 3 and pLKO.1 U6 puro HERV-K(HML-2) gRNA 10 constructs which express the HERV-K(HML-2) gRNAs upon induction with doxycycline through a Tetracycline Response Element (TRE) was lentivirally transduced to H9 TRE-dCas9-VP64 human embryonic stem cell line, selected with antibiotics and validated for HERV-K(HML-2) transcription activation using several methods.

H9 TRE dCas9-VP64 control

The pLKO.1 U6 puro control gRNA constructs which express the CAG control gRNA upon induction with doxycycline through a Tetracycline Response Element (TRE) was lentivirally transduced to H9 TRE-dCas9-VP64 human embryonic stem cell line, selected with antibiotics and validated using several methods.

2.2 Methods

2.2.1 Tissue Culture

2.2.1.1 Cell culture and storage

HEK 293T cell line

The HEK 293T adherent cell line (ATCC® CRL-11268™) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Sodium Pyruvate and 1% Penicillin/Streptomycin(100µg/mL) antibiotic in a T75 cell culture flask at 37°C incubator in a humidified atmosphere saturated with 5% CO_2. Cells were split and passaged at 60-70% growth confluency. The growth media was removed, cells were washed with 1X PBS and incubated for 5 minutes at 37°C with 1.5mL of 0.05% Trypsin-EDTA for detachment. Once the cells were detached, they were resuspended in growth media and processed either for seeding or passaging in a ratio of 1:10/T75 flask.

For proper storage of HEK 293T cell line, a confluent T75 flask of cells were detached, collected in a 15mL falcon tube, spun down at 900 rpm for 5 minutes at room temperature and resuspended in 5mL of DMEM. The cells were counted using a hemocytometer and $2x10^6$ /mL cells were frozen per cryovial in 1mL of freezing media (DMEM+10% FBS+10% DMSO). The cryovials were labelled indicating the name of the cell line, the passage number, the date of freezing and stored at -80°C and liquid nitrogen.

The cells were collected from liquid nitrogen or -80°C and thawed in a 37°C water bath. The cells were then transferred to 2mL of prewarmed DMEM in a 15mL falcon tube and spun down at 900rpm for 5 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 5mL of DMEM and transferred to a T25 cell culture flask. Next day, the medium was changed to fresh DMEM medium and the cells were transferred and cultured in T75 flask as mentioned above.

Stem cell lines (iPS and hES cell lines)

Induced pluripotent stem cell or iPS (#HMGU1) cell line was obtained from the iPS core facility of Helmholtz zentrum Munich. The human embryonic cell line used was WA09 (H9). iPS cells and hES cells were cultured in Essential 8™ Flex Basal media (E8 Flex) supplemented with 2% Essential 8™ Flex supplement. For culturing of stem cells, Vitronectin protein was used as a coating material which provides an adherent surface for feeder free growth of stem cells and maintains the pluripotency levels in higher passages. A 10cm cell culture dish was coated with 8mL of recombinant vitronectin (r-VTN) protein in the ratio 1:100 in 1X PBS for 1 hour at room temperature. 60-70% confluent cells in 10cm dishes were

passaged by removing the growth media, washing with 10mL of 1X PBS and incubating with 8mL of EDTA based detaching agent (0.5M EDTA+5M NaCl+1XPBS, filter purified) at 37°C for exactly 2 minutes. The detaching agent was removed, and the cells were detached using 8mL of E8 Flex growth media. 2mL of the cell suspension was transferred to vitronectin coated 10cm cell culture dish supplied with 8mL of E8 flex basal media. The cells were always passaged and seeded in clumps to maintain the pluripotency and to avoid spontaneous differentiation of the stem cells. Media was changed every alternate day. The pluripotency levels of the cells in culture were routinely authenticated for markers Nanog and OCT-4 and checked for mycoplasma contamination.

For proper storage of iPS and hES cell lines, a confluent 10cm cell culture dish of cells was detached, collected in a 15mL falcon tube, spun down at 1200rpm for 5 minutes at room temperature and resuspended gently as clumps in 5mL of freezing media (E8 flex +20% Knock out serum +10% DMSO). The cryovials were labelled indicating the name of the cell line, the passage number, the date of freezing and stored at -80°C and liquid nitrogen.

The cells were collected from liquid nitrogen or -80°C and thawed at 37°C water bath. The cells were then transferred to 2mL of prewarmed E8 flex media in a 15mL falcon tube and spun down at 1200rpm for 5 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended only once in 2mL of E8 flex and transferred in clumps to a 10cm cell culture dish coated with vitronectin supplied with 8mL of media. Next day, the medium was changed to fresh E8 flex medium and the cells were routinely maintained as described above.

2.2.1.2 Transfection

HEK293T cells were seeded at a density of $2x10^5$ cells/10cm cell culture dishes. Next day, transfection was performed using Xtreme Gene DNA transfection reagent. 1µg of DNA was added to 250µL of Opti-MEM media along with 8µL of transfection reagent. The mixture was incubated for 30 minutes at room temperature and added to the cells and kept for 72 hours at 37°C incubator.

2.2.1.3 Viral Transduction

HEK 293T cells was used to produce Vesicular stomatitis virus G protein pseudotyped lentiviral particles. HEK 293T cells were seeded at $2x10^6$ cells per 7 mL DMEM in a 10 cm cell culture dish. The next day, cells were transfected with 1µg packing plasmid psPAX2, 1.5µg VSV-G envelope expressing plasmid pMD2.G and 1µg lentiviral transfer plasmid using

FuGENE X-treme gene HP DNA transfection reagent (Roche) as described above. The cells were stored in 100% humidity and 5% CO_2 at 37°C for approximately 72 hours.
iPS and hES cells were seeded into vitronectin coated 6-well cell culture dishes at a confluency of 60%, in patches. The next day, the virus containing media from HEK293T cells were harvested, filtered using a Millex HV 0.45 μm filter and concentrated to a volume of 0.5mL using Amicon Ultra-15, PLHK Ultracel-PL Membran, 100kDa ultracentrifugation falcon tubes and centrifuged at 4000 rpm for 10 min. A cationic polymer, Polybrene was added to the virus suspension in a concentration of 1μg/mL to increase the transduction efficiency and the mixture was transferred into one 6-well of iPSCs and hES cells. The cells were incubated in virus for 24 hours at 37°C and then washed off with 1x PBS. Transduced cells were selected with appropriate antibiotic selection markers and maintained as routine cultures.

2.2.2 Neuronal differentiation

Differentiation into cortical neurons and midbrain dopaminergic neurons

The differentiation of human embryonic stem cells and its derivatives (H9-CRISPRa, H9-CRISPRi, H9- CRISPRa-control, H9-CRISPRa-HERV-K(HML-2), H9-CRISPRi-control, H9-CRISPRi-HERV-K(HML-2), H9-TRE-CRISPRa-control, H9-TRE-CRISPRa-HERV-K(HML-2) were differentiated into cortical and dopaminergic neuronal lineage according to the established protocols from MKSCC, NewYork, USA.The Human pluripotent stem cells (hPSCs) were differentiated into cortical neurons (Maroof et al; 2013, Qi et al; 2017) or midbrain dopamine (mDA) neurons (Chambers et al; 2009, Kriks et al; 2011) following optimized versions of previously published protocols (Kriks et al; 2011, Qi et al; 2017). The protocols are explained briefly in the sections below.

Coating plates for differentiation

The day before coating thaw Matrigel or Geltrex at 4°C on ice. The next day, dilute Matrigel (1:25) or Geltrex (1:30) with ice cold DMEM/F12 and add Matrigel-DMEM/F12 dilution to the plates (0.5ml/well for 24-well plate or 2ml/well for 6-well plate) for cortical and midbrain dopaminergic differentiations, respectively. Seal the plates with parafilm and store at 4°C o/n.

Coating plates for replating with PO/Lam/FN

Dilute Poly-L-ornithine hydrobromide (PO) to 15µg/ml in PBS. Add 0.5ml/well for 24- well plate and incubate over night at 37°C / 5% CO2. Next day, remove the PO solution and wash the plates 3 times with 1X PBS. Afterwards dilute Laminin I and Fibronectin at 2µg/ml in PBS and add 0.5mL/well of a 24-well plate. Incubate the plates at 37°C over night.

2.2.2.1 Cortical neuron differentiation

For both types of differentiation (Cortical Neuron and Midbrain Dopaminergic Neuron) sets of abbreviations and timing are listed in tables below

Table 2. 16 Small molecule abbreviation

Small molecule	Abbreviation
LDN-193189	LDN
SB-431542	SB
XAV-939	XAV
CHIR-99021	CHIR
Y-27632	ROCKi

Table 2. 17: Cortical Neuron Differentiation: days relative to time of media change

Day	Duration (hr)
Day 0-5	0-120
Day 5-10	120-240
Day 10-20	240-480
Day 20-30	480-720
Day 30-60	720-1440

Briefly, hESCs were dissociated into single cells using Accutase, and plated at high density on Matrigel or Geltrex coated wells. For cortical neuron patterning, cells were cultured in Essential 6 medium in the presence of LDN193189, SB431542 and XAV939 (until day 5). From day 5 to 10, cells were cultured in the presence of TGFβ and BMP inhibitors to trigger cortical precursors for cortical neuron differentiation.

2.2.2.2 Midbrain Dopaminergic Neuron (mDA) Differentiation

Table 2. 18: Midbrain dopaminergic neuron differentiation: days relative to time of media change

Day	Duration (hr)
Day 0-4	0-96
Day 4-7	96-168
Day 7-10	168-264
Day 10-12	264-312
Day 12-60	312-1440

To induce midbrain floor plate precursors for mDA neuron differentiation cells were culture in the presence of LDN193189, Sonic hedgehog and CHIR99021 (until day 10). Afterwards cells were maintained in mDA differentiation media, 1% Pen/Strep, L-Glutamine, B27-Vitamin A supplemented with DAPT, BDNF, GDNF, cAMP, AA and TGF-β3 as described previously (Kriks et al., 2011; Qi et al., 2017).

2.2.3 Organoid Culture

Forebrain Organoids were generated from human embryonic stem cells and stable cell lines following the modified protocol from Zhou et al (Zhou et al; 2017).

Embryonic stem cells were grown in 10cm dishes and detached using Accutase as described previously.

Cells were harvested and counted as described previously. 9000 cells/well was seeded in a 96-welled V-bottomed cell culture plate (Sbio prime surface 96V plate MS-9096VZ) in 100ul E8 flex medium+ 1:1000 Y-drug.The plate was centrifuged at 3000rpm,10 minutes and incubated at 37°C incubator. This was counted as day -1 and the next day as Day 0.

From D0-D4- change media daily to LSBX E6 media. Change media using a multi-channel pipette.

From D5-D18 change media daily to LSB E6 media.

At D18 the organoids were transferred to 10cm dishes (without any coating) in Organoid media and grown further inside the incubator on a shaker to oxygenate the growing organoids. Shaking was continued throughout the culturing period.

After day 18, change the media every 5^{th} day and supply fresh 12mL of organoid media/10cm dish.

The organoids were harvested anywhere from D40-D100.

Medias

LSBX E6- Essential 6 medium (Gibco, A1516401)+ LDN (1:5000)+ SB (1:1000)+ XAV (1:5000)

LSB E6- Essential 6 medium (Gibco, A1516401)+ LDN (1:5000)+ SB (1:1000)

Organoid differentiation media (For 1L)

Table 2. 19: Details of organoid differentiation media (For 1L)

Ingredients	Provider	Concentration
DMEM F-12	Gibco, 11320033	50%
Neurobasal media	Gibco, 21103049	50%
100x N2 supplement	Gibco, 17502048	0.5X(5mL)
Human Insullin	Sigma, 91077C 100MG	0.025%
L-Glutamine	Gibco, 25030081	5mM
MEM-NEAA	Gibco, 11140050	0.7mM
Penicillin-Streptomycin	Gibco,15140122	50U/mL
2-mercaptoethanol	Sigma,M6250	55uM
50x B27 supplement without Vit A	Gibco, 12587010	1X(10mL)

Harvesting the organoids

The organoid media was carefully aspirated out from the 10cm dish. The tip of a 1mL pipette tip was cut and 10 organoids were transferred to a 50mL falcon containing 1X PBS. The PBS was pipetted out and the organoids were fixed in 20mL of 4% PFA in the 50mL falcon overnight at 4°C.Next day, the PFA was removed and 30mL of 30% Sucrose solution was added to the 50mL falcon and the organoids were kept on a rotating shaker overnight at 4°C or until they settle down at the bottom of the falcon. 1% Sodium Azide was added to the sucrose solution and the falcon tube was sealed with parafilm and stored at 4°C until sectioning.

2.2.4 Molecular biology Methods

2.2.4.1 Cloning

2.2.4.1.1 Designing of single guide RNA

In order to establish CRISPR activation and CRISPR interference system of different HERV families and selected candidate genes, single guide RNAs were designed using software, Optimised CRISPR design tool (www.mit.crispr.edu) or CRISPR-ERA (www.crispr-era.stanford.edu). The LTR sequences of different HERV families or gene sequences of the candidate genes were entered to the webtool and the predicted sgRNA sequences were analysed

for optimal location from transcription start site (TSS) for activation or interference of transcription. A total of four sgRNAs per HERV family or gene were chosen and a combination of two sgRNAs were used for experiments.

2.2.4.1.2 Vector design

For the cloning of sgRNAs, a lentiviral plasmid, pLKO.1-puro U6 sgRNA BfuA1 stuffer (Addgene plasmid # 50920; http://n2t.net/addgene:50920; RRID: Addgene_50920) was used. The puromycin cassette was replaced with selection markers, hygromycin and blasticidin and fluorescent markers, GFP and mCherry sequences and named as pLKO.1-Hyg U6 sgRNA BfuA1 stuffer, pLKO.1-Blas U6 sgRNA BfuA1 stuffer, pLKO.1-GFP U6 sgRNA BfuA1 stuffer and pLKO.1-mCherry U6 sgRNA BfuA1 stuffer. Single guide RNAs were cloned into all of these vectors for further use.

2.2.4.1.3 PCR amplification

Hygromycin, Blasticidin, GFP and mCherry sequences were amplified using Expand high Fidelity PCR amplification Kit as given in Table 2.20 from 100ng template DNA using specific primers designed using the custom oligos primer design tool (Life Technologies) flanking the restriction sites for BamH1 and Kpn-I in the forward and reverse primers, respectively. The PCR protocol was done according to the conditions given in Table 2.21.

Table 2. 20:Details of Polymerase Chain Reaction (PCR)

Mix 1	
10mM dNTP mix	1.0 ul
Forward primer	1.0 ul
Reverse primer	1.0 ul
DNA template	1.0 ul
dd H_2O	21.0 ul
Final Volume	25.0 ul
Mix 2	
Expand High Fidelity 10x buffer	5.0 ul
Expand High Fidelity enzyme mix	0.75 ul
dd H_2O	19.25 ul
Final Volume	25.0 ul

Table 2. 21:Details of PCR reaction conditions with Expand High Fidelity protocol

Step		**Cycles**	**Temperature**	**Time**
Initiatial denaturation		1	94°C	2 min
Amplification	Denaturation	20x	94°C	15s
	Annealing		62°C	30 s
	Elongation		72°C	2min
Final Elongation			72°C	7 min
Cooling		Elongation	4°C	∞

2.2.4.1.4 PCR purification

The PCR products were separated on a 2% Agarose gel, checked for the right size of the amplicon and purified using Machery-Nagel Nucleospin purification kit following manufacturer's protocol.

2.2.4.1.5 sgRNA oligo annealing

The upper and lower strands of lyophilized oligos were reconstituted to a final concentration of 100pm using nuclease free water or TRIS-EDTA buffer. From this, 10pm oligos were used for annealing using annealing buffer, as given in Table 2.22.

Table 2. 22:Details of oligo annealing

Oligo upper strand (10pm)	1.0 µL
Oligo lower strand (10pm)	1.0 µL
10x Annealing buffer	1.0 µL
Water	7.0 µL

The annealing reaction mixture was incubated at 95°C for 5 minutes, the heat block was switched off and cooled gradually to room temperature overnight.

2.2.4.1.6 Restriction digestion

For cloning of the selection markers and fluorescent markers, the pLKO.1-puro U6 sgRNA BfuA1 stuffer was digested with BamH1 and Kpn-I and purified using Nucleobond PCR

purification kit (Table 2.23). For cloning of the sgRNAs, the vectors were digested using BfuA1 restriction enzyme (Table 2.24) and the linearized product was gel extracted using Nucleobond Gel extraction Kit.

Table 2. 23:Details of restriction digestion for cloning of selection markers

pLKO.1-puro U6 sgRNA BfuA1 stuffer	1.0 µg
10x NEB CutSmart Buffer	3.0 µL
BamH1 NEB HF	1.0 µL
Kpn-I NEB HF	1.0 µL
Water	24.0 µL

The reaction mixture was incubated at 37°C for 1 hour.

Table 2. 24:Details of restriction digestion for cloning of single guide RNAs (sgRNA)

pLKO.1-puro U6 sgRNA BfuA1 stuffer	1.0 µg
10x NEB 3 Buffer	3.0 µL
BfuA1 NEB	2.0 µL
Water	24.0 µL

The reaction mixture was incubated at 50°C for 3 hours followed by gel extraction.

2.2.4.1.7 Gel extraction

Digested vector was loaded in a 2 % agarose gel and resolved. The linearized vector was extracted from the gel using Mackery-Nagel DNA isolation kit following manufacturer's protocol.

2.2.4.1.8 Agarose Gel Electrophoresis

Gels with agarose concentrations of 1 % and 2 % were used for electrophoresis. The percentage of the agarose content was determined based on the size of the DNA-fragment. A 1 % ethidium bromide solution was added in a 1/10000 dilution to the gel (dissolved in 1 x TAE buffer). Electrophoresis was carried out at 80 to 120 V in 1 x TAE buffer. A 2-log DNA ladder from New England Biolabs was used as the size comparison standard.

2.2.4.1.9 Ligation

The insert mass was calculated for 50ng of the vector concentration in the ratio 1:3 as follows. Insert mass = 3x (Insert length/vector length) x vector mass.

The ligation was carried out using Roche ligation kit following manufacturer's instructions given in Table 2.25 and Table 2.26 for selection markers and sgRNAs, respectively.

Table 2. 25:Ligation conditions for cloning of selection markers

Insert	1.0 µL (diluted as per calculation)
Vector	1.0 µL (for 50ng)
DNA dilution buffer	2.0 µL
2X Ligase buffer	5.0 µL
T4 DNA ligase	1.0 µL

The ligation reaction was performed by incubating at 16°C overnight.

Table 2. 26:Ligation conditions for cloning of single guide RNAs (sgRNA)

Insert (annealed oligos)	4.0 µL
Vector	1.0 µL
DNA dilution buffer	2.0 µL
2X Ligase buffer	8.0 µL
T4 DNA ligase	1.0 µL

The ligation reaction was performed by incubating at 16°C overnight.

The ligation mix in case of sgRNA cloning was digested with 1µL BfuA1 restriction enzyme before proceeding for transformation.

2.2.4.1.10 Transformation

5µL of the ligated product was added to 50µL of either E-coli DH5α (Invitrogen) or XL10 Gold (Agilent technologies) competent cell strains and incubated on ice for 30 minutes followed by heat shock at 42°C for 45 seconds and snap cooling on ice for 2 minutes. The bacteria were revived by adding 250µL of SOC medium into the ligated product-competent cell mixture and grown at 37°C for 1 hour. The bacteria were spread plated on LB-Agar ampicillin resistant plates and grown in a 37°C bacterial incubator for 16 hours. The efficiency of transformation was calculated in colonies formed per unit (cfu) using the formula

Transformation Efficiency (in cfu) = No of colonies on the plate/ DNA transformed in µg/ total dilution before plating

2.2.4.1.11 Plasmid DNA Mini Preparation

Bacterial colonies from the LB-agar Ampicillin plates were picked and cultured in LB media supplied with 50mg/mL of ampicillin antibiotic in a bacterial shaker at 37°C for 16 hours. The bacterial culture was pelleted down, and the plasmid DNA was isolated using Mackery-Nagel plasmid DNA isolation kit following the manufacturer's protocol.

2.2.4.1.12 Sequencing

The plasmid DNA was isolated, and concentration of DNA was measured using spectrophotometry by NanoDrop. 50-100ng of DNA in a total volume of 15µL along with 10µL volume of primers with a 10pm concentration was sent for sequencing at Eurofins Genomics. The DNA was also verified by restriction mapping.

2.2.4.1.13 Plasmid DNA Maxi Preparation

Following the confirmation of the clones by sequencing and restriction mapping, the plasmid DNA was cultured on a larger scale and a maxi preparation was performed using NucleoBond Maxi DNA kit by Mackery-Nagel, following the manufacturer's protocol. This DNA was further used for all experiments.

2.2.4.2 Genomic DNA isolation

Cells from HEK293T and transduced iPS and hES cell lines were collected in a 1.5mL microcentrifuge tube and centrifuged at 10000rpm for 10minutes at 4°C. Genomic DNA was isolated from pelleted cells using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol.

2.2.4.3 Polymerase Chain Reaction

To verify the integration of dCas9 into the genome of hES cells after viral transduction, a Polymerase Chain Reaction(PCR) was performed using the genomic DNA from the transduced cells as template. RNA polymerase II (RPII), the global housekeeping gene was used as an internal control for the reaction. Reaction mixtures were pipetted as follows using the GoTaq® DNA Polymerase (Promega), Table 2.27.

Table 2. 27:Composition of reaction mixture for GoTaq PCR

Reagent	**Amount**
gDNA	50 ng
5X Green GoTaq® Reaction Buffer	10.0 µL
10M dNTPs	1.0 µL
10 µM forward primer	1.0 µL
10 µM reverse primer	1.0µL
GoTaq® DNA Polymerase	0.25 µL
Nuclease-free H_2O	to 50 µL
Total	50 µL

The PCR reactions were run according to Table 2.28

Table 2. 28:Reaction condition for GoTaq PCR

Step		**Cycles**	**Temperature**	**Time**
Initiation		1	94°C	30 s
Amplification	Denaturation	32	94°C	2 min
	Annealing		57°C (RPII and dCas9 primers)	45 s
	Elongation		72°C	30 s
Final Elongation		1	72°C	5 min
Cooling		1	4°C	∞

The PCR products were loaded onto a 1% Agarose gel with 10 ng/mL EtBr in 1x TAE buffer. Electrophoresis was carried out at 80 to 120V in 1x TAE buffer. A 2-log DNA ladder from New England Biolabs was used as the size comparison standard.

2.2.4.4 RNA isolation

HEK 293T cells

Cells from HEK293T were collected in a 1.5mL microcentrifuge tube and centrifuged at 10000 rpm for 10 minutes at 4°C. Total RNA was isolated from pelleted cells using the Qiagen

QIAshredder and Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Bound RNA was eluted in 30 µL RNAse free H_2O. The concentration was measured in Nanodrop and the RNA was stored at -80°C.

Human Embryonic Stem Cells (hES)

Cells from transduced hES cell lines were collected in a 1.5mL microcentrifuge tube and centrifuged at 10000rpm for 10 minutes at 4°C. Pelleted cells were resuspended thoroughly in 1 mL TRIZOL reagent. 200 mL Chloroform was added, and the suspension was vortexed vigorously for 2 minutes. After incubation for 5 min at RT, the tubes were centrifuged at 12000 rpm for 15 min at 4°C. The aqueous phase was transferred into a new 1.5 mL tube without disturbing the interphase and organic phase. 500 µL of 2-propanol was added and the suspension was mixed and incubated for 10 minutes at room temperature and precipitated at -20°C for 1 hour. The sample was then centrifuged at 12000 rpm for 45 min at 4°C. The RNA pellet was washed with 1 mL of 75 % Ethanol at 7500 rpm for 5 minutes at 4°C, after discarding the 2-propanol. The RNA pellet was left for air-drying after discarding the ethanol. Finally, the RNA was dissolved in 30µL nuclease-free water at 55°C for 15 minutes and the concentration of the RNA was measured with the Nanodrop 2000 (Thermo Fisher Scientific). The RNA was stored at -80°C.

2.2.4.5 DNAse digestion

The DNA contamination of the RNA samples which was isolated using Qiagen RNeasy Mini Kit DNA was removed using Qiagen DNAse after precipitation and binding of the RNA onto the spin column. 70µL of RDD buffer and 10µL of DNAse was added to the spin column and incubated for 20 minutes followed by washing using the wash buffer provided in the Qiagen RNeasy Mini Kit.

For the RNA samples isolated using TRIZOL reagent, the DNA digestion was carried out using the RQ1 RNase-Free DNase Kit (Promega) following the supplier's instructions as given in Table 2.29.

Table 2. 29:Composition of reaction mixture for DNAse digestion

Reagent	**Amount**
RNA	1.0µL (diluted to 1µg/µL)
RQ1 DNAse buffer	1.0µL
RQ1 DNAse	1.0µL
Nuclease free water	7.0µL
Total	10.0µL

The reaction was incubated at 37°C for 30 minutes. The reaction was terminated by adding 1µL of RQ1 stop solution and incubated at 65°C for 10 minutes. The digested RNA was stored at -80°C or proceeded for reverse transcription.

2.2.4.6 Reverse transcription

1µg of DNase-treated RNA was reverse transcribed using random hexamers with the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's protocol. A RNAse-H digestion was performed to remove the residual untranscribed RNA, thereby confirming the integrity of the synthesized cDNA. The synthesized cDNA was stored at -20°C or proceeded for qRT-PCR.

2.2.4.7 Quantitative Real Time PCR (qRT-PCR)

A quantitative real- time RT-PCR was performed with the synthesized cDNA in Roche LightCycler 480 System, using Roche LC480 DNA Master SYBR Green and standard LightCycler protocol (Roche Diagnostics, Mannheim). Real-time RT-PCR experiments for each HERV family and candidate genes were performed in triplicate. The reaction set up was done as follows.

Table 2. 30:Composition of reaction mixture for qRT-PCR

Reagent	**Amount**
LightCycler® 480 SYBR Green I Master	5.0µL
10 µM forward primer	0.5 µL
10 µM reverse primer	0.5 µL
Nuclease-free H_2O	3.0 µL
Total	9.0 µL

9 µL of the reaction mixture from the master mix was aliquoted into each well of a 96-well qPCR plate. 1µL of cDNA were used as template in each well. Quantitative real-time RT-PCR was performed with LightCycler® 480 System (Roche). The transcript levels of the target genes were normalized to that of the global housekeeping gene RNA polymerase II. Cycling conditions can be seen in Table 2.31.

Table 2. 31:Cycling conditions in the LightCycler® 480 System (Roche) for qRT-PCR

Step	Temperature	Time / Acquisition rate
Initial denaturation	95°C	5 min
Amplification 45 cycles	95°C	10s
	60°C	10s
	72°C	10s
Melting curve	95°C	5s
	65°C	1 min
	Increase continuously to 97°C	Acquire images of fluorescence measurement every 0.11°C

Relative quantification

The relative expression of target genes in relation to the reference gene was calculated using the 'delta Cp' or 'delta delta Ct method'(Livak and Schmittgen; 2001). The difference in the Cp values were determined by

$$\Delta Cp = (Cp \text{ target gene}) - (Cp \text{ housekeeping gene})$$

$$\text{The fold change} = 2^{-(\Delta Cp)}$$

This method gives the relative mRNA transcript levels of our gene of interest in reference to a constant mRNA expression considered as reference, which is RNA polymerase II.

2.2.5 Biochemical Methods

2.2.5.1 Isolation of protein from cell lines

HEK 293T cells

HEK 293T cells were seeded in 6 well cell culture dishes or 60mm cell culture dishes for protein isolation. Cells were washed in 1X PBS, trypsinized and harvested by centrifugation. The cell pellet was washed with 1 mL 1x PBS and centrifuged at 13,000 rpm for 5 min at 4°C. PBS was discarded, and the cells were resuspended in 200 µL lysis buffer with a protease inhibitor cocktail. The cell suspension was incubated on ice for 3 hours, thoroughly vortexed and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was transferred to a new 1.5mL microcentrifuge tube. For western blot analysis, 30 µL of the supernatant were mixed with 10µL (4x) NuPAGE™ Lamellie sample buffer (Invitrogen) and boiled at 95 °C for 5 min.

hES cells

Old medium was removed from a 60% confluent 10cm dish and the cells were washed twice with 10 mL 1x PBS. 1mL of (4x) NuPAGE™ sample buffer (Invitrogen) supplied with 150 mM DTT was directly added to the dish and the cells were thoroughly removed with a cell scraper. The whole cell lysate was transferred to a 1.5mL microcentrifuge tube and boiled at 95°C for 5 min.

2.2.5.2 Western blotting

Protein lysates were loaded onto 10% SDS-gels that were cast according to Table 2.32.
For size reference, 6µL PageRuler^{TM} Prestained Protein ladder (Thermo Fisher Scientific) was used.

Table 2. 32:Composition of stacking gel and 10% separation gel for SDS PAGE.

Reagent	Stacking gel	Resolving gel (10%)
H_2O	1.4 mL	1.6 mL
30% acrylamide mix	330 µL	2 mL
1.5 M Tris (pH = 8.8)	-	1.3 mL
1 M Tris (pH = 6.8)	250 µL	-
10% SDS	20 µL	50 µL
10% APS	20 µL	50 µL
TEMED	2 µL	2 µL

The gels were submerged in 1x TGS buffer and run at a constant voltage of 60V per gel until the ladder started resolving and at 120V for 90 min until the desired size was resolved in the ladder.
Protein was transferred from the gel onto a 0.45 µm Nitrocellulose membrane by running the transfer for 60 min at 330 mA in 1x Towbin buffer. Unspecific binding sites were blocked by shaking the Nitrocellulose membrane in 5 % skim milk powder in PBST (1x PBS with 1 % Tween) for 1h. Following three washing steps for 10 min in PBST, the membranes were put into the respective antibody solutions. As an internal control GAPDH was used. Antibodies were diluted in in 5 % skim milk powder in PBST and rolled at 4 °C overnight. The next day, the membranes were washed in PBST three times for 10 min before a 1:10,000 dilution of HRP-conjugated secondary α-mouse antibody (CalBiochem) in 5 % skim milk powder in PBST was added for 1 h at RT. For developing, Pierce^{TM} ECL Western blotting substrate (Thermo Fisher Scientific) was used. The blots were left to incubate for 4 min. Bands were

detected on CL-XPosure™ film (Thermo Fisher Scientific) by an enhanced chemiluminescence system (Perbio Science, Bonn, Germany).

2.2.5.3 Immunostaining

hES cell lines

For immunostaining of hES cell lines, cells were grown as patches in Vitronectin coated 6 welled cell culture dishes. The cells were washed once with 1X PBS and fixed with 4% PFA for 15 min at room temperature. Cells were permeabilized with 1ml 0.3% Triton-X-100 in 1X PBS for 10 minutes and washed twice with 1mL 0.15% Triton-X-100 in 1X PBS at room temperature. Blocking was done in 0.5mL of 0.15% Triton-X-100 supplied with 5% BSA (40mg/mL) and 10% Fetal Bovine Serum (FBS) for 60 minutes at room temperature in a horizontal shaker. The cells were incubated with desired antibodies in 0.15% Triton-X-100 supplied with 5% BSA (40mg/mL) and 10% Fetal Bovine Serum (FBS) o/n at 4°C. Next day, the cells were washed three times with 1mL of 0.15% Triton-X-100 and incubated with specific secondary antibodies in 0.5mL of 0.15% Triton-X-100 supplied with 5% BSA (40mg/mL) and 10% Fetal Bovine Serum (FBS) for 2 hours. 90 minutes post incubation, nuclear stain DAPI was added in 1:10000 dilution. Cells were washed three times with 0.5mL of 0.15% Triton-X-100 and imaged using EvOs microscope or Zeiss AX microscope.

Differentiated Neurons

For immunostaining of cultured neurons differentiated from hES cells, the neurons were fixed using 4% PFA at specific time points, supplied with adequate amount of 1X PBS, sealed with parafilm and stored at 4°C until use. The immunostaining was done as described above.

Forebrain Organoids

For immunostaining, the forebrain organoids generated from hES cells were harvested at specific time points and stored in 30% Sucrose solution, sealed with parafilm and stored at 4°C until use. Cryosectioning was done using Leica 1850 UV cryostat and sections with a thickness of 20 microns were collected in a 1.5mL microcentrifuge tube. Permeabilization, washing and antibody incubation was done in microcentrifuge tubes as described above. Post incubation with secondary antibody, DAPI staining and washing, the sections were dried on a clean glass slide, mounted using immumount, sealed by coverslips and imaged using a Zeiss AX10 microscope.

2.2.5.4 Image analysis

The image analysis was performed in collaboration with Institute of Toxicology, Helmholtz zentrum Munich using Columbus high-content imaging and analysis software version 2.8.0 (PerkinElmer Life Sciences).

2.2.5.5 Calcium Imaging of Cortical Neurons

hPSC-derived neural stem cells were plated onto PO/Lam/FN coated 0.5mm black ΔT dishes (#10199-774*, VWR)* and used for calcium imaging as described (Jason et al; 2019). At day 60 of cortical neuronal differentiation, neurons were incubated with 5μM of Fura-2 (#F1221, Thermo Fisher Scientific) for 30 minutes at 37° C. Cultures were perfused with normal Tyrode's solution (pH 7.4) and supplemented with glutamate (100μM), ATP (30μM) or KCl (65mM) for 1 minute. Images were taken every 30 seconds at 340 and 380 nm. Images were analyzed by calculating the signal ratio between 380/340 using FIJI (ImageJ).

2.2.6 Quantification and statistical analysis

Three independent biological replicates were performed if not otherwise indicated. For organoids three independent biological replicates were performed and three organoids per time point were sectioned and stained. *p* values were calculated by unpaired two-tailed Student's t test if not otherwise specifically indicated (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Values are presented as mean ± SD.

3. Results

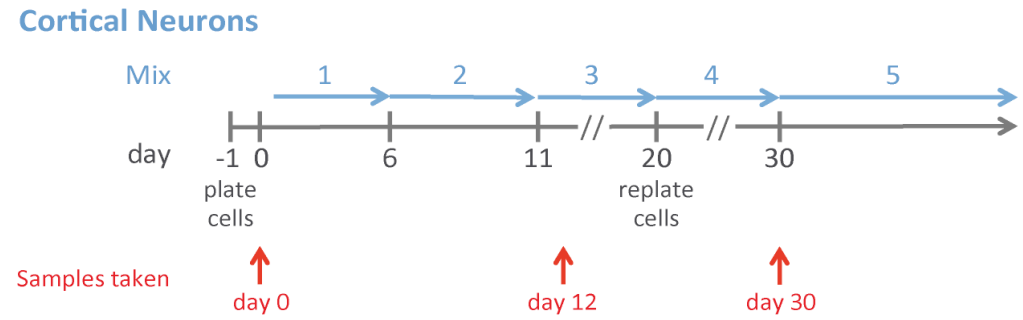
Recent studies suggest the active roles of endogenous elements in cellular gene regulation, immunological harmony, neurological homeostasis and many more (Geis and Goff; 2020, Göke et al; 2015, Douville and Nath; 2017, Küry et al; 2018, Deniz et al; 2020). The main aim of this study was to analyze in detail the functional role of human endogenous retrovirus, in particular HERV-K(HML-2) in cortical neuronal differentiation and human forebrain development.

3.1 Endogenous retroviral families are differentially regulated during early neuronal development.

Endogenous retroviruses are tightly regulated in our genome. However, they play crucial roles in orchestrating critical cellular mechanisms during early development. In order to investigate whether there are differences in HERV expression levels in distinct neuronal subtypes, we differentiated hESCs into cortical and dopaminergic neurons. **Figure 3.1A** and **Figure 3.2A** depict the individual complex protocols for differentiation into the two lineages in which we investigated the expression of several HERV groups at various time points of differentiation. Numerous markers were explored to control for proper differentiation into cortical neurons **(Figure 3.1B)** or dopaminergic **(Figure 3.2B)**. The differentiating neurons were harvested at indicated time points for total RNA. Transcript levels of different neuronal markers were analyzed to establish the proper progression of the cultured cells to the directed neuronal lineage. To monitor efficient differentiation into cortical neurons, the expression of FOXG1 (forebrain marker), MAP2 (neuronal marker), EMX2 (dorsal marker) and KI67 (proliferation marker) were analyzed **(Figure 3.1B).** To follow efficient differentiation into dopaminergic neurons, the expression of the floor plate marker FOXA2, the midbrain marker LMX1A, the tyrosine hydroxylase TH and the transcription factor NURR1 were analyzed. As expected, all markers showed an increase in expression over the course of differentiation **(Figure 3.2B).** FOXG1 transcript levels show steady increase throughout the differentiation timeline, suggesting the apparent fate of the directed differentiation into cortical neurons. MAP2 transcript levels are increased as differentiation progresses. This suggests the enrichment of neuronal cells in the culture. The increase of EMX2 transcripts in the later time points of the experiment provides further confirmation that the differentiating neurons are directed towards the cortical lineage.

The transcript levels of Ki67 goes down after day 0 of differentiation, suggesting that the pluripotent cells are pushed towards neuronal lineage, commencing the development of neural progenitors and ceasing of cell proliferation.

A



B

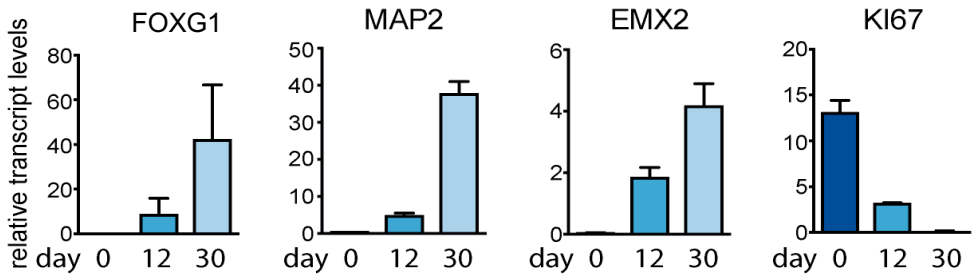
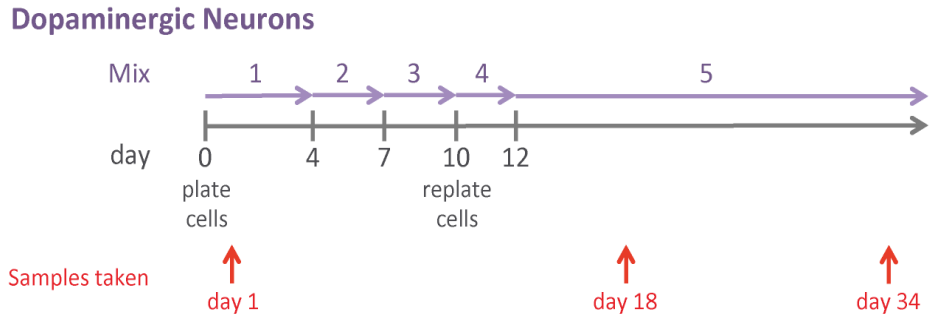


Figure 3.1-Differentiation of human embryonic stem cells (hES) into cortical neuronal lineage. *Panel A- Schematic showing the protocol for directed differentiation into cortical neuronal lineage. Panel B- Total RNA was isolated from hES cells differentiating into cortical neurons and subjected to a qRT-PCR for specific markers.*

The FOXA2 transcription factor transcript levels show steady increase throughout the differentiation timeline, suggesting the apparent fate of the directed differentiation into midbrain dopaminergic neurons. The LIM- homeodomain transcription factor LMXA1 plays a critical role in the development and survival of midbrain dopaminergic neurons. The transcript levels of LMXA1 increases throughout the differentiation timeline, suggesting the direction of the differentiation into midbrain dopaminergic neurons. The increased transcript levels of TH suggest the quality and neurotransmission ability of the generated midbrain dopaminergic neurons through directed differentiation. The mRNA transcripts of the orphan nuclear receptor transcription factor, Nurr-I which is responsible for the proliferation and development of dopaminergic neurons is increased, suggesting that the matured neurons at the later time points of differentiation, day 60 is in mDA lineage. The transcript levels of the pluripotency marker,

OCT-4 remains high in the start of differentiation and decreases as the differentiation progresses.

A



B

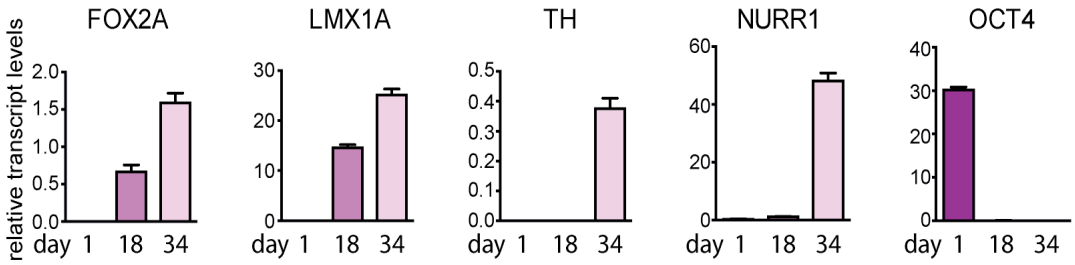


Figure 3.2 Differentiation of human embryonic stem cells (hES) into dopaminergic neuronal lineage. *Panel A- Schematic showing the protocol for directed differentiation into dopaminergic neuronal lineage. Panel B- Total RNA was isolated from hES cells differentiating into dopaminergic neurons and subjected to a qRT-PCR for specific markers.*

To analyze the expression pattern of different HERV families during neuronal differentiation, hES cells were differentiated into cortical and dopaminergic neuronal lineages. The expression levels of HERV-K (HML2), HERV-H and HERV-W was monitored over a period of 60 days of differentiation. Excitingly, in the course of differentiation the expression of various HERV groups changed **(Figure 3.3 and Figure 3.4)**, when compared to the initial state. This fact indicates that HERVs may be involved in the process of neuronal differentiation. Of important note, these changes were not seen for all HERVs, thereby proving a degree of specificity of the observed effects towards certain HERV groups and not an overall transcriptional phenomenon. Interestingly, only HERV-K(HML-2) behaved differently in its expression when differentiated into dopaminergic or cortical neurons. While HERV-K(HML-2) is strongly downregulated in dopaminergic neurons, it is upregulated in cortical neurons during differentiation (Refer to

HERV-K(HML-2) panel in **Figure 3.3 and Figure 3.4**). Altogether, it is apparent that the expression pattern of distinct HERVs change in the course of neuronal differentiation.

As seen in **Figure 3.3**, in the case of cortical neurons generated from hES cells, it was observed that the transcript levels of HERV-K(HML2) family of endogenous retroviruses were completely silenced at the earlier time points of differentiation but expressed in later stages.

The HERV-H transcript levels were high at day 0 during the start of differentiation, but then there was practically no detection of the transcripts in later stages. HERV-H is known to establish the mark for pluripotency and provides binding sites for key transcription factors to establish pluripotency in cells. Thus, loss of HERV-H leads to differentiation of stem cells.

The HERV-W transcript levels were not affected by the differentiation.

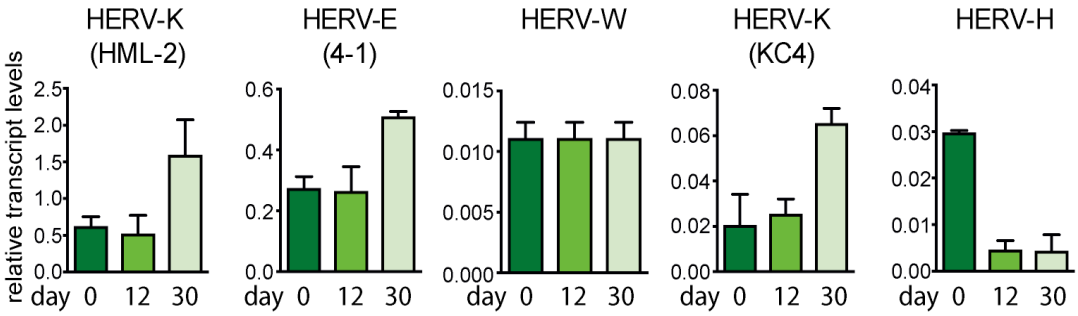


Figure 3.3-Differential regulation of HERV families during cortical neuron differentiation. *Total RNA was isolated from hES cells differentiating into cortical neurons and subjected to a qRT-PCR of different HERV families*

As shown in **Figure 3.4**, in the case of dopaminergic neurons generated from hES cells, it was observed that the transcript levels of HERV-K(HML2) family of endogenous retroviruses were active at the earlier time points of differentiation but silenced in later stages.

The HERV-H transcript levels were high at day 0 during the start of differentiation, but then there was practically no detection of the transcripts in later stages as the cells proceed for differentiation from the naïve stage established by HERV-H.

The HERV-W transcript levels were not affected by the differentiation.

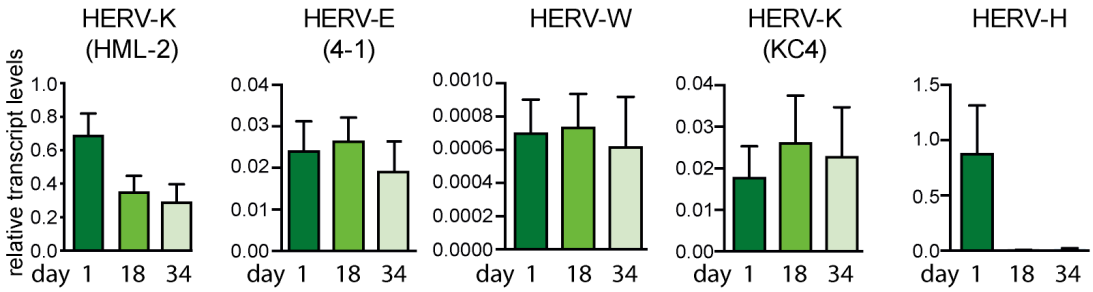


Figure 3.4-Differential regulation of HERV families during dopaminergic neuron differentiation. *Total RNA was isolated from hES cells differentiating into dopaminergic neurons and subjected to a qRT-PCR of different HERV families*

3.2 Establishment of CRISPR mediated activation (CRISPRa) and CRISPR mediated interference (CRISPRi) system of Human Endogenous Retroviruses (HERVs) in human embryonic stem cells.

As shown in the previous section, there is a differential regulation of HERV-K(HML2) family of endogenous retroviruses in the early stages of neuronal differentiation. This observation prompted us to investigate more on the influence of HERV-K(HML-2) elements in the context of early neuronal development.

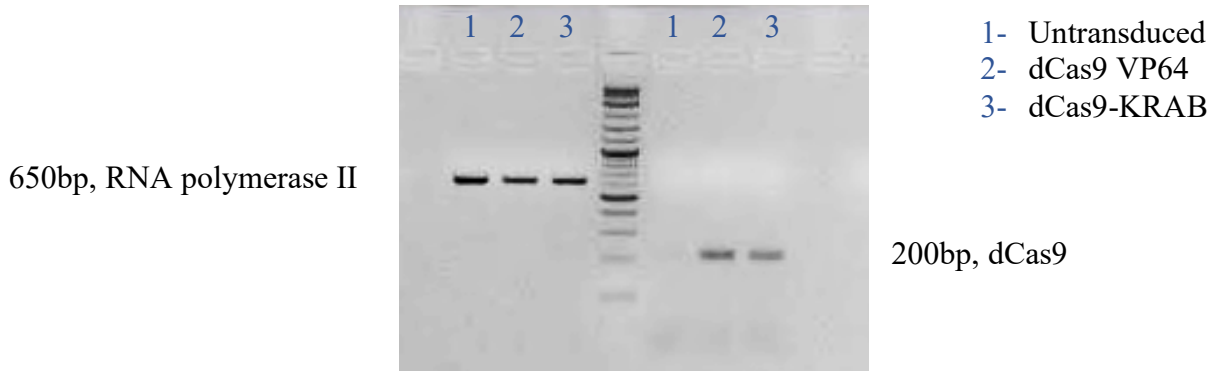
In order to manipulate the mRNA levels of HERV-K(HML-2) LTRs in human embryonic stem cells, we used the novel CRISPR activation and CRISPR interference for transcriptional activation and repression, respectively. The systems were first established in HEK293T cells, to avoid screening and standardization of protocols in stem cells.

3.2.1 dCas9 fusion constructs can be successfully integrated in hES cells for CRISPRa, and CRISPRi

As a first step, hES cells were lentivirally transduced with the pHAGE EF1alpha dCas9-VP64 and pHAGE EF1alpha dCas9-KRAB plasmids to establish cell lines which stably express the dCas9 fusion proteins for CRISPRa and CRISPRi. The lentiviral transduction is explained in detail in section 2.2.1.3.

The integration of the dCas9 gene into the genome of hES cells were checked by a genomic DNA PCR. As depicted in **Figure 3.5A**, stable integration of dCas9 was observed in the transduced cells but not in the untransduced hES cells respectively. The dCas9 gene was observed at a fragment size of 200bp. RNA polymerase II was used as an internal control for the PCR experiment which appeared around a fragment size of 650bp.

A



B

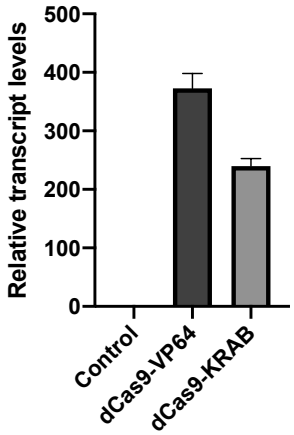


Figure 3.5-dCas9 integration in hES cells. *A) Genomic DNA was isolated from hES cells transduced with dCas9-VP64 and dCas9-KRAB fusion constructs and subjected to a Polymerase Chain reaction (PCR) experiment. B) qRT-PCR showing the transcript levels of dCas9. Data shown are result of 3 independent sets of experiments (n=3).*

Total RNA was isolated from the transduced cells and the cDNA was synthesized and subjected to a qRT-PCR to check for the mRNA transcript levels of dCas9 gene after transduction. A global housekeeping gene, RNA polymerase II was used as the reference gene to calculate the relative expression levels as explained in section 2.2.4.7.

As shown in **Figure 3.5B**, increased transcript levels of dCas9 gene were observed in the transduced cells but not in the untransduced hES cells, suggesting a successful transcription of the dCas9 fusion constructs in hES cells.

3.2.2 Integration of dCas9 fusion constructs in hES cells does not change the pluripotency levels of the cells

To analyze the effect of dCas9 fusion constructs on the pluripotency levels of hES cells, an immunofluorescence staining was performed for the expression of the pluripotency markers Nanog and OCT-4 on the H9 embryonic stem cell line which stably express dCas9-VP64 or dCas9-KRAB through an EF1 alpha promoter.

The protein expression of Nanog and OCT-4 in the dCas9-VP64 and dCas9-KRAB transduced hES cells is comparable to normal pluripotent untransduced hES cells **(Figure 3.6A).**

To further confirm the observation from the immunofluorescence staining of the effect of dCas9 fusion constructs on the pluripotency levels of hES cells, a qRT-PCR was performed to analyze the transcript levels of two early pluripotency markers, Nanog and OCT-4 **(Figure 3.6B)**.

In **Figure 3.6B**, the dCas9-VP64 and dCas9-KRAB transduced hES cells shows similar levels of mRNA expression of Nanog and OCT-4 compared to normal pluripotent untransduced hES cells. These results suggest that addition and integration of fusion constructs into the hES cell genome does not change the pluripotency levels of the cells.

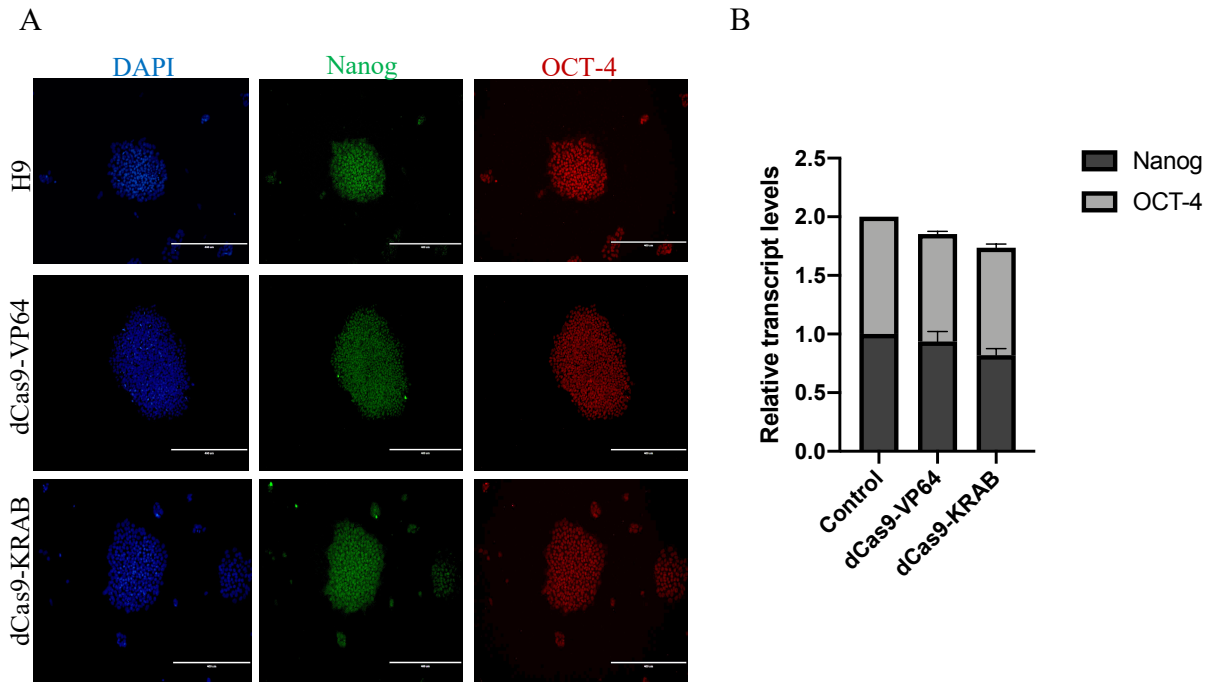


Figure 3.6-dCas9 integration does not change the pluripotency of cells. *A) Immunofluorescence staining of H9, H9dCas9-VP64 and H9dCas9-KRAB cells for Nanog and OCT-4. B) qRT-PCR showing the transcript levels of Nanog and OCT-4 in H9, H9dCas9-VP64 and H9dCas9-KRAB cells (n=3).*

3.2.3 HERV-K(HML-2) elements can be activated and repressed in hES cells using CRISPRa and CRISPRi

hES dCas9-VP64 and hES dCas9-KRAB cell lines which stably expresses the dCas9 fusion constructs for activation and repression of transcription of desired genes were successfully established. Single guide RNAs targeting HERV-K(HML-2) LTRs were transduced into the stable cell lines expressing the dCas9 fusion constructs and checked for the activation and

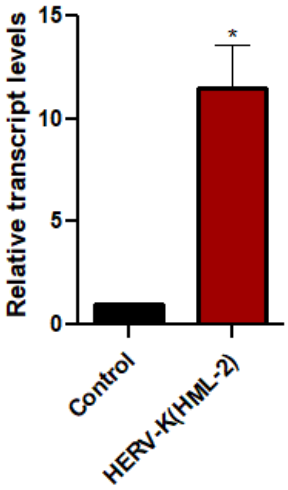
repression of transcription of HERV-K(HML-2) LTRs. (For cloning of sgRNAs and transduction, see section 2 2.4.1 and 2.2.1.3).

cDNA was synthesized from the total RNA isolated from the transduced cells and the was subjected to a qRT-PCR to analyze mRNA transcript levels of HERV-K(HML-2) LTRs after transduction. A global housekeeping gene, RNA polymerase II was used as the reference gene to calculate the relative expression levels as explained in section 2.2.4.7.

As shown in **Figure 3.7A**, transduction of sgRNAs into the hES dCas9-VP64 cell lines resulted in around 10-fold increase in the transcripts of HERV-K(HML-2) LTRs in hES cells, when compared to control gRNA transduction, suggesting that CRISPR activation system can be successfully utilized to activate HERV-K(HML-2) transcription in hES cells.

As shown in **Figure 3.7B**, transduction of HERV-K(HML-2) sgRNAs into hES dCas9-KRAB cell lines resulted in more than 50% repression of transcript levels of HERV-K(HML-2) in comparison with control gRNA transduction, proving that CRISPR interference system can be successfully utilized to repress HERV-K(HML-2) transcription in hES cells.

A



B

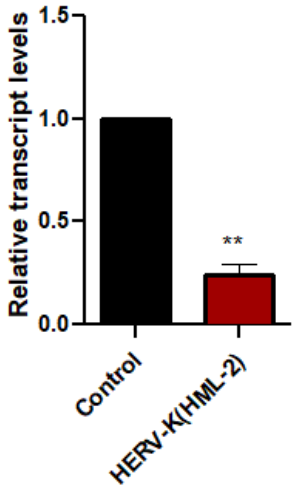


Figure 3.7 CRISPR regulation of HERV-K(HML-2) *A) CRISPR mediated activation of HERV-K(HML-2) in hESCs. B) CRISPR mediated repression of HERV-K(HML-2) in hESCs. Figure shows results of 3 independent sets of experiments (n=3).*

3.3 Activation of HERV-K(HML-2) reveals impaired cortical differentiation pattern in human embryonic stem cells

Towards understanding the influence of HERV-K(HML-2) activation on early neural development, HERV-K(HML-2) activated human embryonic stem cells were differentiated into cortical neurons for a period of 60 days as described in section 2.2.2.

3.3.1 MAP2 and Synapsin-I levels are decreased during HERV-K(HML-2) activation in cortical neurons

To analyze the impact of HERV-K(HML-2) activation on cortical neuron differentiation, an immunofluorescence assay was performed for the global neuronal marker, MAP2 and a pre-synaptic marker, Synapsin-I proteins. Differentiating cortical neurons were stained for MAP2 and Synapsin-I at time points day 41 and day 60 and imaged for the protein expression **(Figure 3.8A and 3.8B)**. Interestingly, the expression level of MAP2 in HERV-K(HML-2) activated cortical neurons were considerably lesser compared to that of the control neurons. There were lesser branching of the neurons and the neurite length appeared to be shorter than the normal neurons. Similarly, in the case of Synapsin-I, HERV-K(HML-2) activation caused overall decrease in the expression levels when compared to normal controls.

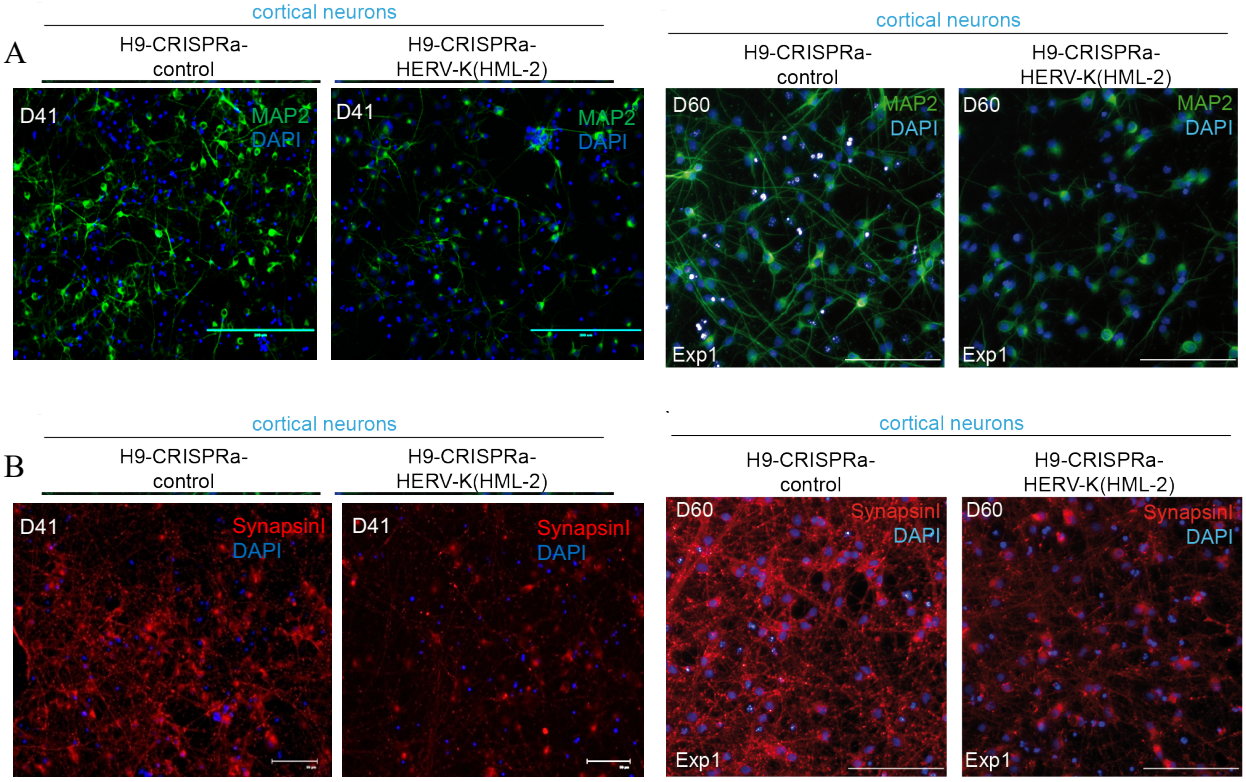


Figure 3.8 Decreased MAP2 and Synapsin-I upon HERV-K(HML-2) LTR activation. *Immunofluorescence staining showing the MAP2 (Panel A) and Synapsin-I (Panel B) expression in cortical neurons generated from control and HERV-K(HML-2) activated human embryonic stem cells at day 41 and day 60. Scale- 100uM.*

To further confirm the observations of decreased MAP2 and Synapsin-I expression levels upon activation of HERV-K(HML-2) in cortical neuron differentiation, a qRT-PCR was performed on RNA isolated from different time points, day 0,18, 27, 41 and 60 of cortical neuron differentiation to analyse the transcript levels of neuronal markers MAP2 and presynaptic marker, Synapsin-I.

As shown in **Figure 3.9A**, the overall MAP2 levels of HERV-K(HML-2) activated cortical neurons are significantly reduced when compared to the control cortical neurons. Similarly, the relative expression levels of Synapsin-I **(Figure 3.9B)** also decreased when there is activation of HERV-K(HML-2) LTRs. This suggest that activation of HERV-K(HML-2) endogenous elements results in an impaired cortical neuron differentiation pattern.

As shown in **Figure 3.9C**, the Cas9 expression levels from the CRISPRa system remains the same and the HERV-K(HML-2) levels **(Figure 3.9D)** in activated cells are still increased and maintained throughout the time period of differentiation. This further confirms that activation of HERV-K(HML-2) has long standing impacts on cortical differentiation.

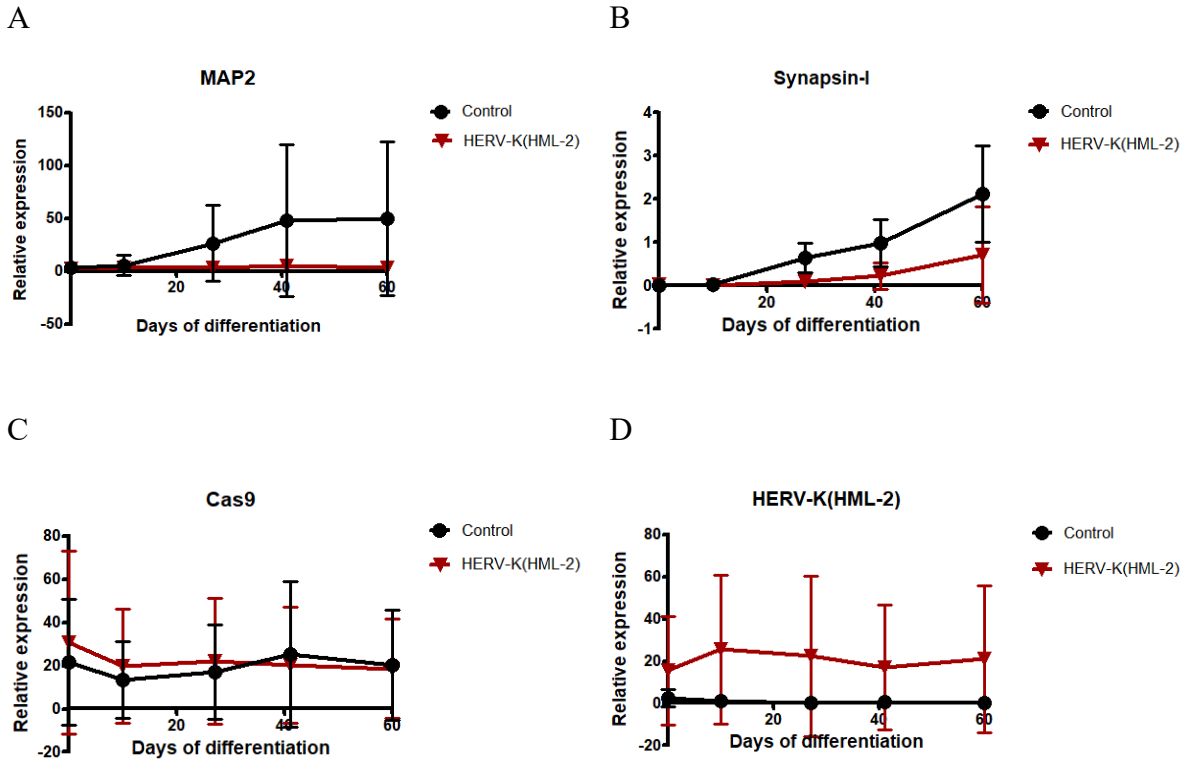


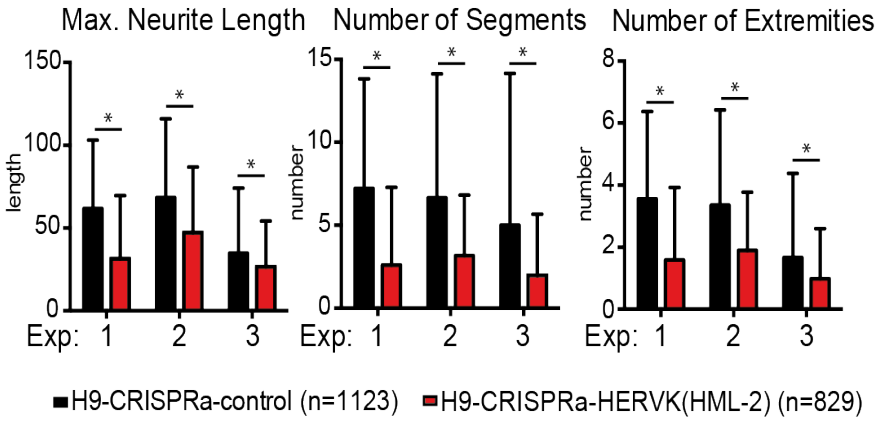
Figure 3.9 – Decreased MAP2 and Synapsin-I levels upon HERV-K(HML-2) LTR activation. *qRT-PCR experiment showing the relative expression levels of A) MAP2, B) Synapsin-I, C) Cas9 and D) HERV-K(HML-2) in cortical neurons at different time point relative to RNA polymerase-II values. Data shown are results of three independent experiments (n=3).*

During differentiation, along with the expression levels of MAP2 and Synapsin-I, the overall morphology of the differentiating neurons was also studied using high content image analysis using the Columbus software to quantify the MAP2 and Synapsin-I stainings, as shown in section 2.2.5.4. As depicted in **Figure 3.10A**, at day 60, the HERV-K(HML-2) activated

cortical neurons showed significant reduction in number of segments of neurons, number of extremities observed and maximum neurite length of the neurons.

As shown in **Figure 3.10B**, the total number of spots and spot intensity of the Synapsin-I staining was drastically decreased in HERV-K(HML-2) activated neurons suggesting that activation of HERV-K(HML-2) results in notable morphological changes in developing neurons.

A



B

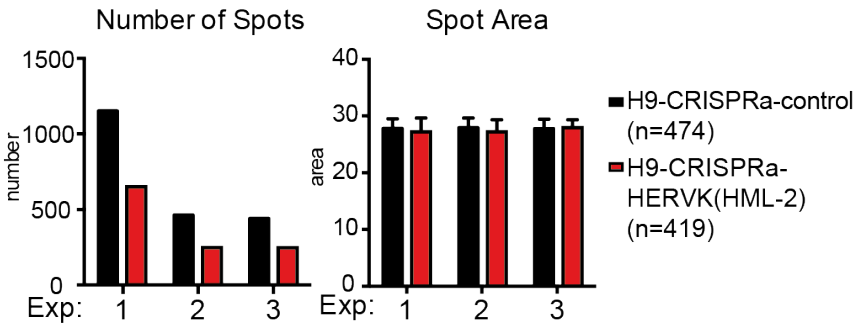


Figure 3.10 - Morphology of cortical neurons changes during HERV-K(HML-2) activation. *Quantification using high content image analysis showing Neurite length as well as number of segments and extremities during MAP2 staining and spot number and spot area during Synapsin-I staining in HERV-K(HML-2) activated and control cortical neurons. Three independent differentiations were quantified.*

3.3.2 Activation of HERV-K(HML-2) reduces the functionality of cortical neurons

To understand the impact of HERV-K(HML-2) activation on the functionality of cortical neuron, a calcium imaging was performed on the developing cortical neurons from control and HERV-K(HML-2) activated embryonic stem cells, as explained in section 2.5.5.5. At day 45 of the differentiation, cells were treated with the calcium dye Fura-2 and imaged for calcium levels upon stimulation **(Figure 3.11 A and Figure 3.11 B).** A glutamate pulse generates a calcium response, which results in a calcium peak. (Tchieu et al., 2019). An increase in Ca^{2+}

levels were detected in control cortical neurons, but the calcium response, as determined by Fura-2 fluorescence peak, was weaker in neurons, where HERV-K(HML-2) levels were elevated (**Figure 3.11A**). We quantified the strength of the calcium response at the time point 20 min (time point of main peak (T_{20}) and observed a significant reduction in calcium response in HERV-K(HML-2) activated cortical neurons compared to the control (**Figure 3.11B**), suggesting that activation of HERV-K(HML-2) impairs functionality of differentiated cortical neurons.

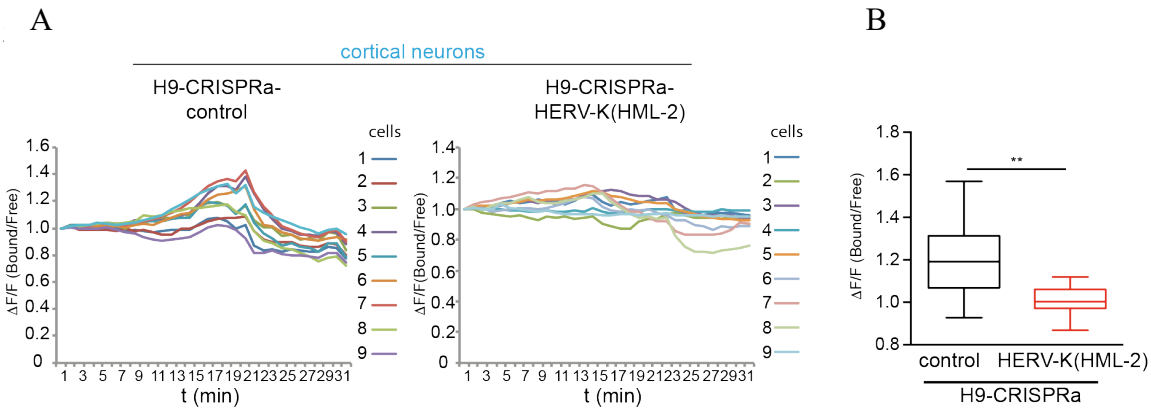


Figure 3.11 – Reduced functionality of cortical neurons upon HERV-K(HML-2) activation*. A) Calcium imaging in control and HERV-K(HML-2) activated cortical neurons at D45. B) Quantification of calcium response at T_{20} in control and HERV-K(HML-2) activated neurons. Two independent experiments were conducted and quantified (n-2).*

3.3. MAP2 and Synapsin-I levels are unaffected during HERV-K(HML-2) activation in dopaminergic neurons

Previous experiments suggested a decreased MAP2 and Synapsin-I levels upon HERV-K (HML-2) activation. Next step was to analyse the impact of HERV-K(HML-2) activation on dopaminergic neuron differentiation. To this end, HERV-K(HML-2) activated human embryonic stem cells were subjected to a directed differentiation into dopaminergic neuronal lineage and monitored over a period of 55 days. At day 55 of differentiation, an immunofluorescence assay was performed for the global neuronal marker, MAP2 and a pre-synaptic marker, Synapsin-I proteins. As shown in **Figure 3.12A**, the overall MAP2 levels of HERV-K(HML-2) activated dopaminergic neurons are comparable to that control dopaminergic neurons. Similarly, the relative expression levels of Synapsin-I are also similar to the control neurons when there is an activation of HERV-K(HML-2) LTRs **(Figure 3.12B).** This suggest that activation of HERV-K(HML-2) endogenous elements does not affect the

efficiency of dopaminergic neuron differentiation and it can be concluded that the observed phenotype of drastic reduction in overall MAP2 and Synapsin-I expression levels upon HERV-K(HML-2) activation seems to be cortical specific.

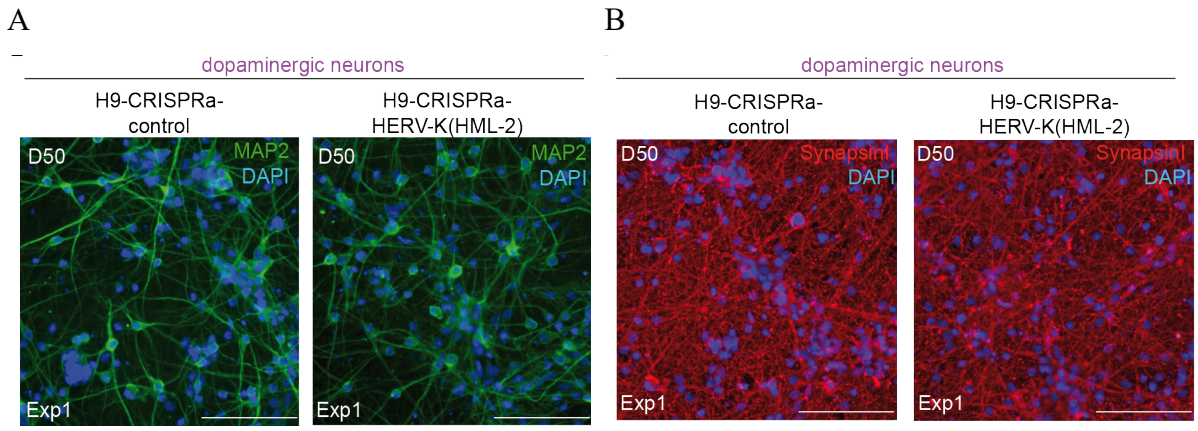


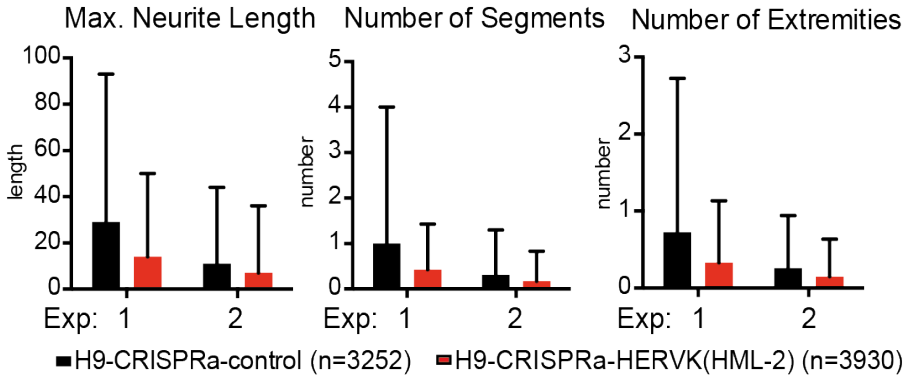
Figure 3.12- Dopaminergic neurons upon HERV-K(HML-2) LTR activation *Immunofluorescence staining showing the MAP2 (Panel A) and Synapsin-I (Panel B) expression in dopaminergic neurons generated from control and HERV-K(HML-2) activated human embryonic stem cells at day 50. Scale- 100uM.*

During the dopaminergic neuronal differentiation, along with the expression levels of the neuronal marker and pre-synaptic marker, the overall morphology of the differentiating neurons was also quantified and studied using high content image analysis using Columbus software on immunofluorescence stainings **(Figure 3.13A-B).**

As depicted in **Figure 3.13A**, at day 55 of two independent differentiation experiments, the HERV-K (HML-2) activated dopaminergic neurons stained for MAP2 expression levels MAP2 showed no significant reduction in number of segments of neurons, number of extremities observed and maximum neurite length of the neurons in comparison with the control differentiation.

As shown in **Figure 3.13B**, the total number of spots and spot intensity of the Synapsin-I staining was also similar in HERV-K (HML-2) activated neurons suggesting that activation of HERV-K(HML-2) has little or no impact on dopaminergic neurons.

A



B

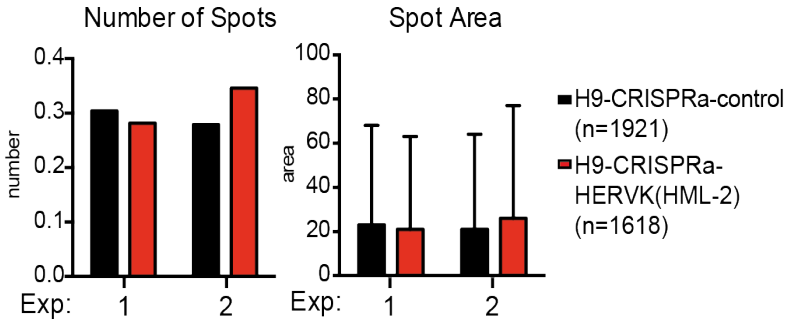


Figure 3.13- Morphology of dopaminergic neurons does not change during HERV-K(HML-2) activation. *Quantification using high content image analysis of Neurite length as well as number of segments and extremities during MAP2 staining (Panel A) and spot number and spot area during Synapsin-I staining (Panel B) in HERV-K(HML-2) activated and control cortical neurons. Two independent differentiations were quantified.*

3.3.4 MAP2 and Synapsin-I levels are unaffected during HERV-K(HML-2) repression in cortical neurons

From the above explained experiments, it was clear that an activation of HERV-K(HML-2) LTRs during cortical neuronal differentiation results in a drastic reduction in overall MAP2 as well as Synapsin-I expression levels. Thus, an obvious question of the effect of repression of HERV-K(HML-2) during cortical neuronal differentiation and its impact on the neuronal phenotype arises. To this end, a CRISPR interference system was generated as explained in section 3.2.3 and a directed differentiation into cortical neuron lineage was conducted along with control cells over a period of 60 days.

To understand the effect of repression of HERV-K(HML-2) LTRs on cortical neuron differentiation, an immunofluorescence staining was performed for MAP2 on differentiating cortical neurons at D41 and D60.

As shown in **Figure 3.14**, the overall MAP2 levels of HERV-K(HML-2) repressed cortical neurons are comparable to that of the expression levels of control cortical neurons. This suggest

that repression of HERV-K(HML-2) endogenous elements does not affect the efficiency of cortical neuron differentiation and it can be concluded that the observed phenotype of decreased MAP2 in cortical neuron differentiation occurs as a result of HERV-K (HML-2) activation.

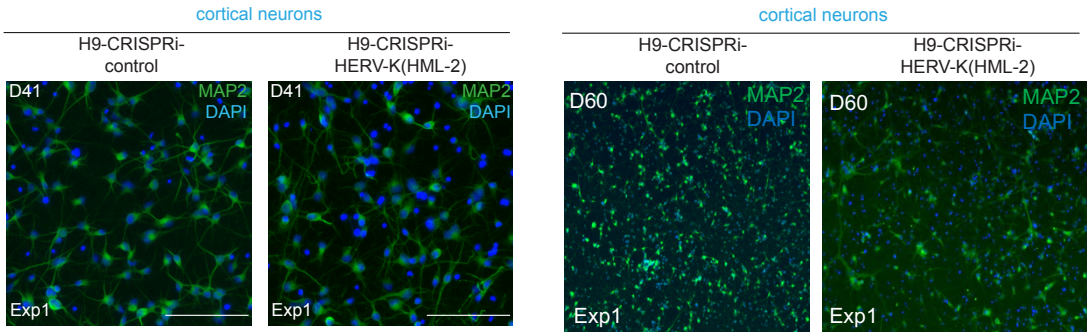


Figure 3.14-MAP2 expression is not affected in cortical neurons upon HERV-K(HML-2) repression. *Immunofluorescence staining showing the MAP2 expression in cortical neurons generated from control and HERV-K(HML-2) repressed human embryonic stem cells at day 41 and day 60. Scale- 100uM. Data shown are results of two independent differentiation experiments (n=2).*

To further confirm the observations from immunofluorescence staining on mRNA level, a quantitative real time PCR was performed with cDNA synthesized from specific time points day 0, 41 and 60 of differentiating cortical neurons in order to analyze the transcript levels of neuronal markers MAP2 **(Figure 3.15 A)** and Synapsin-I **(Figure 3.15 B).** There was no apparent change in the levels of MAP2 and Synapsin-I transcripts suggesting that HERV-K(HML-2) repression has no impact on cortical neuron differentiation, as shown in **Figure 3.15 A-B.**

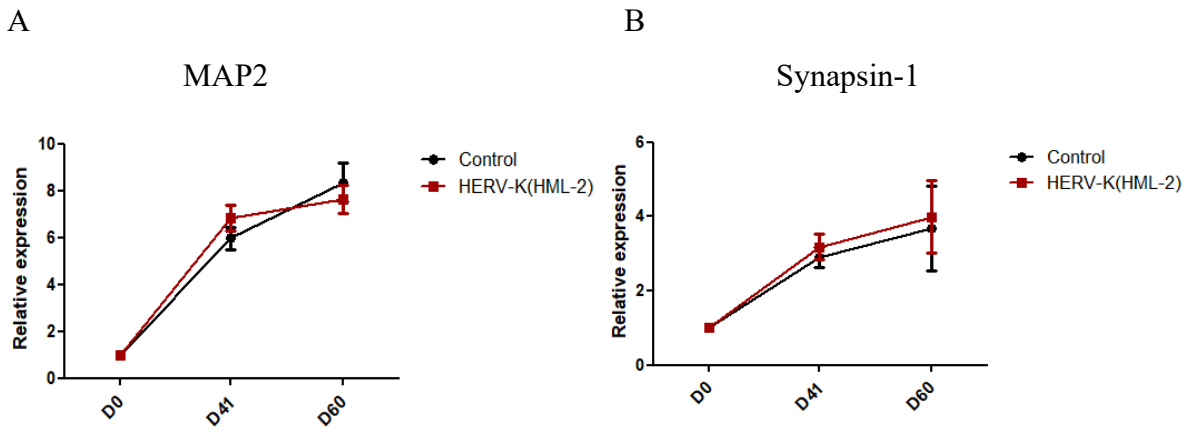


Figure 3.15- MAP2 and Synapsin-I expression is not affected in cortical neurons upon HERV-K(HML-2) repression. *qRT-PCR showing the MAP2 (Panel A) and Synapsin-I (Panel B) transcript levels in cortical neurons generated from control and HERV-K(HML-2) repressed human embryonic stem cells at*

day 41 and day 60 relative to RNA Polymerase II values. Data shown are results of two independent differentiation experiments (n=2).

During the cortical neuronal differentiation of HERV-K(HML-2) repressed stem cells, along with the expression levels of the neuronal marker and pre-synaptic marker, the overall morphology of the differentiating neurons was also quantified and studied using high content image analysis using Columbus software on immunofluorescence stainings.

As depicted in **Figure 3.16**, at day 60 of two independent differentiation experiments, the MAP2 staining of HERV-K(HML-2) repressed cortical neurons showed no significant reduction in number of segments of neurons, number of extremities observed and maximum neurite length of the neurons in comparison with the control differentiation.

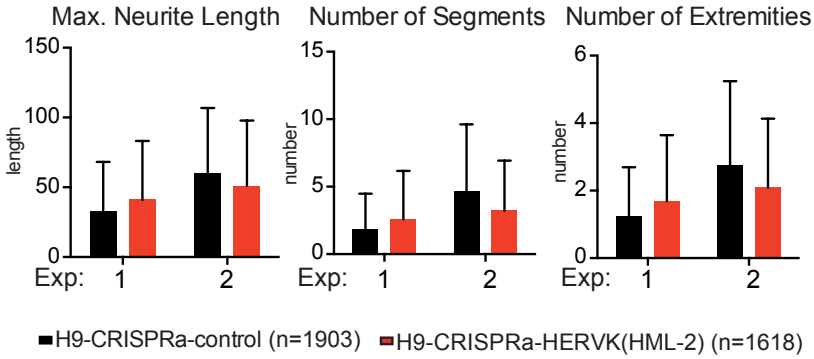


Figure 3.16-Morphology of cortical neurons does not change during HERV-K(HML-2) repression. *Quantification using high content image analysis of Neurite length as well as number of segments and extremities during MAP2 staining were quantified by high- content image analysis in HERV-K(HML-2) activated and control dopaminergic neurons. Two independent differentiations were quantified (n=2)*

3.4 Forebrain organoids from HERV-K(HML-2) activated embryonic stem cells reveal cortical malformation

To further elucidate the effect of transcriptional activation of HERV-K(HML-2) LTRs on forebrain development, 3D culture of control and HERV-K(HML-2) activated cells were performed to generate forebrain organoids, as described in section 2.3.3. As shown in **Figure 3.17**, a significant reduction in the overall diameter and size was observed in organoids generated from HERV-K(HML-2) activated cells in comparison with controls.

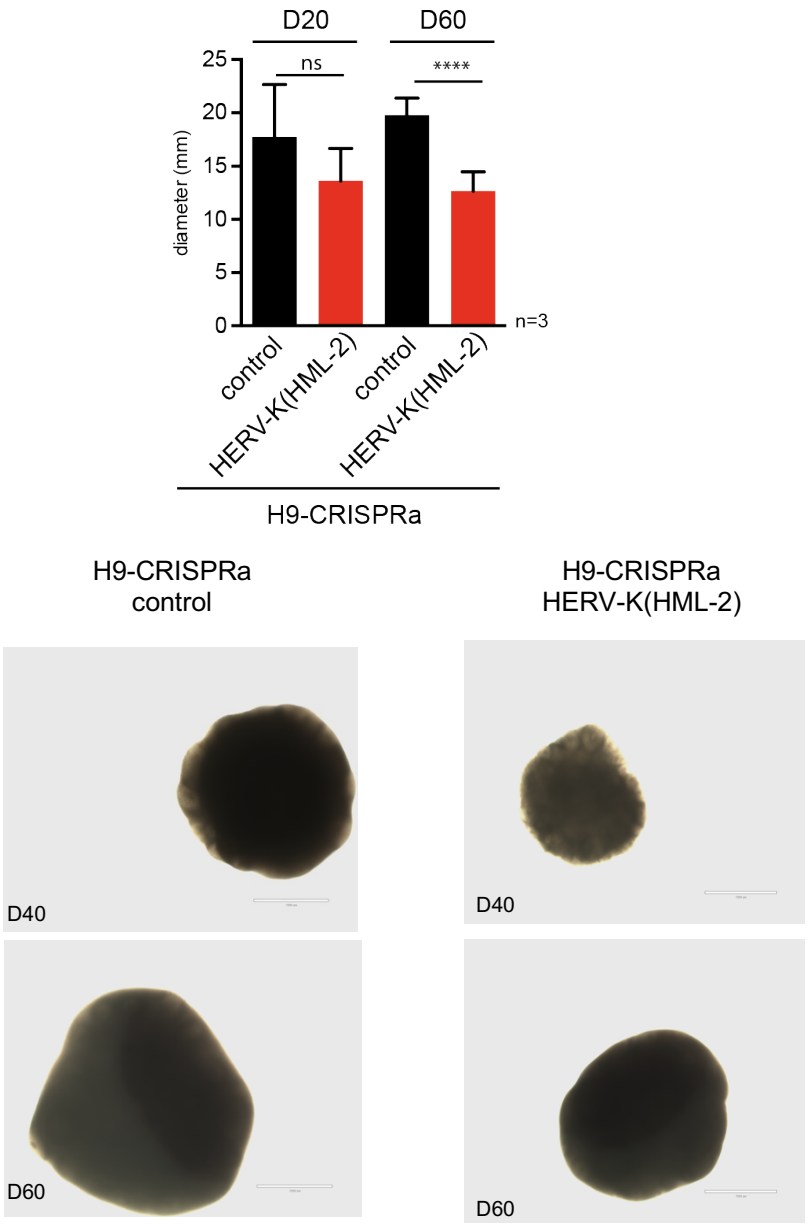


Figure 3.17 - Generation of forebrain organoids from HERV-K(HML-2) activated hES cells. *Forebrain organoids from HERV-K(HML-2) activated human embryonic stem cells showing significant difference in overall size and diameter in comparison with controls.*

Following the generation and culturing of organoids until day 60, the laminar brain patterning was analyzed using different markers specific for brain layers. In this context, SOX2 and PAX6 was used to stain for the pluripotent and proliferating ventricular zone of the organoids, TBR2, CTIP2, TBR1 and SATB2 markers were used to represent the layer formation of the brain in the order of first formed layers stained by TBR2 to deep matured layers stained by CTIP2

TBR1 and SATB2. To further investigate the radial projection of the neurons from the growth cone, Vimentin staining was performed.

As shown in **Figure 3.18A and 3.18B**, there is an unorganized and diffused distribution of CTIP2+ and TBR1+ neurons in HERV-K(HML-2) activated organoid sections at day 40 and day 60 in comparison with the controls where the CTIP2+ and TBR1+ layers were distributed in a sharp organized fashion.

Interestingly, activation of HERV-K(HML-2) transcription in the organoids had less or no impact in the organization and distribution of the SOX2+ and PAX6+ neural progenitors, as shown in **Figure 3.17A and 3.17B**.

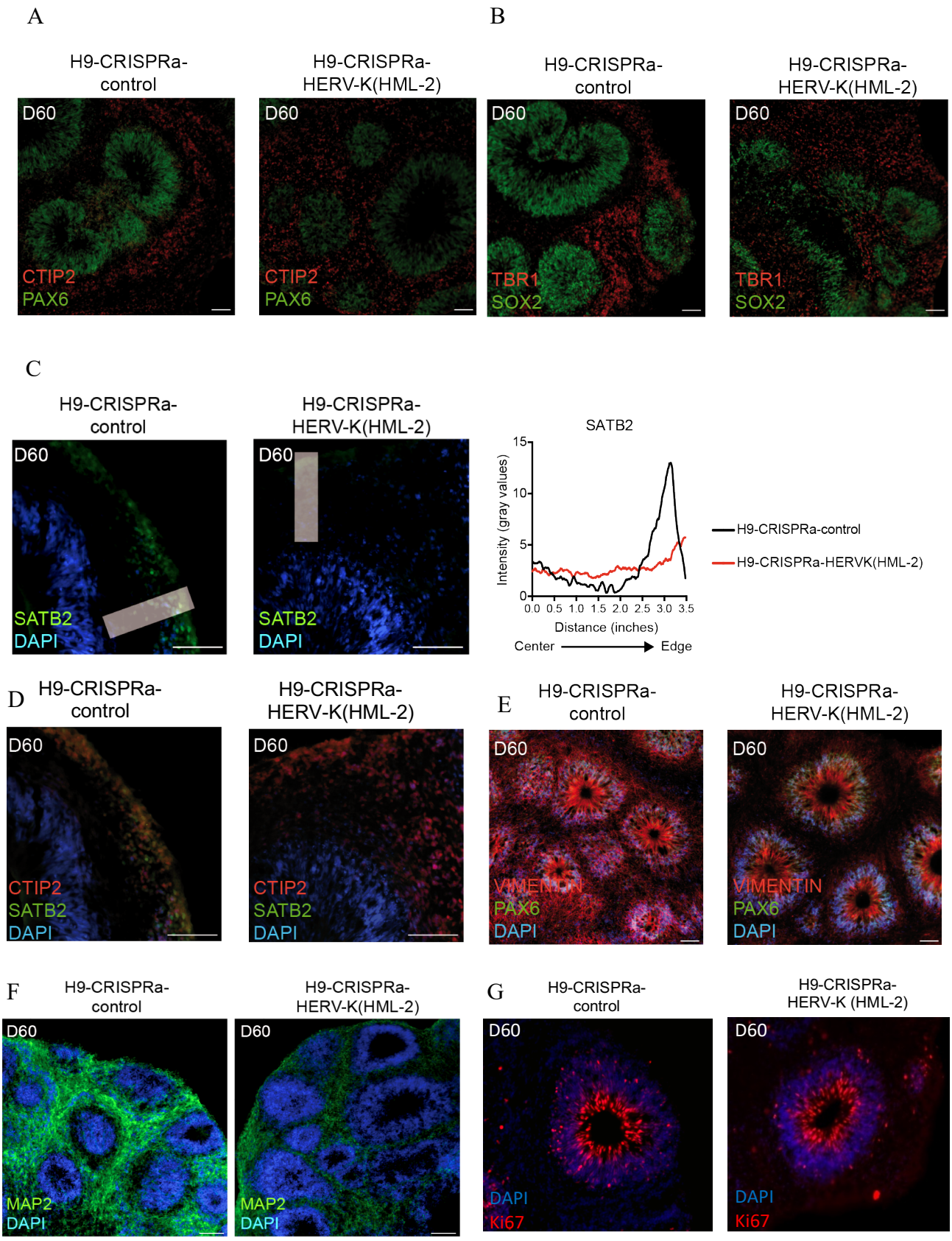
The late born neural layer of SATB2+ neurons formed a perfect layer in the control organoids but was completely absent in the HERV-K(HML-2) activated organoids. It could be suggesting either a delayed or a wrong developmental pattern of the brain upon HERV-K(HML-2) transcription activation, as shown in **Figure 3.18C.**

Vimentin marks the epithelial to mesenchymal transition (EMT) of neuroepithelia to radial glial cells (RG). Stronger vimentin staining with polarized morphology and basal processes, branched basal end feet was observed in control organoids in comparison with HERV-K (HML-2) activated organoids, as shown in **Figure 3.18E.**

MAP2 is a ubiquitous neuronal marker which stains for neuronal cells and plasticity. A decrease in the overall MAP2+ neurons were observed in HERV-K(HML-2) activated organoids in comparison with the control organoids, as shown in **Figure 3.18F**, suggesting that there is a reduced neuronal profile upon HERV-K(HML-2) transcriptional activation.

Ki67 is a marker which stains for the proliferation of cells. Overall similar Ki67+ neurons were observed in HERV-K(HML-2) activated organoids in comparison with the control organoids, as shown in **Figure 3.18G**, suggesting that there is a no proliferation differences upon HERV-K(HML-2) transcriptional activation.

A Propidium Iodide (PI) staining is widely used to detect viable, apoptotic and necrotic cells and Caspase 3 is a popular death protease of the intrinsic apoptotic pathway. Overall, no differences were observed in HERV-K(HML-2) activated organoids in comparison with the control organoids, as shown in **Figure 3.18H and 3.18I**, when stained with PI and Caspase3, suggesting that there is no proliferation differences and cell death happening in the organoids upon HERV-K(HML-2) transcriptional activation.


A
H9-CRISPRa-control
H9-CRISPRa-HERV-K(HML-2)
D60
CTIP2
PAX6
B
H9-CRISPRa-control
H9-CRISPRa-HERV-K(HML-2)
D60
TBR1
SOX2
C
H9-CRISPRa-control
H9-CRISPRa-HERV-K(HML-2)
D60
SATB2
DAPI
SATB2
Intensity (gray values)
Distance (inches)
Center
Edge
H9-CRISPRa-control
H9-CRISPRa-HERVK(HML-2)
D
H9-CRISPRa-control
H9-CRISPRa-HERV-K(HML-2)
D60
CTIP2
SATB2
DAPI
E
H9-CRISPRa-control
H9-CRISPRa-HERV-K(HML-2)
D60
VIMENTIN
PAX6
DAPI
F
H9-CRISPRa-control
H9-CRISPRa-HERV-K(HML-2)
D60
MAP2
DAPI
G
H9-CRISPRa-control
H9-CRISPRa-HERV-K (HML-2)
D60
DAPI
Ki67

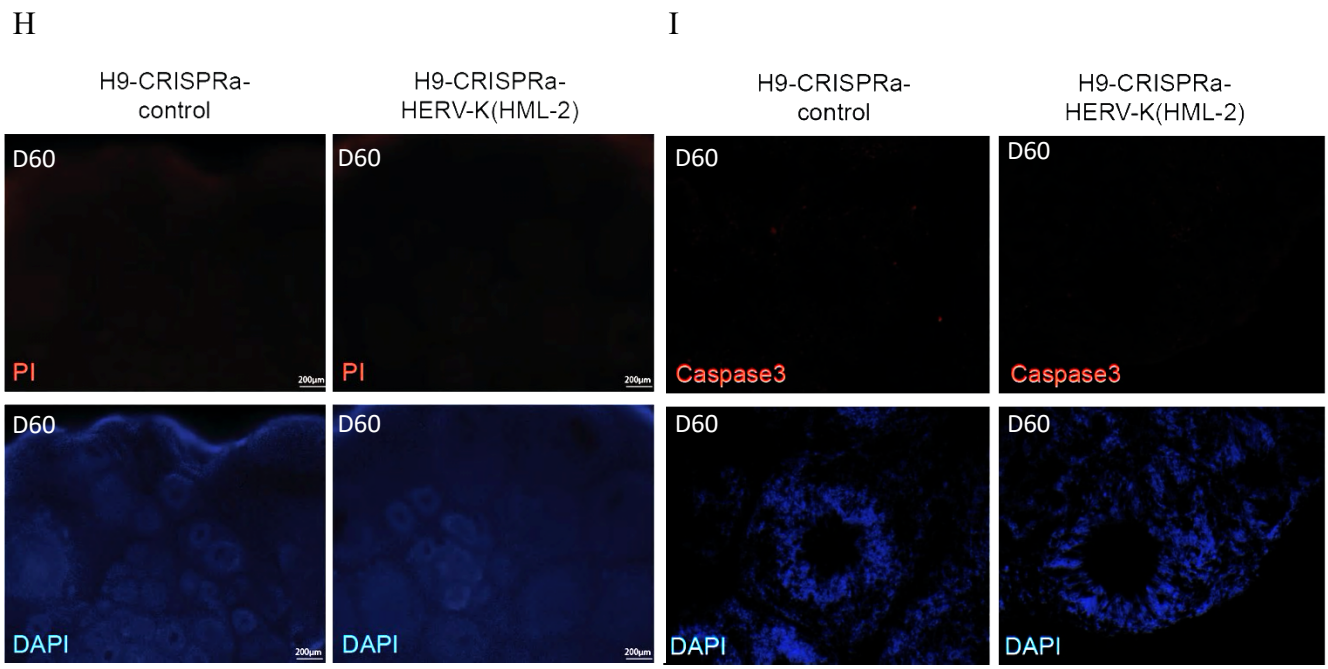


Figure 3.18- CRISPR activation of HERV-K(HML-2) impairs organization of brain layers in forebrain organoids.

Control and HERV-K(HML-2) activated forebrain organoids at day 60 was sectioned and stained for different brain layer markers, CTIP2 (Panel A), TBR1 (Panel B), SATB2 (Panel C), CTIP2 and SATB2 (Panel D), Vimentin (Panel E), MAP2 (Panel F), Ki67 (Panel G), PI (Panel H) and Caspase 3 (Panel I).

3.5 Establishment of an inducible CRISPR activation and Repression system for HERV-K(HML-2) LTRs

So far, the experiments conducted on human embryonic stem cells revealed that the CRISPR activation of HERV-K(HML-2) LTRs resulted in severe impairment of cortical differentiation and development. In order to decipher the specific stage of cortical differentiation influenced by HERV-K(HML-2) LTR transcriptional activation, an inducible CRISPR cell line for timely induction of HERV-K (HML-2) transcription was generated as mentioned below.

3.5.1 dCas9 fusion constructs can be successfully integrated in hES cells for inducible CRISPRa and inducible CRISPRi

For generating the inducible system of CRISPR activation and interference, named as inducible CRISPR activation (iCRISPRa) and inducible CRISPR interference (iCRISPRi), lentiviral transduction of hES cells were done using pHAGE TRE dCas9-VP64 and pHAGE TRE dCas9-KRAB plasmids. The lentiviral transduction is explained in detail in section 2.2.1.3.

As explained previously, the integration of dCas9 gene into the genome of human embryonic stem cells were verified using a genomic DNA PCR using the genomic DNA of hES cells which were subjected to lentiviral transduction of the dCas9 fusion constructs and subsequent selection using Geneticin antibiotic. As depicted in **Figure 3.19A**, stable integration of dCas9

was observed in the transduced cells but not in the untransduced hES cells respectively. The dCas9 gene was observed at a fragment size of 200bp. RNA polymerase II was used as an internal control for the PCR experiment which appeared around a fragment size of 650bp.

To further confirm the successful transduction of dCas9 in hES cells, total RNA was isolated from the transduced cells and the cDNA was synthesized and subjected to a qRT-PCR to check for the mRNA transcript levels of dCas9 gene after transduction **(Figure 3.19B).** The transduced cells were treated with doxycycline for 72 hours to induce the expression of dCas9 through the Tetracycline Response Element, prior to proceeding for RNA isolation. A global housekeeping gene, RNA polymerase II was used as the reference gene to calculate the relative expression levels, as explained in section 2.2.4.7.

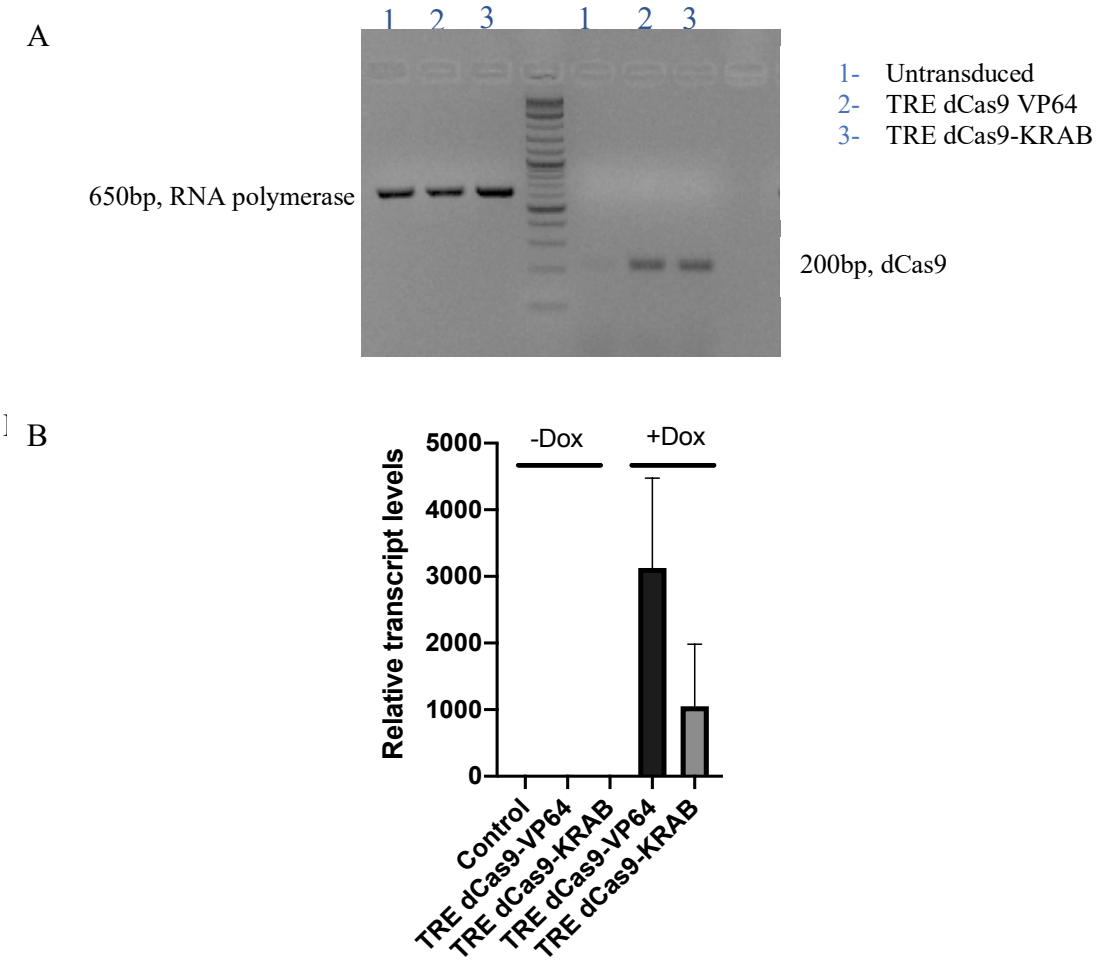


Figure 3.19- dCas9 integration in hES cells. *A) Genomic DNA was isolated from hES cells transduced with dCas9-TREVP64 and dCas9-TREKRAB fusion constructs and subjected to a Polymerase Chain reaction (PCR) experiment. B) qRT-PCR showing the transcript levels of dCas9 upon induction with doxycycline. Data shown are result of 3 independent sets of experiments (n=3).*

3.5.2 Integration of dCas9 fusion constructs in hES cells does not change the pluripotency levels

To analyze the effect of dCas9 fusion constructs on the pluripotency levels of hES cells, a qRT-PCR was performed to analyze the transcript levels of two early pluripotency markers, Nanog and OCT-4 on the H9 embryonic stem cell line which stably express TRE dCas9-VP64 or TRE dCas9-KRAB which expressed dCas9 through a Tetracycline Response Element when subjected to doxycycline treatment for a time period of 72 hours.

In **Figure 3.20** the TRE dCas9-VP64 and TRE dCas9-KRAB transduced hES cells shows similar levels of mRNA expression of Nanog and OCT-4 compared to normal pluripotent untransduced hES cells. These results suggest that addition and integration of fusion constructs into the hES cell genome does not change the pluripotency levels of the cells.

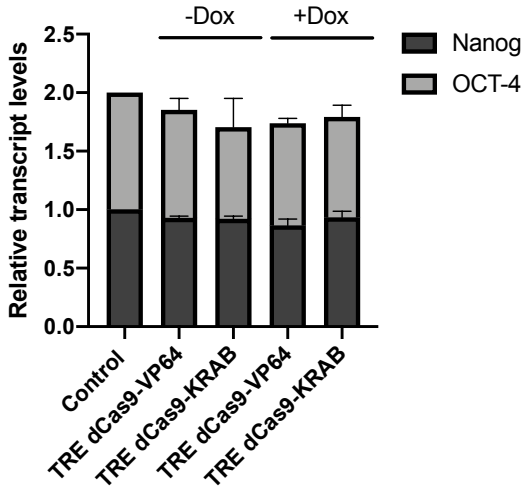


Figure 3.20- dCas9 integration does not change the pluripotency of cells. *qRT-PCR showing the transcript levels of Nanog and OCT-4 in H9, H9dCas9-VP64 and H9dCas9-KRAB cells (n=3).*

3.5.3 HERV-K(HML-2) elements can be activated and repressed in hiPS cells using iCRISPRa and iCRISPRi

Towards establishing a stable cells line through which activation and repression of HERV-K(HML-2) LTRs can be induced at desired time points, single guide RNAs targeting the HERV-K(HML-2) LTRs were transduced into hES TRE dCas9-VP64 and hES TRE dCas9-KRAB stable cell lines. (For cloning of sgRNAs and transduction, see section 2 2.4.1 and 2.2.1.3).

cDNA synthesized from the total RNA isolated from the transduced cells was subjected to a qRT-PCR to analyze mRNA transcript levels of HERV-K(HML-2) LTRs after transduction. The transduced cells were selected with Geneticin antibiotic and treated with doxycycline for 72 hours to induce the expression of dCas9 through the Tetracycline Response Element, prior

to proceeding for RNA isolation. A global housekeeping gene, RNA polymerase II was used as the reference gene to calculate the relative expression levels as explained in section 2.2.4.7. As depicted in **Figure 3.21A**, transduction of sgRNAs into the hES TREdCas9-VP64 cell lines and induction of dCas9 through a Tetracycline response Element with doxycycline resulted in around 8-fold increase in the transcripts of HERV-K(HML-2) LTRs in hES cells, when compared to control sgRNA transduction and without doxycycline induction, suggesting that inducible CRISPR activation system can be successfully utilized to activate HERV-K(HML-2) transcription in hES cells.

Transduction of HERV-K(HML-2) sgRNAs into hES TRE dCas9-KRAB cell lines and induction of dCas9 with doxycycline resulted almost 50% repression of transcript levels of HERV-K(HML-2) in comparison with no doxycycline induction and control sgRNA transduction, as shown in **Figure 3.21B**, proving that inducible CRISPR interference system can be successfully utilized to repress HERV-K(HML-2) transcription in hES cells.

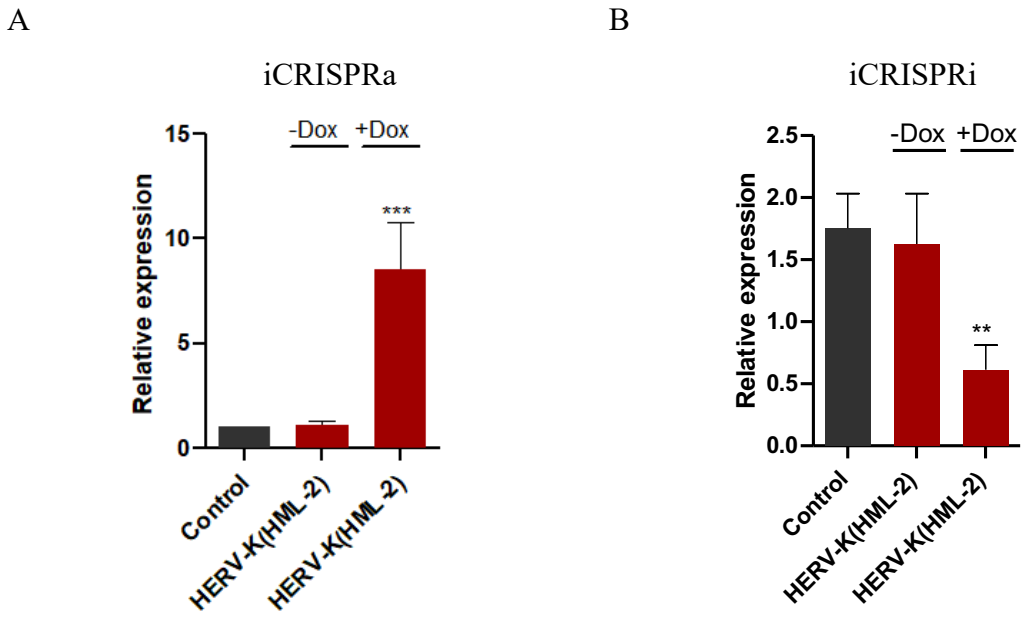


Figure 3.21 - Inducible CRISPR activation/ CRISPR interference of HERV-K(HML-2) in hESC. *Quantitative Realtime PCR (qRT-PCR) experiment showing the (iCRISPRa) and repression (iCRISPRi) in transcription of HERV-K(HML-2) LTRs post doxycycline induction in transduced cells relative to RNA polymerase II mRNA transcript levels. n=3.*

3.6 Induction of HERV-K(HML-2) transcription activation at early stages of cortical differentiation negatively impacts cortical development

The cortical neuron differentiation involves different stages from pluripotent embryonic stem cells to neuroectoderm to neural progenitors and immature neurons. In order to determine the

stage of differentiation affected by the activation of HERV-K(HML-2), tetracycline mediated transcriptional activation of HERV-K(HML-2) LTRs were obtained using doxycycline treatment at day 5, day 10 and day 21 of cortical differentiation corresponding to early neuroectoderm, neural progenitors and maturing neurons, respectively **(Figure 3.23A).**

3.6.1 MAP2 levels are aberrant when HERV-K(HML-2) transcription is induced in early stages

In order to understand the effect of early induction of HERV-K(HML-2) transcription on cortical development, differentiating cells were treated with doxycycline at day 5, day 10 and day 21 continuously over a period of 55 days.

An immunofluorescence staining was performed for MAP2 at day 55 on the differentiating cortical neurons which expressed HERV-K(HML-2) at specific timepoints, as mentioned above. Two independent sets of experiments were performed along with the control and checked for the change in expression levels of MAP2 protein.

As shown in **Figure 3.22**, HERV-K(HML-2) induction at day 5 and day 10 resulted in drastic reduction of MAP2 expression and overall level of differentiation. In comparison, the induction of HERV-K(HML-2) at day 21 when the cells have already differentiated into immature neuron state does not have any effect. This suggest that activation of HERV-K(HML-2) endogenous elements in the early stages of cortical neuron differentiation results in drastic degeneration of neurons and heavily impaired progression of cortical differentiation.

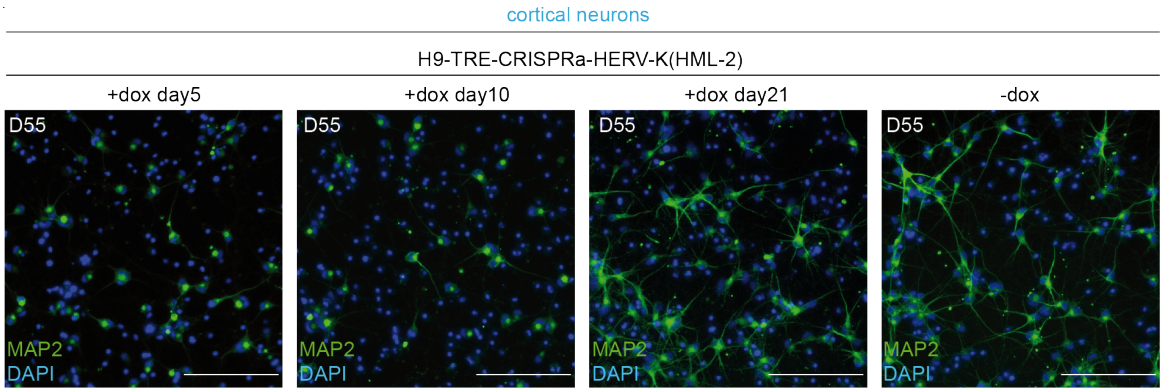
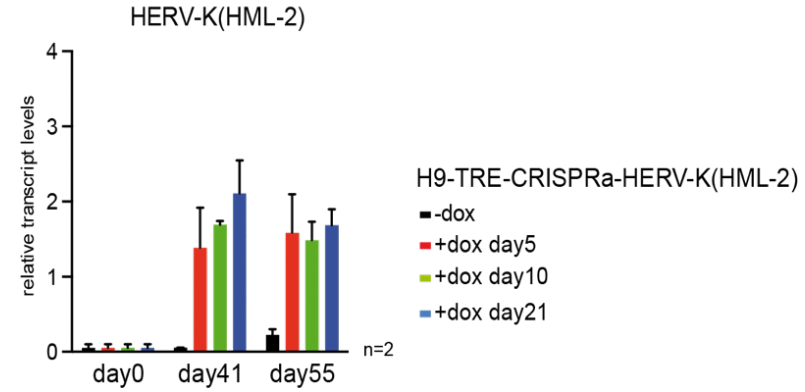


Figure 3.22- MAP2 levels are impacted upon inducible expression of HERV-K(HML-2) *Immunofluorescence staining of cortical neurons generated from H9-TRE-CRISPRa-HERV-K(HML-2) cells with HERV-K(HML-2) transcriptional activation at day 5, day10 and day21 for MAP2 expression at D55. Cortical neurons treated without doxycycline (-dox) served as control. Scale bars, 100 µm.*

To further confirm the observations of the immunofluorescence staining, total RNA was isolated at day 55 from two sets of treatment conditions. A quantitative real time PCR (q-RT PCR) experiment was conducted to check for the transcript levels of MAP2 in the doxycycline treated samples **(Figure 3.23B).** Samples without doxycycline treatment was considered as

controls for the experiment. The transcript levels of HERV-K(HML-2) was also monitored to check for the induction of HERV-K(HML-2) through inducible CRISPRa **(Figure 3.23A).**

A



B

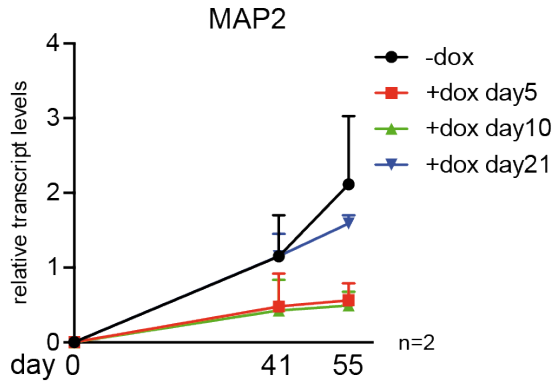


Figure 3.23 - MAP2 levels are impacted upon inducible expression of HERV-K(HML-2). *qRT-PCR showing the transcript levels of HERV-K(HML-2) (Panel A) and MAP2 (Panel B) during the course of cortical differentiation in TRE-HERV-K(HML-2) activated and control cells. Two biological replicates of differentiations are shown(n=2).*

During differentiation, along with the expression levels of the neuronal marker MAP2, the overall morphology of the differentiating neurons was also quantified and studied using high content image analysis using Columbus software on immunofluorescence staining.

As depicted in **Figure 3.24**, at day 55 of two independent differentiation experiments, the induction of HERV-K(HML-2) at early stages of differentiation ie, day 5 when there is neuroectoderm formation and day 10 when there is commencement of neural progenitor formation, resulted in decreased MAP2 expression along with severe reduction in number of segments of neurons, number of extremities and maximum neurite length of the neurons in

comparison with the differentiation of cells with HERV-K(HML-2) induced at day 21 when the immature neurons are established and non-induced control differentiation.

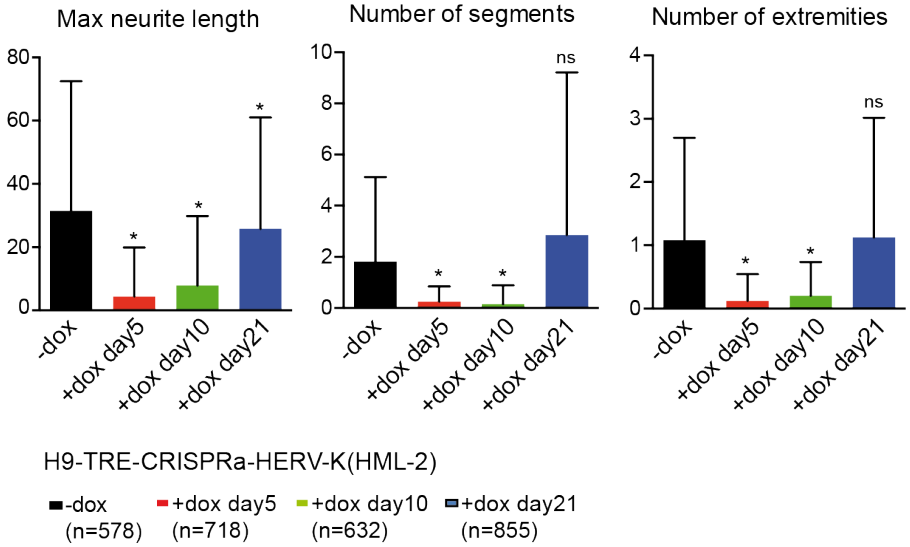


Figure 3.24-Morphology of neurons change upon inducible expression of HERV-K(HML-2). *Quantification using high-content image analysis of neurite length as well as number of segments and extremities in TRE-HERV-K(HML-2) activated and control cortical neurons. Two independent differentiations were quantified (n=2).*

3.7 Impaired cortical neuron differentiation upon HERV-K(HML-2) activation is not driven via the expression of viral ENV and GAG genes

Substantial studies have gone into deciphering the mechanism behind the numerous biological implications upon activation of endogenous elements.

To understand the role of viral genes, ENV and GAG in the phenotype of decreased MAP2 levels upon HERV-K(HML-2) activation, an immunofluorescence staining for ENV and GAG protein expression was conducted in pluripotent cells with overexpression of HERV-K(HML-2) and controls as wells as day 60 of HERV-K(HML-2) activated cortical neurons and controls. As evident from **Figure 3.25A and 3.25 B**, there was no change in the expression levels of ENV and GAG proteins, suggesting that the impaired cortical differentiation phenotype upon HERV-K(HML-2) activation is not driven by expression of Virus encoded proteins, but rather a regulatory role of the LTRs.

To further confirm the immunofluorescence results, total RNA from the differentiating cortical neurons from embryonic stem cells with activated transcription of HERV-K(HML-2) were isolated at day 60 of differentiation. A quantitative real time PCR was performed to analyze the transcript levels of different viral encoded genes, ENV and GAG. Viral genes showed no apparent changes in control as well as HERV-K(HML-2) activated neurons suggesting that the

neurodegenerative phenotype upon HERV-K(HML-2) activation is not driven by viral genes, as shown in **Figure 3.25C and 3.25D.**

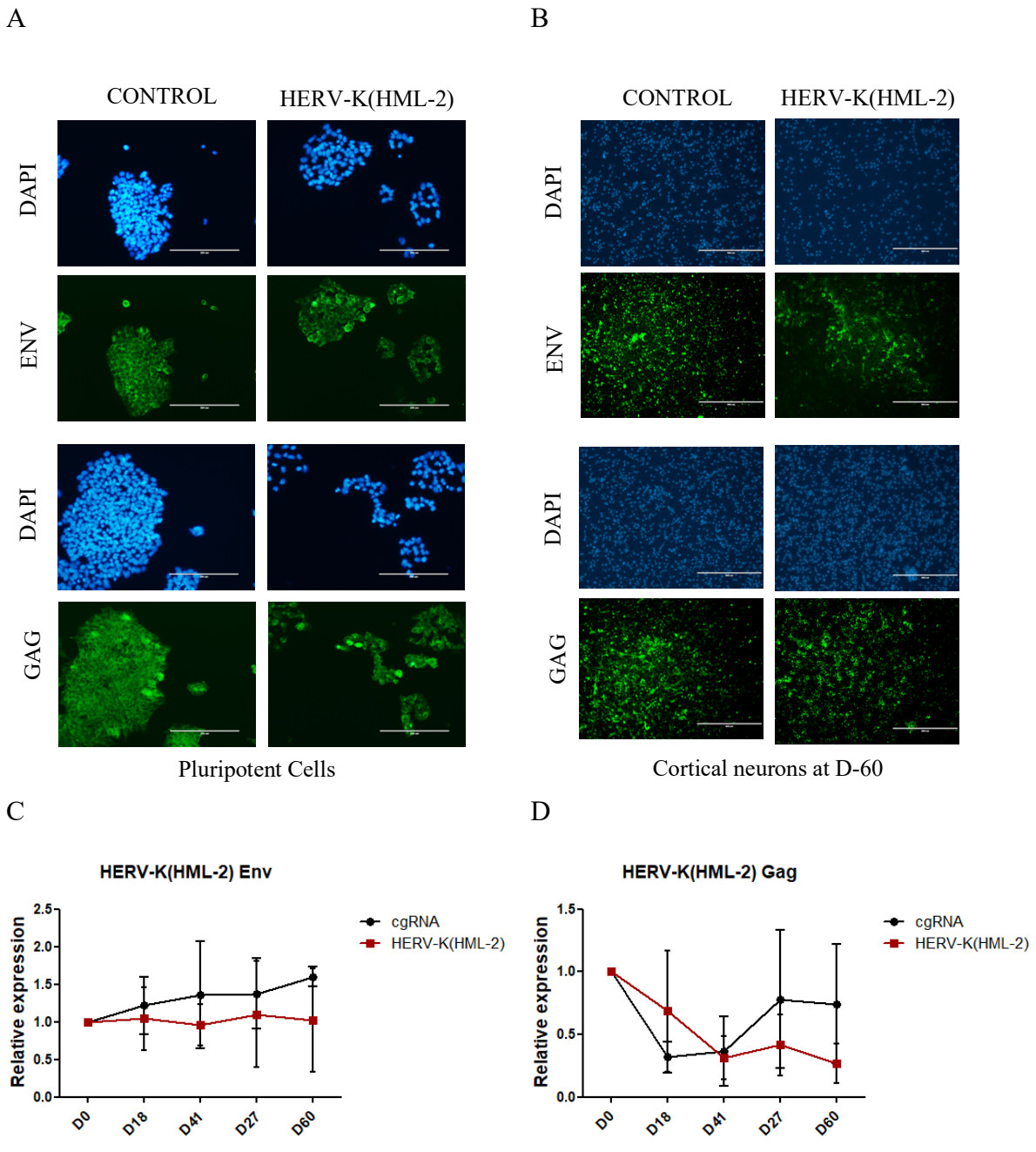


Figure 3.25- HERV-K(HML-2) ENV and GAG genes do not drive the impaired cortical phenotype
A-B) Immunofluorescence staining for HERV-K(HML-2) ENV and GAG expression in pluripotent cells and cortical neurons. C-D) qRT-PCR showing the HERV-K(HML-2) ENV and GAG transcript levels in cortical neurons generated from control and HERV-K(HML-2) activated cortical neurons at day 60 relative to RNA Polymerase II values.

3.8 Cell death mechanisms do not contribute to the impaired cortical neuron differentiation upon HERV-K(HML-2) activation

As an essential step towards understanding the mechanisms behind the decreased cortical outcome upon HERV-K(HML-2) activation in embryonic stem cells and subsequent differentiation into cortical neuronal lineage, an analysis of different cell death mechanisms was investigated in the context of HML-2 LTR activated cortical neurons at D60. HERV-K(HML-2) activated cortical neurons were treated with a pan-caspase inhibitor, Z-VAD-FMK, a ferroptosis inhibitor, ferrostatin and a necrosis inhibitor, necrostatin.

An immunofluorescence staining was performed on the HERV-K(HML-2) activated cortical neurons treated with the cell death inhibitors. The HERV-K(HML-2) activated neurons developed the phenotype of lesser MAP2 levels. However, the treatment with the compounds did not revert the phenotype induced by HERV-K(HML-2) activation **(Figure 3.26),** suggesting that the impaired cortical differentiation phenotype upon HERV-K(HML-2) activation is not driven by the apoptotic or necrotic signaling pathways, but rather a regulatory role of the LTRSs.

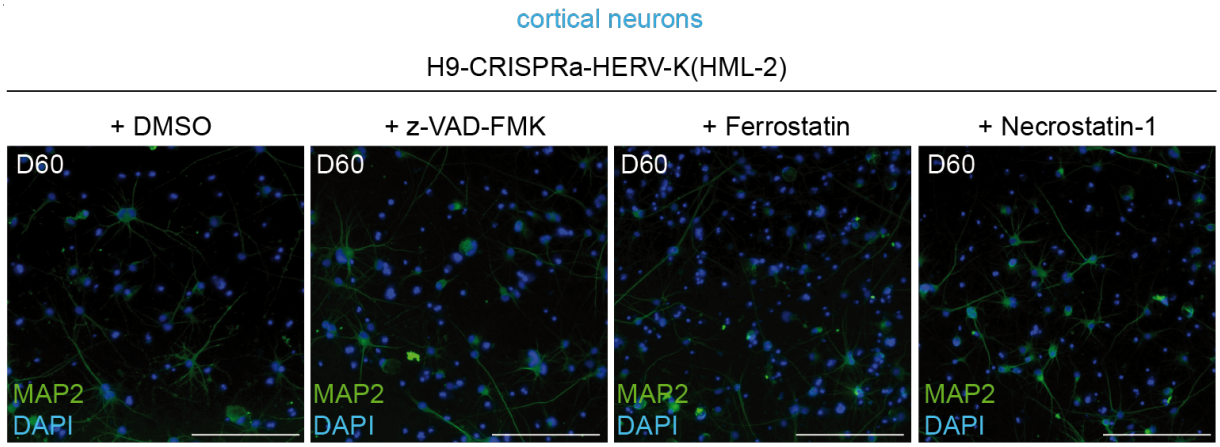


Figure 3.26- HERV-K(HML-2) activation do not trigger cell death pathways*. Immunofluorescence staining of MAP2 on HERV-K(HML-2) activated cortical neurons treated with cell death inhibitors.*

3.9 Whole genome RNA sequencing reveals differential regulation of neural development-related genes in cortical neurons due to HERV-K(HML-2) activation

As a primary step towards understanding the molecular mechanism behind the impaired cortical neuron differentiation marked by decreased MAP2 and Synapsin-I expression phenotype upon HERV-K(HML-2) activation and the influence on global gene expression profile, a whole genome RNA sequencing was performed on total RNA isolated from HERV-K(HML-2) activated and control cortical neurons at timepoints D0, D10, D27, D41 and D60.

A Principal Component Analysis (PCA) was performed on the RNA sequencing dataset for bidirectional promoter long noncoding RNA, miRNA, antisense RNA, lnc RNA and protein coding genes to identify the similarity and differences between the control cells and HERV-K (HML-2) activated cells.

As shown in **Figure 3.27A-3.27C**, only protein coding genes were affected by the activation of HERV-K(HML-2) during differentiation, and not the miRNA, lnc RNA or sense and antisense RNAs **(Figure 3.27C)**. At D0, the control cells and activated cells clustered together, indicating that there are considerably less unspecific patterns of gene profile due to transcriptional activation of HERV-K(HML-2) **(Figure 3.27A).** The separation and localization of control cells and HERV-K(HML-2) activated cells become prominent during differentiation progression at D10, D27, D41 and D60, suggesting that the changes in protein coding genes arise from HERV-K(HML-2) transcriptional activation **(Figure 3.27B).**

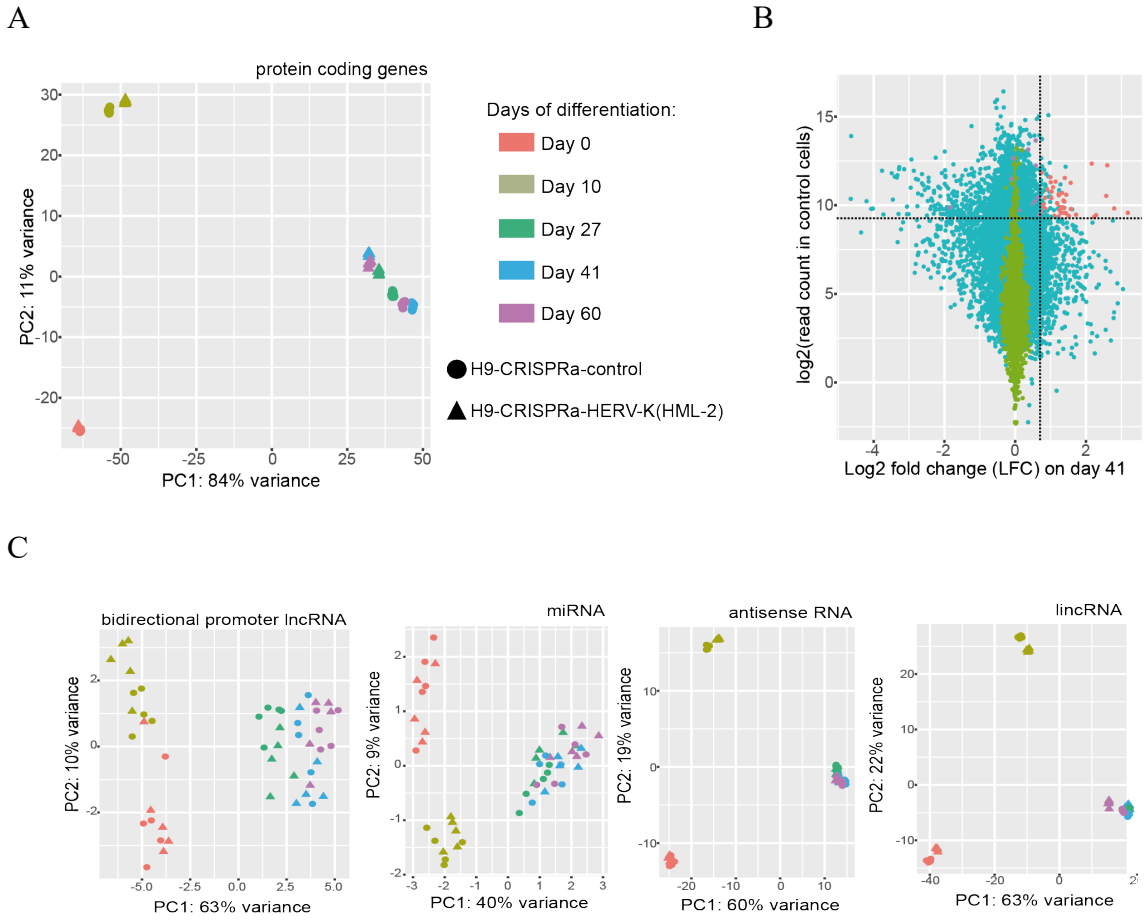
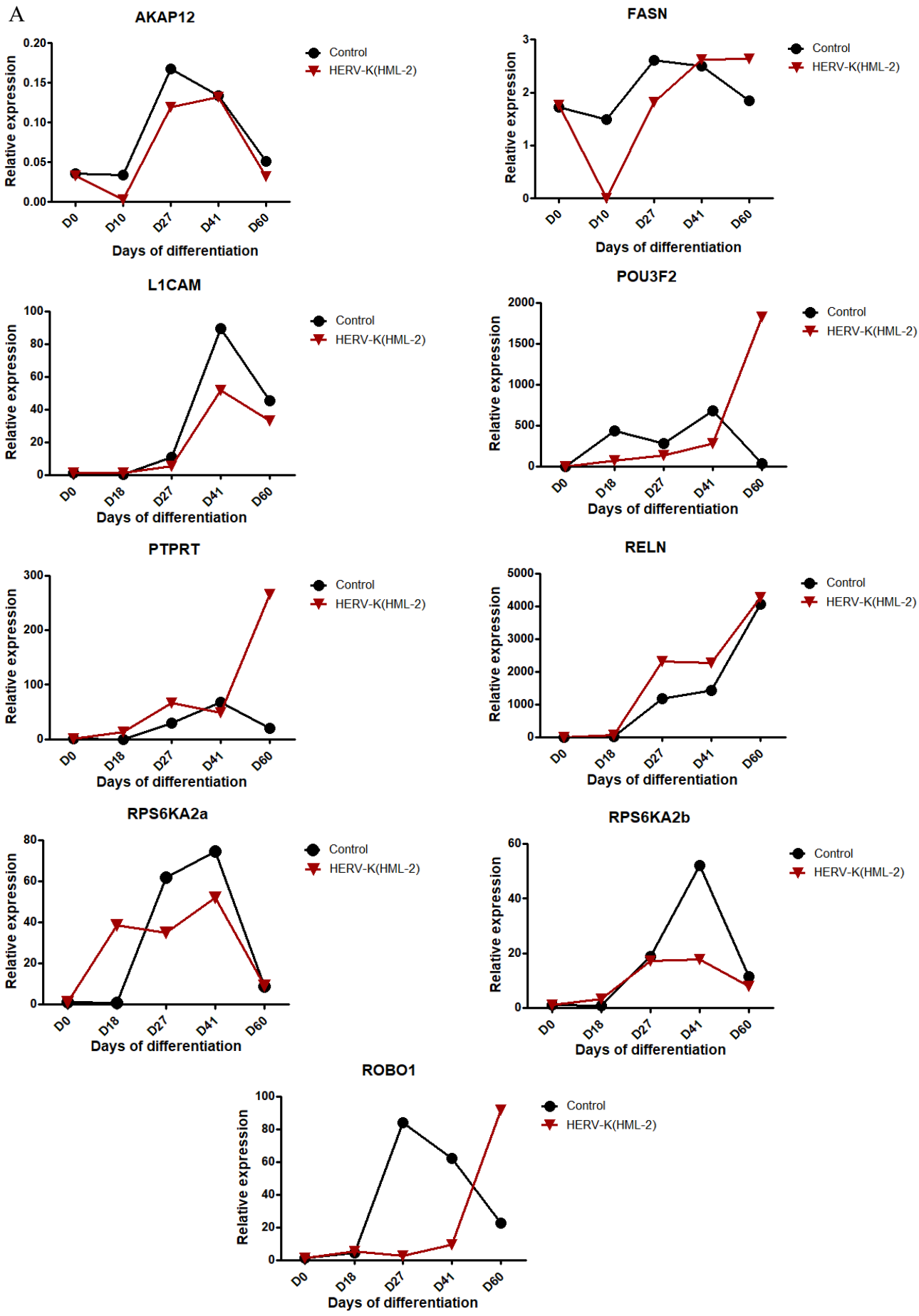


Figure 3.27 - Analysis of HERV-K(HML-2) activated cortical neurons using whole genome RNA-seq. *A-B) Identification of the 28 most significantly upregulated protein coding genes in the H9-dCas9-VP64-gRNA (HERK-K(HML-2) cells compared with the H9-dCas9-VP64-gRNA (control) cells. C)Bidirectional promoter lnc RNA, miRNA, antisense RNA, lincRNA showed no difference upon HERV-K(HML-2) activation in cortical neurons.*

3.9.1 Neuron related genes are upregulated during HERV-K(HML-2) activation

In the detailed bioinformatic analysis of the RNA sequencing dataset, a total of 28 genes were identified which exhibited the strongest upregulation in HERV-K(HML-2) activated neurons in comparison to control cells (Supplementary Table 1). A quantitative real time PCR experiment was performed on the RNA isolated from the differentiating cortical neurons at different time points in order to confirm the observations from the whole genome RNA sequencing. To this end, qRT-PCR was done on a total of 14 out of the 28 upregulated genes. As shown in **Figure 3.28A**, upregulation at transcript level was observed only in certain genes. Interestingly, among the identified upregulated genes, four gene candidates were differentially expressed upon HERV-K(HML-2) activation. In particular, Calsyntenin 2 (CLSTN2), Chordin Like 1 (CHRDL1), EPH Receptor A4 (EPHA4) and Neurotrophic Tyrosine Receptor Kinase 3 (NTRK3) were consistently highly upregulated at days 41 and 60 during three independent cortical differentiation experiments as shown by qRT-PCR, **in Figure 3.28B.**

A


AKAP12
Relative expression
0.20
0.15
0.10
0.05
0.00
D0
D10
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
FASN
Relative expression
3
2
1
0
D0
D10
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
L1CAM
Relative expression
100
80
60
40
20
0
D0
D18
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
POU3F2
Relative expression
2000
1500
1000
500
0
D0
D18
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
PTPRT
Relative expression
300
200
100
0
D0
D18
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
RELN
Relative expression
5000
4000
3000
2000
1000
0
D0
D18
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
RPS6KA2a
Relative expression
80
60
40
20
0
D0
D18
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
RPS6KA2b
Relative expression
60
40
20
0
D0
D18
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)
ROBO1
Relative expression
100
80
60
40
20
0
D0
D18
D27
D41
D60
Days of differentiation
Control
HERV-K(HML-2)

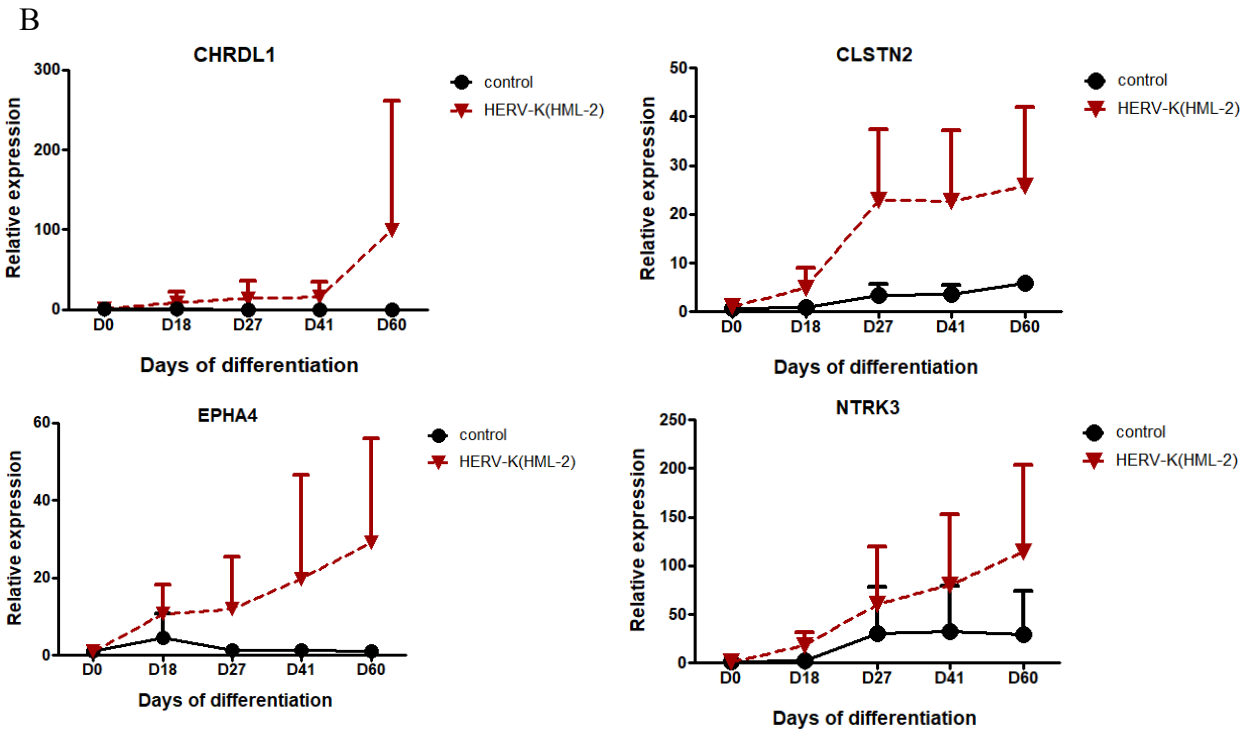


Figure 3.28- Upregulation of neuron-related genes upon HERV-K(HML-2) activation. *qRT-PCR of selected genes from the RNA -seq data (Panel A) and genes with consistent upregulation in three independent differentiation experiments (Panel B).*

3.9.2 Gene network of upregulated genes show distinct interaction profile

An interaction profile of the upregulated genes was generated using commercially available software programmes and multifactorial database CIDeR, in collaboration with Institute of Genome-Oriented Bioinformatics, Technical University of Munich and Institute of Experimental Genetics, HelmholtzZentrum, Munich. CIDeR is a manually curated cloud-based resource database which gives the information regarding the interaction of distinct biomolecules and metabolic, neurodegenerative and cardiovascular diseases (Lechner et al; 2012). Each node of interaction was verified by published experimental evidence. Interestingly, 25 out of the 28 identified up-regulated genes are interconnected to each other, and are directly or indirectly related to neurodegenerative diseases, processes, or phenotypes via SNP associations, expression or regulation **(Figure 3.30).** Nearly all gene-gene and gene-disease interactions included in this network were reported to occur in the human brain or in animal disease models (mouse, rat). Overall, these findings demonstrate that HERV-K(HML- 2) transcriptional activation affects the expression of specific cellular genes implicated in neurogenesis and neurological disorders.

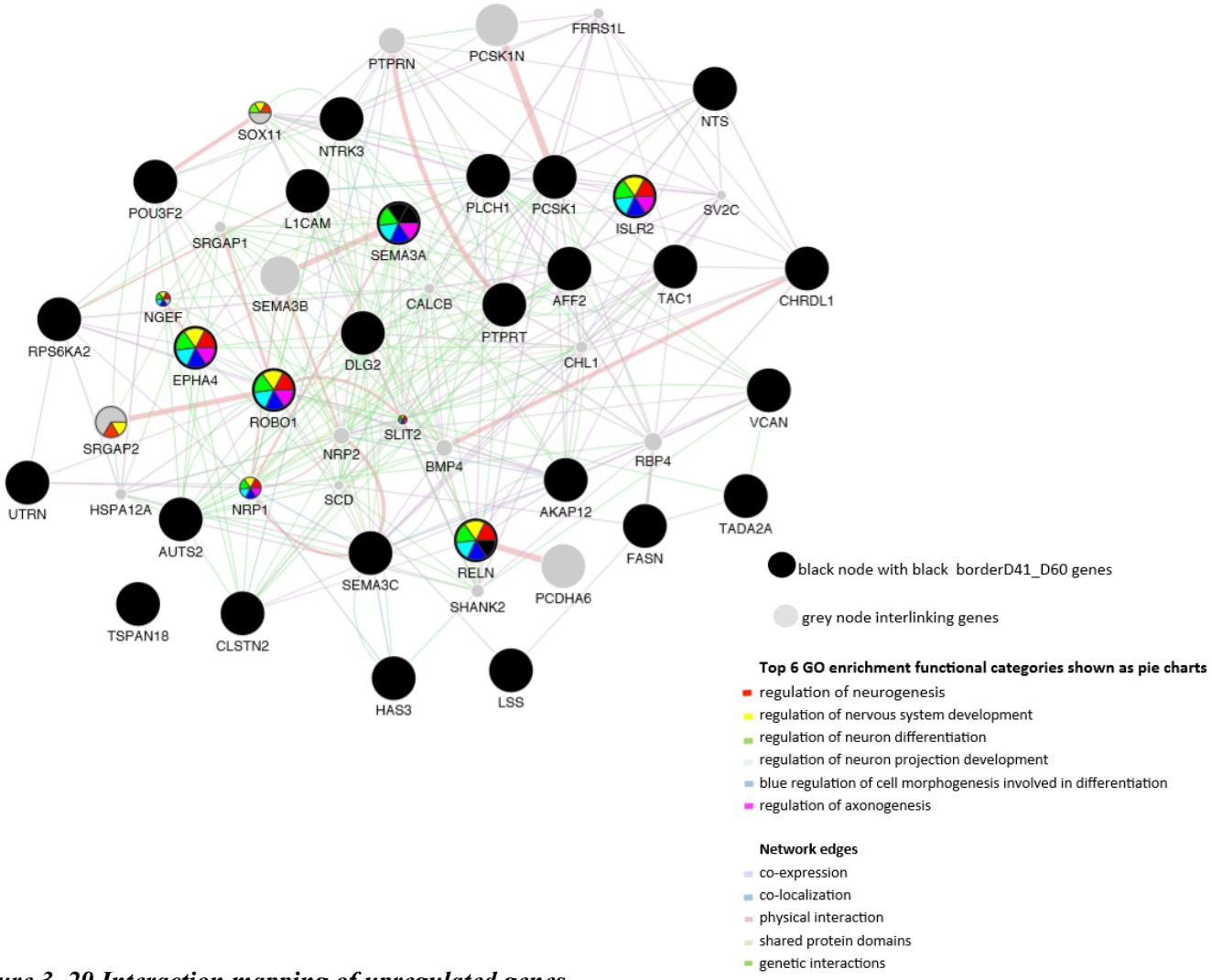


Figure 3. 29 Interaction mapping of upregulated genes

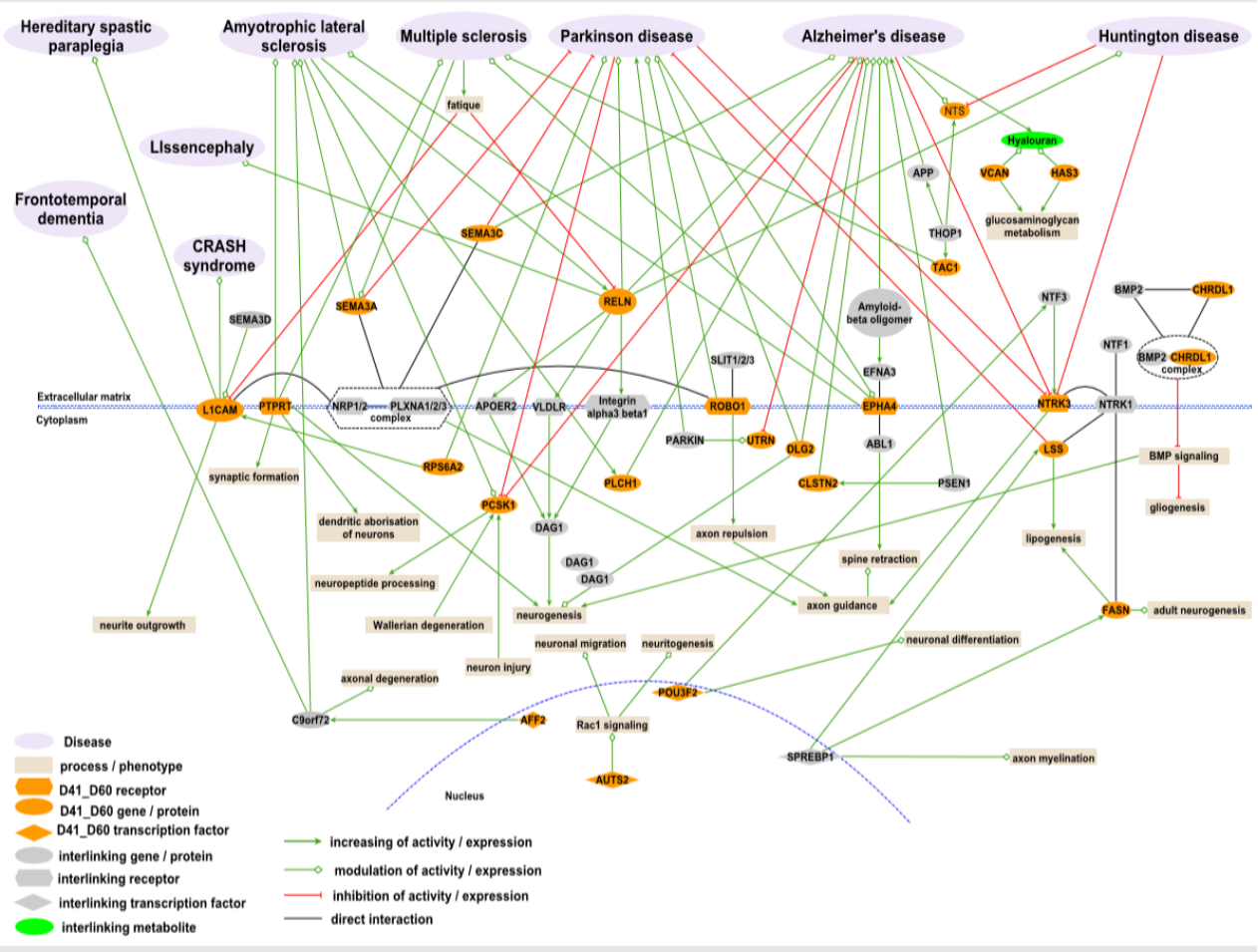


Figure 3.30- Interaction profile of upregulated genes using CIDeR database

A Gene ontology analysis revealed strong association of identified genes with neuron related biological processes **(Figure 3.31A).** In addition to gene ontology analysis, a disease enrichment analysis **(Figure 3.31 B)** was performed, and it showed steady association of the upregulated genes to the progression of several neurodegenerative diseases, suggesting that activation of HERV-K(HML-2) LTR transcription can be a key factor in the manifestation of several nervous system related disorders.

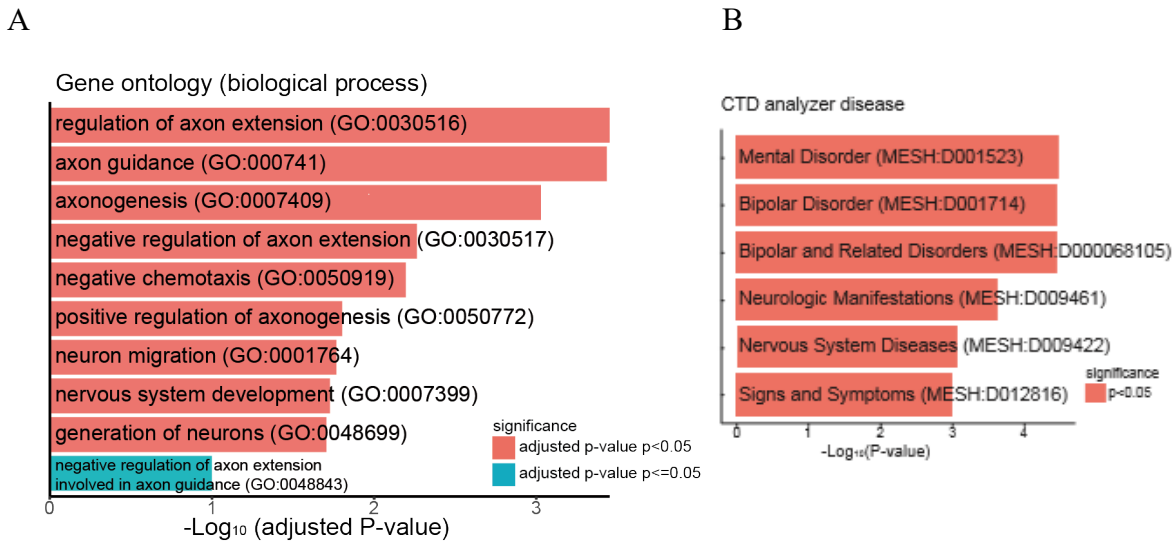


Figure 3.31 Gene ontology analysis for the biological processes (Panel A) Disease enrichment analysis which relates to neurodegenerative disorders (Panel B).

3.10 Activation of candidate genes in human embryonic stem cells using CRISPRa

Following the extensive bioinformatic analyzes of the whole genome RNA sequencing data of the HERV-K(HML-2) activated cortical neurons and controls, certain genes were selected which shows strongest upregulation during HERV-K(HML-2) activation in the differentiation time period. These genes, CHRDL1, CLSTN2, EPHA4 and NTRK3 were subjected to elaborate literature study and were reported to be involved in different stages of neuronal development. Next step was to activate these genes in the embryonic stage and monitor the influence of these genes in the differentiation pathway and to analyze if the activation of one or more of these genes would impart a HERV-K(HML-2) associated phenotype of cortical neurons. In this regard, next step was to introduce the synthesized sgRNA combination targeting the selected genes for CRISPRa into human embryonic stem cells.

Single guide RNAs targeting genes were designed and cloned into pLKO.1U6-puro BfuA1 sgRNA stuffer lentiviral plasmid as described previously. These plasmids carrying the gRNA sequences was transduced into the stable cell lines expressing the dCas9 fusion constructs and checked for the activation of transcription of respective genes. As shown in **Figure 3.32**, a qRT-PCR from total RNA isolated from these cells showed an average of around 3fold activation of the transcription of the genes in embryonic stage, suggesting that these cell lines possessed high transcription rates of selected genes, which was actively transcribed in cortical neurons upon HERV-K(HML-2) LTR activation.

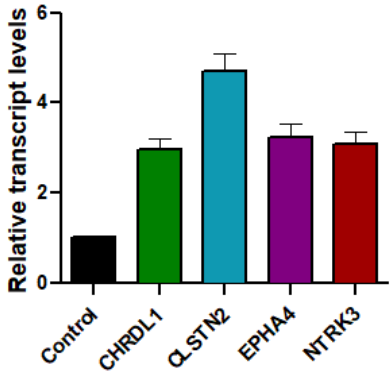


Figure 3.32 - CRISPR activation of candidate genes *H9-dCAS9-VP64 expressing cells were used to generate cells transcriptionally activating CLSTN2, CHRDL1, EPHA4 and NTRK3, respectively. Activation of the genes in human embryonic stem cells was analyzed by qRT-PCR (n=2).*

3.10.1 Activation of genes in hES cells does not change the pluripotency levels

To analyze the effect of activation of the genes, CHRDL1, CLSTN2, EPHA4 and NTRK3 in hES cells, a qRT-PCR was performed to analyze the transcript levels of two early pluripotency markers, Nanog and OCT-4.

As shown in **Figure 3.33**, the dCas9-VP64 transduced hiPS cells shows similar levels of mRNA expression of Nanog and OCT-4 compared to normal pluripotent untransduced hES cells. These results suggest that addition and integration of fusion constructs and activation of these genes using CRISPRa into the hES cell genome does not change the pluripotency levels of the cells.

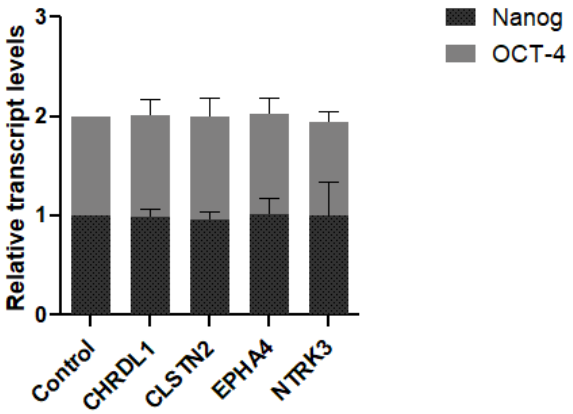


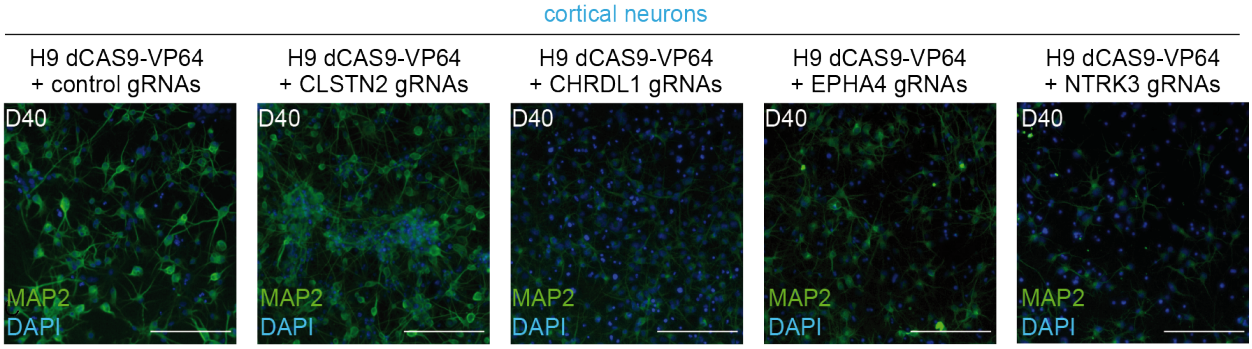
Figure 3.33 - CRISPR activation of candidate genes does not change pluripotency levels *qRT-PCR showing the transcript levels of pluripotency markers Nanog and OCT-4 in H9-dCas9-VP64 CLSTN2, CHRDL1, EPHA4 and NTRK3 cells (n=2).*

3.11 Activation of NTRK3 in embryonic stem cells reveal an impaired differentiation trajectory in cortical neurons

To understand the underlying mechanism of decreased MAP2 and Synapsin-I expression patters and weakened neuronal morphology upon HERV-K(HML-2) LTR activation, an immunofluorescence staining was performed on the differentiating cortical neurons from four different embryonic stem cells with activated transcription of CHRDL1, CLSTN2, EPHA4 and NTRK3 for the neuronal marker, MAP2 **(Figure 3.34A-B).** The transcription activation of these genes was achieved using CRISPRa, as shown in the previous section in **Figure 3.32.**

At day 40 and day 60, as shown in **Figure 3.34 A and B**), the overall MAP2 levels of the cortical neurons from four different cell lines overexpressing each gene, CHRDL1, CLSTN2, EPHA4 and NTRK3 were monitored. As shown in **Figure 3.34 A and B**, there is no apparent change in the levels of MAP2 expression in cortical neurons derived from embryonic stem cells harboring overexpression of CHRDL1, CLSTN2 and EPHA4, in comparison to control cells. Interestingly, cortical neurons with an activated expression of NTRK3 gene showed a decrease in the MAP2 levels consistent to the phenotype observed in the case of cortical neurons derived from embryonic stem cells with HERV-K(HML-2) activation (refer to **Figure 3.8A**). This suggest that activation of NTRK3 transcription leads to a malfunctioning in cortical differentiation progression and the resultant phenotype of cortical neurons is almost identical to that of HERV-K(HML-2) activation, suggesting an interplay of NTRK3 and HERV-K(HML-2) in the progression of cortical neuron differentiation upon activation of HERV-K(HML-2) LTRs.

A



B

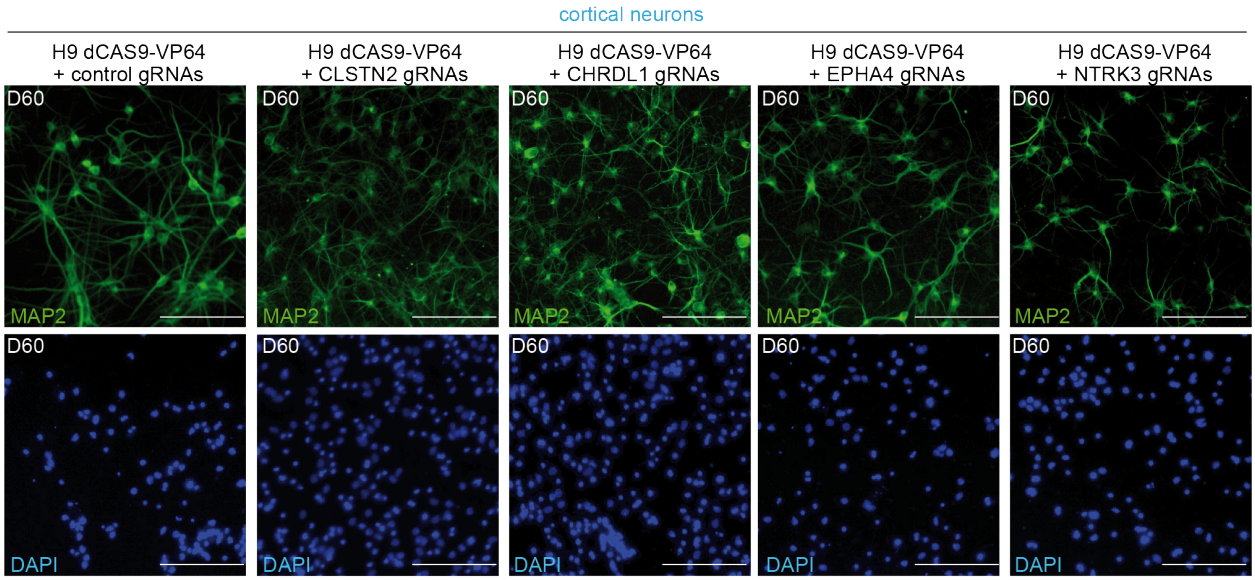


Figure 3.34 - Activation of NTRK3 transcription in stem cells leads to decreased MAP2 expression in cortical neurons. *Immunofluorescence staining of MAP2 expression at day 40 and day 60 in transcriptionally activated CLSTN2, CHRDL1, EPHA4 or NTRK3 neurons and control cells (n=2). One representative experiment shown. Scale bars, 100 μm.*

To further confirm the observations from immunostaining at transcript level, total RNA from differentiating cortical neurons from embryonic stem cells with activated transcription of selected candidate genes, CHRDLI, CLSTN2, EPHA4 and NTRK3 were isolated at day 0, 41 and 60 of differentiation. A quantitative real time PCR was performed to analyze the transcript levels of neuronal marker MAP2. A reduced transcript level of MAP2 was observed in NTRK3 activated cortical neurons, as shown in **Figure 3.35**. Other genes showed no decrease in the MAP2 mRNA levels, suggesting that NTRK3 activation results in a phenotype which we observed similar to HERV-K (HML-2) activation.

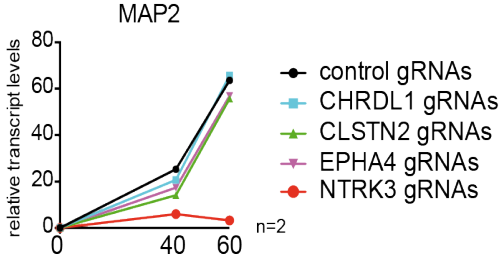


Figure 3.35 - Activation of NTRK3 transcription in stem cells leads to decreased MAP2 expression in cortical neurons, *MAP2 mRNA levels were quantified by qRT-PCR in transcriptionally activated CLSTN2, CHRDL1, EPHA4 or NTRK3 neurons and control cells (n=2). One representative experiment shown.*

In addition to accessing the MAP2 expression levels in differentiating cortical neurons, the overall morphology of the generated neurons from cells with activated transcription of candidate genes, CHRDL1, CLSTN2, EPHA4 and NTRK3 was also monitored and studied using high content image analysis of MAP2 stainings from immunofluorescence experiment with Columbus software. As shown in **Figure 3.36**, the neurite outgrowth in case of NTRK3 activated cortical neurons were drastically reduced in comparison with controls. However, CHRDL1, CLSTN2 and EPHA4 activation did not have any effect in the neuron structure and morphology, suggesting that NTRK3 activation critically affects the cortical neuron differentiation and healthy progression of the differentiation and development of cortical neurons.

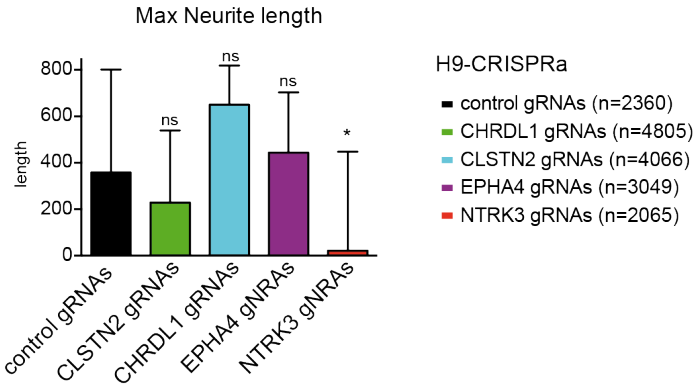


Figure 3.36 - Activation of NTRK3 transcription in stem cells leads to malformed neurites in cortical neurons. *Quantification using high content image analysis of Neurite length, number of segments and extremities in CLSTN2, CHRDL1, EPHA4 and NTRK3 activated and control cortical neurons at day 60. Two independent differentiations were quantified (n=2).*

4. Discussion

4.1 Neurodegeneration associated with HERV activation

It has been shown in this study that activation of HERV-K(HML-2) LTRs using CRISPR activation in human embryonic stem cells and subsequent differentiation into cortical neuron lineage results in a decreased expression levels of global neuronal marker MAP2 and pres-synaptic marker Synapsin-I and impaired establishment of deep layers in forebrain organoids. Several studies have shown the impact of HERV activation in contexts related to nervous system and neurodegenerative diseases. Research conducted on the brain biopsy samples of Amyotrophic lateral Sclerosis patients and transgenic mice models overexpressing HERV-K envelope protein showed that envelope protein might be the contributing agent to motor neuron degeneration. The transgenic mice models were characterized by decreased motor cortex volume, synaptic activity and beading of neurites (Li et al; 2015) Increased expression levels of HERV-K were observed in Human Immunodeficiency Virus infected patients, especially in brain tissues which are marked by HIV replication reservoirs (Vincendeau et al; 2015, Albright, Soldan, and Gonzlez-Scarano; 2003). There are also reports of a neuropathological interference between Amyotrophic Lateral Sclerosis and HIV associated encephalitis directed by the formation of neurotoxin, Trans-activation responsive (TAR) DNA binding Protein-43 (TDP-43) deposits in neurons (Douville and Nath; 2014). In earlier studies connecting ALS pathology and Human Endogenous Virus-K association, it has been shown that TDP-43 regulates the expression of HERV-K by binding to the long terminal repeat of the virus (Douville and Nath; 2014, Douville and Nath; 2017, Alfahad and Nath; 2013). Implications of the contribution of HERV-K activation in other neurodegenerative diseases like Alzheimer's disease, Schizophrenia, Bipolar disorder and Ageing is controversially discussed (Balestrieri et al; 2015, Nevalainen et al; 2018, Frank et al; 2005).

In our study, activation and repression of HERV-K(HML-2) LTRs were achieved using the CRISPR activation and CRISPR interference technologies, respectively to understand the functional role of HERV-K(HML-2) elements in neuronal development and pathology of several neuron-related disorders. Constitutive transcriptional activation of HERV-K(HML-2) was established in human embryonic stem cells and subsequently subjected to directed differentiation into cortical neuron lineage. We observed a consistent reduction in the expression levels of the global neuronal marker protein, MAP2 during the course of differentiation in HERV-K(HML-2) activated neurons. At day 41 and day 60 of differentiation, the HERV-K(HML-2) transcript levels remained the same, but MAP2 transcription was

impaired. We also analyzed for the transcription pattern of Synapsin-I, which is essential to maintain and manage the connectivity and communication of neurons. The Synapsin levels were also affected by activation of HERV-K(HML-2) transcription at day 41 and day 60 of cortical neuron differentiation. This it can be concluded that HERV-K(HML-2) activation impaired the growth and development of neurons. Additionally, we also analyzed the morphological characters of the developing neurons and we could see that there were significant alterations in the overall health, appearance and quality of the neurons from control and HERV-K(HML-2) activated cells. The shortness of the processes, lack of the extensive branching which is the characteristic feature of human neuronal network, the reduced number of neurons and the lack of synaptic activity appears to be the end results of active transcription of HERV-K(HML-2) LTRs in these cells. We also performed a directed differentiation into dopaminergic neuronal lineage with HERV-K(HML-2)+ and control cells. In contrast to cortical neurons, the dopaminergic neuron identity was not affected by the active transcription of HERV-K(HML-2). The MAP2 and Synapsin-I levels and the morphology of the generated neurons did not suggest impaired cortical development. Thus, we could conclude that the activation of HERV-K(HML-2) LTR transcription leads to a cortical specific phenotype in human embryonic stem cells.

The forebrain organoids generated from HERV-K(HML-2)+ and control cells revealed further insights into the role of HERV-K (HML-2) in laminar brain pattern formation. As established from the mRNA transcript levels and protein levels of MAP2 during cortical neuron differentiation, the organoids from HERV-K(HML-2) activated cells showed significant decrease in size compared to controls. There have been studies on cerebral atrophies following traumatic brain injuries which exhibits shrinkage of brain volume, thinning of cortex etc. Over a period of time, these traumatic events may lead to long term structural and functional changes in the brain, characterized by gradual loss of neurons and neuronal connections (Harris, de Rooij, and Kuhl; 2019). It is interesting to point out that there is a clear correlation of the neurodegenerative pathways in the case of chronic traumatic encephalopathy and Alzheimer's Disease (Harris, de Rooij and Kuhl; 2019, Turner et al; 2016, Bedem and Kuhl; 2017). The surface area of brain has been reported to be decreasing during aging process and decline of cognitive sharpness (Lemaitre et al; 2012, Hogstrom et al; 2013, Choi et al; 2019). These studies and the data from this study which reveals a decrease in the overall diameter of the generated organoids upon HERV-K(HML-2) activation could potentially suggest a decreased cortical volume. Additionally, sections from HERV-K(HML-2)+ organoids at day 60 showed decreased levels of MAP2 staining compared to controls. MAP2+ neurons were considerably

decreased in HERV-K(HML-2) + organoids, which could be due to the decreased neuronal connectivity during organoid maturation. Thus, decrease in the overall size of the organoid and MAP2+ neurons upon HERV-K(HML-2) activation can be read together and it can be suggested that activation of HERV-K(HML-2) during forebrain organoid formation can result in the decrease of cortex volume and neuronal network formation, which is essential for normal brain function in homosapiens. In order to further look into the role of HERV-K(HML-2) in the intricacies of brain patterning and deep layer formation during development, forebrain organoids from control and HERV-K(HML-2)+ cells were sectioned and stained at day 40 and day 60 for CTIP2, TBR1, TBR2 and SATB2, along with PAX6 and SOX2. The normal patterning of forebrain layers from inside is SATB2-TBR1-CTIP2-TBR2-PAX6/SOX2. There are generally six different brain layers formed in human brain in development. In HERV-K(HML-2) activated organoids, there was an absence of the deep layer SATB2+ neurons. Additionally, TBR1+ and CTIP2+ neuronal layers were unorganized and unevenly distributed. This could be read together with the neuronal degeneration promoted by HERV-K(HML-2) activation and the impact of HERV-K(HML-2) on brain development. There were no obvious changes observed in the neural rings stained by SOX2 and PAX6 in control and HERV-K(HML-2) activated neurons. This could be due to the fact that activation of HERV-K(HML-2) impacts the migration of the neurons and establishment of brain layers which are crucial in the development of the complexity of human brain. The complete absence of SATB2+ neurons at day 60 in HERV-K(HML-2) activated organoids points towards an apparent delay in the migration and maturation of neurons in the brain. The Vimentin+ neurons in HERV-K(HML-2) activated organoids revealed shorter basal radial glia cells (RG) processes with improper basal endfeet branching. This observation of defective RG progenitors further substantiates the fact that HERV-K(HML-2) activation impairs neuronal migration and layer formation.

As discussed before, HERV-K(HML-2) activation has been reported to be associated with several neurodegenerative disease conditions, most of which are directly associated with impaired cognitive functionality and defective brain morphology. Cerebral cortex establishes a smooth layer of neurons which proliferates and forms the ventricular zone and migrates through radial glial fibers. Our observations suggest a cortical specific manifestation of impaired neuronal development upon transcriptional activation of HERV-K(HML-2). Furthermore, forebrain organoid experiments point towards the impact of HERV-K(HML-2) activation on neuronal migration and layer formation. Human brain is composed of about 100 billion neurons (Herculano-Houzel; 2009). Throughout the evolutionary time scale of life from insects to mammals to lower order primates to homosapiens, the most distinctive

morphological feature of brain is the expansion of cerebral cortex (Kaas; 2013, Hey; 2005). The cortical gray matter volume in humans is about 50% of the total brain volume. The total cerebral cortex volume of the humans is the highest among the living and is about 80% of the entire brain and only 40% in mice (Herculano-Houzel; 2009, Hofman; 1988, Azevedo et al; 2009, Hofman; 2014). This clearly establishes the involvement of cerebral cortex in the evolution of higher order cognitive functionality and supra intelligence of homosapiens. The fact that endogenous retroviral elements are still actively transcribed in lower order animals (Waterston et al; 2002) and the regulation of these elements are loosened in lower order primates (Greenwood et al; 2005) can be definitely speculated and extrapolated in connection to the evolution of human brain. From our results, the presence of HERV-K(HML-2) elements negatively impacts the establishment of the evolved pattern of brain. Thus, the silencing of HERV-K(HML-2) in brain from lower order animals to humans could be considered as an evolutionary leap towards increased brain and cognitive functionality. Our understanding of the geometric, biophysical, molecular, genetic and energy constraints that govern the evolution and functional organization of human brain is still rudimentary (Hofman; 2014). Thus, it can be argued that the active state of HERV elements in several neurodegenerative diseases cannot be considered as mere coincidences but are markers of miswiring of a highly sophisticated neuronal connectome.

4.2 Activation of Human Endogenous Retroviruses and Gene expression

As discussed earlier, endogenous retroviral activation has been associated with the onset and progression of several diseases. These elements are reported to be involved in genomic regulation and chromatin opening during early embryogenesis and development (Gao et al; 2018, Liu et al; 2016). Transcriptional repression of endogenous retroviruses is reported to be mediated by DNMT-1 dependent cytosine methylation (Tie et al; 2018, Howard et al; 2008). However, these studies do not provide a conclusive proof of regulation and molecular mechanism behind the activation of HERV LTRs and how these elements manifest as a cause-effect entity in neurogenesis and degenerative disorders. There are several agents, which are discussed to be activating agents of HERV LTR transcription such as ultraviolet radiations, exogenous viruses to host cellular mechanisms of epigenetic regulation. DNA methylation, histone modifications, and regulatory factors such as transcriptional factors, cytokines and small noncoding RNAs are agents that can activate HERV transcription. Widespread presence of the so called solitary LTRs which are the results of recombination and deletion of the 5’ and 3’LTRs are established to be influencing host cellular regulation. LTR mediated control of

cellular gene expression happens either via LTRs acting as alternative promoters or enhancers or by acting as binding sites for transcription factors and regulatory proteins. Rarely, full-length LTR transcripts drive the expression of certain genes and pathways.

The regulation of HERVs in our genome has been mainly attributed to the large family of KRAB Zinc finger proteins, KAP-1. Most cell types have shown to express KAP-1 or TRIM28, notably during early developmental stages in embryonic stem cells and during brain development (Grassi et al; 2019, Rowe et al; 2010, Herzog et al; 2011). ZFPs are known to be the largest class of transcriptional regulators of vertebrates dating back to around 420 million years (Yang, Wang, and Macfarlan; 2017). Non-transcriptional restriction of endogenous retroviruses via KRAB ZFPs have been reported in the case of HIV-1, affecting the virion assembly (Emerson and Thomas; 2011). The presence of evolutionarily younger KRAB ZFPs in the vicinity of intracisternal A particles (IAPs), one of the most active transposable elements in mice further substantiates the idea that coevolution of TEs and KRAB ZFPs is the major driving force behind ERV domestication (Wolf et al; 2020, Göke and Ng; 2016). Recent studies suggest that HERVs are responsible for the transcriptional regulation of several KRAB ZnF genes located near tumor vicinity (Ito et al; 2020). KAP1 or TRIM28 mediated repression of endogenous retroviral elements in brain has been reported previously and this has been considered absolutely essential for the regulation of several transcriptional networks involved in mammalian brain development and neuronal differentiation. Knockdown of TRIM28 resulted in the upregulation of genes located near the activated ERVs (Brattås et al; 2017, Fasching et al; 2015). So far, nothing much is known about the intricacies of the regulatory networks established by the repression of HERVs by KAP1 or the activation of HERVs by loss of KAP1 or any other epigenetic regulatory factor. A recent study on the regulatory role of HERV-K(HML-2) on stem cell function showed that the envelope protein of HERV-K(HML-2) from Chromosome 12 and 19 interacted with CD98HC via mTOR signaling cascade in order to maintain the stemness. Epigenetic silencing of HML-2 Env resulted in loss of stemness and enhanced differentiation pathways (Wang et al; 2020). However, these data addresses only the loss of function of Env gene from the two loci which harbor the full length ORF of the HERV-K(HML-2). Albeit the promising direction of role of Env gene in balancing stemness and differentiation efficiency, a conducive conclusion of how HERV-K(HML-2) activation affects the early developmental patterns is lacking. Additionally, the study does not address the well discussed theme of the impact of solitary LTRs which constitute a major bulk of the transposon milieu.

In our experiments, we targeted the sequences of around 600 LTRs of HERV-K(HML-2) to identify the homology to design single guide RNAs for CRISPR activation. The single guide RNA pair covered around 70% of the HERV-K(HML-2) LTR milieu resulting in a transcriptional activation of about 6-fold. Thus, it should be emphasized that the neurodegenerative phenotype upon HERV-K(HML-2) activation presented in our study is not a result of manipulation of a single LTR sequence but is an outcome of a global regulation of LTRs in the genome.

The whole genome RNA sequencing of the LTR-activated cortical neurons and controls revealed 28 genes which were consistently upregulated during LTR activation. Confirmation of the transcriptional upregulation of these genes in mRNA level and extensive literature study revealed that most of these genes are involved in neurogenesis and development. Out of the many genes, Chordin-like 1(CHRDL-1), Calsyntenin-2 (CLSTN-2), Ephrin Type A Receptor-4 (EPHA-4) and Neurotrophin Kinase Receptor-3 (NTRK-3) were consistently upregulated, related to neuron development and differentiation and additionally one of these genes harbored HERV-K(HML-2) LTRs in a proximal distance which was relevant to impart an influence on the gene expression. Thus, we regulated these genes to further understand the mechanism of impaired cortical neuron differentiation upon HML-2 LTR activation. Thus, LTR-mediated enhanced transcriptional activity and subsequent biological aspects in neuronal differentiation reported in this study reveals a myriad of gene regulatory network which could be via direct LTR control or a regulatory role of HERV-K(HML-2).

4.3 NTRK3 as a central factor in HERV-K(HML-2) LTR activation mediated cortical differentiation and development

Neurotrophins are a large family of closely-knit proteins which majorly function as growth factors for the survival, development and function of both central and peripheral nervous systems (Huang and Reichardt; 2001, Korsching; 1993). These factors regulate critical aspects of nervous system development such as proliferation in neural progenitor cells, morphological establishment and maintenance of individual neurons, apoptosis of neurons following injury and synaptic plasticity (Korsching; 1993, Large et al; 1986, Xie and Yung; 2012). Four different types of neurotrophins have been characterized, Nerve Growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Huang and Reichardt; 2001, Hallbook; 1999). NGF and BDNF has been reported to be involved in placental angiogenesis and maturation (Huang and Reichardt; 2001).

Neurotrophins mediate their actions by binding to tropomyosin-related kinase (Trk) receptors, the p75 neurotrophin receptors or Sortilin family of receptors (Huang and Reichardt; 2003). Several studies have shown the role of Neurotrophic Tyrosine Kinase Receptors in neurogenesis and development. There are mainly three types of neurotrophin receptors, NTRK1, NTRK2 and NTRK3 also known as TrkA, TrkB and TrkC respectively. These receptors mediate the signaling cascades of specific neurotrophins to relay their trophic effects. Specific neurotrophin induces dimerization and activation of neurotrophin receptors which are shown to be crucial for cell proliferation, neuronal survival and differentiation. The role of NTRK3 or TrkC has been studied and the neurotrophin-3 or NT-3 binds to its receptor NTRK3, activating the signaling cascade downstream which is responsible for the development of cortical precursors and cortical neurons. Inhibition of NTRK3 signaling resulted in impaired migration of neurons into cortical plate and reduced cortical precursor cell proliferation, which results in immature neurogenesis (Nikoletopoulou et al; 2010). Another study suggests that TrkA and TrkC prompts the developing neurons to death. Interestingly TrkB plays major role in neuron survival. Thus, TrkA and TrkC acts as dependence receptors modulating the neurons to be trophic factor-dependent for survival (Fagan et al; 1996).

In our experiments, activation of HERV-K(HML-2) LTRs resulted in the consistent upregulation of TrkC or NTRK3. Even though there are contradictory ideas pertaining to the role of NTRK3 in neuronal survival and differentiation, in our hands, the upregulation of NTRK3 was observed in the cortical neurons with LTR activation and perturbed cortical neuron differentiation and development, characterized by decreased expression levels of MAP2 and Synapsin-I and weakened neurite lengths and axonal extension. To address this in a more elaborate manner, we generated an NTRK3 activated stem cell line along with other genes of interest and studied the pattern of cortical differentiation. Our observations suggest an active role of NTRK3 in the phenotype of impaired cortical expansion and maturation evident from the decrease in MAP2 expression levels in cortical neurons. Furthermore, the morphological changes in the cultured neurons upon NTRK3 activation suggests a phenotypic pattern which mimics that of HERV-K(HML-2) activation. Thus, it can be conclusively concluded that NTRK3 plays a detrimental role in cortical differentiation upon HERV-K(HML-2) activation. The inducible activation of HERV-K(HML-2) LTRs using doxycycline and subsequent differentiation of these embryonic stem cells into cortical neurons revealed that activation at early time points of differentiation is crucial for the survival and maturation of the neurons. Induction of HERV-K(HML-2) at day 5 and day 10 of cortical differentiation resulted in impaired growth of neuronal progenitors. Induction of the LTR activation at day 21 post

progenitor state of neurons did not affect the progression of differentiation into a healthy state. Thus, it becomes more evident that the early development and HERV-K(HML-2) silencing is orchestrated in an immensely complicated and precise manner that a perturbance even in a small scale can have drastic impacts. It has been well established that modest alterations in the levels of secreted neurotrophins or polymorphisms in neurotrophic genes result in clinically and biologically relevant neurological impacts (Shen et al; 2018). The differences in the effects of the neurotrophin receptors upon their activation on a neuronal scenario can be attributed to the alternative splicing of these receptors. Differential splicing of TrkA, TrkB and TrkC might result in mRNAs that could be translated into different proteins that can vary in their pattern of expression upon ligand interactions. Thus, these splicing mechanisms and subsequent changes in the downstream signaling pathways can determine the specificity of response to different neurotrophins and their impact on neuronal development. This could be one of the major reasons behind the overlapping effects of neuronal rescue and neuronal death upon neurotrophin activation. In short, a timely precise and well-orchestrated ligand detection and ligand binding is imperative for the development of neurons in our system. We hypothesize that activation of the otherwise silent HERV-K(HML-2) LTRs in the embryonic stage of neurodevelopment results in the mis regulation of the precisely organized neurotrophic signaling cascade and this has deleterious impacts on neural progenitor formation and cortical neuron differentiation. It is a point to be noted that there is a recently integrated HERV-K (HML-2) LTR, named as LTR5_Hs approximately 283kb upstream of the NTRK3 transcription start site. The potential for long distance control of host transcription has been already reported for LTR5_Hs (Fuentes et al; 2018). Several endogenous retroviral LTRs, HERV-K in particular have been reported to be acting as anti-sense promoters (Domansky et al; 2000, Bhardwaj et al; 2015). The LTR5_Hs is located anti-sense to the NTRK3 gene, in plus-strand. Thus, it is possible that this LTR could serve as an antisense promoter which could potentially drive the early transcription of NTRK3 upon activation.

Thus, the cortical phenotype observed upon HERV-K(HML-2) LTR activation could be through a direct control of NTRK3 transcription by LTR5_Hs. However, the precise mechanistic detailing of how HERV-K(HML-2) LTR drives NTRK3 activation and mediates the cortical development and organization needs further studies. Our preliminary data suggests that there is a clear interconnection between the transcription of the LTRs and activation of NTRK3. Many studies have shown that the env protein of HERV-K is majorly responsible for the effects of activation. The study conducted in ALS patients and transgenic mouse models

depict the role of viral env in the disease progression (Li et al; 2015) A recent study on the regulatory role of HERV-K(HML-2) on stem cell function showed that the epigenetic silencing of HML-2 Env resulted in loss of stemness and enhanced differentiation pathways (Wang et al; 2020). However, in our data, there is no correlation of viral env or gag proteins to the observed phenotype. Neither the HERV-K(HML-2) LTR activated pluripotent cells nor the cortical neurons with activated LTRs showed any increase in env or gag transcript levels or protein levels in comparison to the controls. Another recent study conducted in the cerebrospinal fluids of black 6 wild type or the APPPSI mice which is the model for Alzheimer's Disease showed that the RNA from the envelope gene of HERV-K(HML-2) activates both human Toll-like receptor (TLR) 8 and murine Tlr7 which is predominantly expressed in neurons and microglia and resulted in neurodegeneration and apoptosis in these cells (Dembny et al; 2020). In our experiments, activation of HERV-K(HML-2) LTRs caused reduced neuronal functionality and decreased MAP2 and Synapsin expression. However, the data from the HERV-K(HML-2) activated neurons treated with different apoptotic and necrosis inhibitors demonstrated that the impaired cortical neuron phenotype observed upon HML-2 LTR activation is independent of the cell death pathways and is a separate mechanism pivoted by HERV-K(HML-2) LTR activation.

Thus, it is evident that the phenotype of cortical impairment which we observe upon HERV-K(HML-2) activation is not driven by virus encoded proteins or apoptotic signaling mechanisms, but a regulation of cellular gene expression via the LTRs. Modulation of NTRK3 receptor is the major key to the intricacies of neuronal regulation mediated by HERV-K (HML-2) LTRs. Although there is a possibility of HML-2 LTR acting as a direct antisense promoter of NTRK3 transcription, the scope of an enhancer effect of LTRs cannot be ruled out. An experiment to dissect the role of LTR as an enhancer would be a search for an LTR binding factor near the promoter and a CHIP assay to show RNA polymerase II and other active histone modifications at the LTR. Another approach for a direct demonstration of LTR as an enhancer would be a chromosome conformation capture or 3C assay. These experiments are time consuming but will give exact mechanistic detailing of the role of LTR in the activation of NTRK3. Alternative splice variants of HERV-K(HML-2) could act as modulators for the activation of NTRK3 transcription. This could be experimentally proved using methods which can show the splicing of the HERV-K(HML-2) in cortical neurons. Published Chromatin Immunoprecipitation assay (CHIP) data of NTRK3 and analysis of promoters of NTRK3 shows three different promoters for three different transcript variants. A quantitative Realtime

PCR with primers specific to each transcript variant can give an idea of which of the variant is upregulated and which promoter is active.

We have already looked into the transcript levels of interferon activating genes in the differentiated cortical neurons with LTR activation and controls. Increased transcription of Mx alpha, IkB and ISG56 was observed. CHIP assay and sequence studies of NTRK3 revealed an NFkB promoter near the transcription start site. This together with the fact that IkB upregulation could be read together and can be hypothesized that modulation of NTRK3 by HERV-K(HML-2 might be via the NFkB pathway. More detailed analyses and experiments has to be performed to substantiate this speculation.

It has been established thoroughly that HERVs are highly represses by solid epigenetic restriction mechanisms. In addition to the induced changes in the epigenetic regulatory pathways, environmental conditions such as radiations, chemicals, infectious agents, exogenous viruses etc can also act as spontaneous activators of these elements (Vincendeau et al; 2015, Li et al; 2014)). It is interesting to observe that mostly all type of cancers has reported activated levels of ERVs (Chen et al; 2019). Additionally, it is intriguing that most of the ERV reactivation events are directly or indirectly correlated with neurodegenerative diseases, as discussed in detail in previous sections. Any external or internal stimuli which can trigger the alteration of the chromatin state can result in spontaneous reactivation of these endogenous elements. One such observation is regarding the ageing process. Several functional studies point towards the immense role of epigenetic changes in the process of ageing, such as altered histone marks, DNA methylation patterns, introduction of histone variants etc (Pal and Tyler; 2016). These changes invariably lead to widespread genomic instability which can be directly linked to transposon activation. Thus, drawing a connection to the loss of genome integrity during normal senescence and increased manifestation of several cancers, autoimmune and neurodegenerative diseases etc due to reactivation of endogenous retroviruses cannot be an exaggeration. It is a point to be noted that an intricate balance of developmental factors can have long standing relevance in the context of transgenerational inheritance. My findings from this thesis strongly advocates the symphony of neurotrophins and human endogenous retroviruses for a healthy cortical development. A direct correlation of activation of the LTR of a specific HERV group and developmental delay and subsequent neurological manifestation has not yet been conclusively proven. Thus, my thesis stands relevant in the context of differential effect of HERV-K(HML-2) on neuronal lineages, opening the scope for promising studies ahead.

5. Conclusion and perspectives

This study conclusively shows an efficient model of CRISPR-based constitutive and inducible activation and repression of endogenous retroviruses and the effect of HERV-K(HML-2) LTR activation upon cortical neuron differentiation and brain development. It further details on the molecular basis of the observed phenotype of impaired cortical neuron differentiation, reduced neuronal functionality and defective laminar brain patterning upon HERV-K(HML-2) activation and points out a single gene which plays an active role in the manifestation of the phenotype. This data also shows that early activation of HERV-K(HML-2) can be detrimental in the development of cortical neurons and efficiency of neuronal migration and formation of deep layers of the brain. Conclusive experiments have been done to prove that phenotype developed upon HERV-K(HML-2) activation is not driven by the expression of viral proteins but is rather an LTR driven effect on the whole gene regulation.

It could be interesting to establish the exact pathway through which this is established. HERV-K(HML-2) LTR could be acting as an enhancer to regulate the expression of NTRK3. It could also be worthy to study the signaling pathway through which this information is carried and transmitted. Taken together, this study shows that activation of HERV-K(HML-2) results in the upregulation of a neurotrophin specific receptor, NTRK3 and untimely activation of this receptor can cause deleterious effects on the survival and functionality of the neurons, leading to many neuron-related disorders.

6. Summary

It has been reported that approximately 8% of the total genome make up of human beings appear to be contributed by retrotransposable endogenous retroviral elements known as human endogenous retroviruses or HERVs. There are almost 22 different families of HERVs studied, out of which Human endogenous Retrovirus K HML-2 or HERV-K(HML-2) has been studied extensively as the recently integrated and human specific member of the transposon milieu. In this study, I attempted to regulate the transcription of HERV-K (HML-2) LTRs in human embryonic stem cells using the CRISPR activation and CRISPR interference techniques. Transduction of specific single guide RNA pairs generated to target around 600 LTR sequences of the HERV-K(HML-2), along with the dCas9-VP64 fusion construct resulted in around 4-fold increase in the relative mRNA transcript levels of HERV-K(HML-2) LTRs. Subsequent neuronal differentiation experiments conducted in these cell lines along with appropriate controls resulted in an impaired development and differentiation phenotype in cortical neurons upon HERV-K(HML-2) activation, which was evident from the reduced MAP2 and Synapsin-I expression levels and decreased functionality in HERV-K(HML-2) activated cells in comparison with the controls. The morphology of the generated neurons was also affected resulting in shorter branching of neurites and axons. Differentiation experiments in dopaminergic neuronal lineage did not exhibit the observed neuronal phenotype, suggesting the outcome is cortical specific. Repression of HERV-K(HML-2) LTRs did not affect the process of cortical neuron differentiation. Further analysis into this observation using inducible transcriptional activation of HERV-K(HML-2) LTRs using inducible transcriptional activation of HERV-K(HML-2) LTRs by continuous doxycycline treatment from day 5, day 10 and day 21 up to day 60 of neuronal differentiation revealed that activation of HERV-K(HML-2) at early stages of differentiation, when neuroectoderm is established and neural progenitors are formed has a detrimental effect on the efficiency and morphology of cortical neurons.

Furthermore, forebrain organoids generated from control and HERV-K(HML-2) activated neurons showed marked differences in their size, organization of layers and expression of cell type specific protein markers. The HERV-K(HML-2) activated organoids were smaller in size compared to controls and the deep layer organization was more compact and structured in controls whereas in the case of HERV-K(HML-2) activated organoids, the layers were loosely organized with slower and less efficient neuronal migration.

Whole genome RNA sequencing data on HERV-K(HML-2) activated cortical neurons and controls revealed that several neuron related genes such as CLSTN2, CHRDL1, EPHA4, and

NTRK3 were consistently upregulated in cells where HML-2 LTR transcription was activated. Further analyses showed that these genes, along with others are associated with different stages of neurogenesis and with several neurodegenerative diseases. Out of these, NTRK3 transcriptional activation and differentiation into cortical neurons mimicked the debilitated cortical neuron phenotype similar to that observed upon HERV-K(HML-2) activation. Collectively, these findings unravel a unique cell-type specific regulatory mechanism of HERV-K(HML-2) during cortical neuronal differentiation paving the path to elucidate how de-regulation of endogenous elements can drive neurodegenerative disorders.

7. Bibliography

Albright, Andrew V., Samantha S. Soldan, and Francisco González-Scarano. 2003. "Pathogenesis of Human Immunodeficiency Virus-Induced Neurological Disease." *Journal of NeuroVirology*. Taylor and Francis Ltd. https://doi.org/10.1080/13550280390194073.

Alfahad, Tariq, and Avindra Nath. 2013. "Retroviruses and Amyotrophic Lateral Sclerosis." *Antiviral Research*. Antiviral Res. https://doi.org/10.1016/j.antiviral.2013.05.006.

Antony, Joseph M., Andre M. DesLauriers, Rakesh K. Bhat, Kristofer K. Ellestad, and Christopher Power. 2011. "Human Endogenous Retroviruses and Multiple Sclerosis: Innocent Bystanders or Disease Determinants?" *Biochimica et Biophysica Acta - Molecular Basis of Disease*. Biochim Biophys Acta. https://doi.org/10.1016/j.bbadis.2010.07.016.

Antony, Joseph M., Kristofor K. Ellestad, Robert Hammond, Kazunori Imaizumi, Francois Mallet, Kenneth G. Warren, and Christopher Power. 2007. "The Human Endogenous Retrovirus Envelope Glycoprotein, Syncytin-1, Regulates Neuroinflammation and Its Receptor Expression in Multiple Sclerosis: A Role for Endoplasmic Reticulum Chaperones in Astrocytes." *The Journal of Immunology* 179 (2): 1210–24. https://doi.org/10.4049/jimmunol.179.2.1210.

Arru, Giannina, Giuseppe Mameli, Vito Astone, Caterina Serra, Yu-Min Huang, Hans Link, Enrico Fainardi, et al. 2007. "Multiple Sclerosis and HERV-W/MSRV: A Multicentric Study." *International Journal of Biomedical Science : IJBS* 3 (4): 292–97. http://www.ncbi.nlm.nih.gov/pubmed/23675056.

Azevedo, Frederico A.C., Ludmila R.B. Carvalho, Lea T. Grinberg, José Marcelo Farfel, Renata E.L. Ferretti, Renata E.P. Leite, Wilson Jacob Filho, Roberto Lent, and Suzana Herculano-Houzel. 2009. "Equal Numbers of Neuronal and Nonneuronal Cells Make the Human Brain an Isometrically Scaled-up Primate Brain." *Journal of Comparative Neurology* 513 (5): 532–41. https://doi.org/10.1002/cne.21974.

Balestrieri, Emanuela, Claudia Matteucci, Chiara Cipriani, Sandro Grelli, Laura Ricceri, Gemma Calamandrei, and Paola Sinibaldi Vallebona. 2019. "Endogenous Retroviruses Activity as a Molecular Signature of Neurodevelopmental Disorders." *International Journal of Molecular Sciences*. MDPI AG. https://doi.org/10.3390/ijms20236050.

Balestrieri, Emanuela, Francesca Pica, Claudia Matteucci, Rossella Zenobi, Roberta Sorrentino, Ayele Argaw-Denboba, Chiara Cipriani, Ilaria Bucci, and Paola Sinibaldi-Vallebona. 2015. "Transcriptional Activity of Human Endogenous Retroviruses in Human Peripheral Blood Mononuclear Cells." *BioMed Research International* 2015. https://doi.org/10.1155/2015/164529.

Bannert, Norbert, Henning Hofmann, Adriana Block, and Oliver Hohn. 2018. "HERVs New Role in Cancer: From Accused Perpetrators to Cheerful Protectors." *Frontiers in Microbiology*. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2018.00178.

Bannert, Norbert, and Reinhard Kurth. 2004. "Retroelements and the Human Genome: New Perspectives on an Old Relation." *Proceedings of the National Academy of Sciences of the United States of America* 101 (SUPPL. 2): 14572–79. https://doi.org/10.1073/pnas.0404838101.

Baust, Corinna, Wolfgang Seifarth, Herbert Germaier, Rüdiger Hehlmann, and Christine Leib-Mösch. 2000. "HERV-K-T47D-Related Long Terminal Repeats Mediate Polyadenylation of Cellular Transcripts." *Genomics* 66 (1): 98–103. https://doi.org/10.1006/geno.2000.6175.

Bedem, Henry Van Den, and Ellen Kuhl. 2017. "Molecular Mechanisms of Chronic Traumatic Encephalopathy." https://doi.org/10.1016/j.cobme.2017.02.003.

Belshaw, Robert, Anna L. A. Dawson, John Woolven-Allen, Joanna Redding, Austin Burt, and Michael Tristem. 2005. "Genomewide Screening Reveals High Levels of Insertional Polymorphism in the Human Endogenous Retrovirus Family HERV-K(HML2): Implications for Present-Day Activity." *Journal of Virology* 79 (19): 12507–14. https://doi.org/10.1128/jvi.79.19.12507-12514.2005.

Belshaw, Robert, Vini Pereira, Aris Katzourakis, Gillian Talbot, Jan Pačes, Austin Burt, and Michael Tristem. 2004. "Long-Term Reinfection of the Human Genome by Endogenous Retroviruses." *Proceedings of the National Academy of Sciences of the United States of America* 101 (14): 4894–99. https://doi.org/10.1073/pnas.0307800101.

Benachenhou, Farid, Patric Jern, Merja Oja, Göran Sperber, Vidar Blikstad, Panu Somervuo, Samuel Kaski, and Jonas Blomberg. 2009. "Evolutionary Conservation of Orthoretroviral Long Terminal Repeats (LTRs) and Ab Initio Detection of Single LTRs in Genomic Data." Edited by Niyaz Ahmed. *PLoS ONE* 4 (4): e5179. https://doi.org/10.1371/journal.pone.0005179.

Bendiksen, Signy, Inigo Martinez-Zubiavrra, Conny Tümmler, Gunnar Knutsen, Jan Elvenes, Elisabeth Olsen, Randi Olsen, and Ugo Moens. 2014. "Human Endogenous Retrovirus w Activity in Cartilage of Osteoarthritis Patients." *BioMed Research International* 2014. https://doi.org/10.1155/2014/698609.

Berger, Thomas, and Markus Reindl. 2007. "Multiple Sclerosis: Disease Biomarkers as Indicated by Pathophysiology." *Journal of the Neurological Sciences* 259 (1–2): 21–26. https://doi.org/10.1016/j.jns.2006.05.070.

Bhardwaj, Neeru, Meagan Montesio, Farrah Roy, and John M. Coffin. 2015. "Differential Expression of HERV-K (HML-2) Proviruses in Cells and Virions of the Teratocarcinoma Cell Line Tera-1." *Viruses* 7 (3): 939–68. https://doi.org/10.3390/v7030939.

Bjerregaard, B., S. Holck, I. J. Christensen, and L. I. Larsson. 2006. "Syncytin Is Involved in Breast Cancer-Endothelial Cell Fusions." *Cellular and Molecular Life Sciences* 63 (16): 1906–11. https://doi.org/10.1007/s00018-006-6201-9.

Blond, Jean-Luc, Dimitri Lavillette, Valérie Cheynet, Olivier Bouton, Guy Oriol, Sylvie Chapel-Fernandes, Bernard Mandrand, François Mallet, and François-Loïc Cosset. 2000. "An Envelope Glycoprotein of the Human Endogenous Retrovirus HERV-W Is Expressed in the Human Placenta and Fuses Cells Expressing the Type D Mammalian Retrovirus Receptor." *Journal of Virology* 74 (7): 3321–29. https://doi.org/10.1128/jvi.74.7.3321-3329.2000.

Brattås, Per Ludvik, Marie E. Jönsson, Liana Fasching, Jenny Nelander Wahlestedt, Mansoureh Shahsavani, Ronny Falk, Anna Falk, Patric Jern, Malin Parmar, and Johan Jakobsson. 2017. "TRIM28 Controls a Gene Regulatory Network Based on Endogenous Retroviruses in Human Neural Progenitor Cells."

Cell Reports 18 (1): 1–11. https://doi.org/10.1016/j.celrep.2016.12.010.

Brudek, Tomasz, Tove Christensen, Hans Jacob Hansen, Jadwiga Bobecka, and Anne Møller-Larsen. 2004. "Simultaneous Presence of Endogenous Retrovirus and Herpes Virus Antigens Has Profound Effect on Cell-Mediated Immune Responses: Implications for Multiple Sclerosis." *AIDS Research and Human Retroviruses* 20 (4): 415–23. https://doi.org/10.1089/088922204323048168.

Brudek, Tomasz, Pernille Lühdorf, Tove Christensen, Hans Jacob Hansen, and Anné Møller-Larsen. 2007. "Activation of Endogenous Retrovirus Reverse Transcriptase in Multiple Sclerosis Patient Lymphocytes by Inactivated HSV-1, HHV-6 and VZV." *Journal of Neuroimmunology* 187 (1–2): 147–55. https://doi.org/10.1016/j.jneuroim.2007.04.003.

Buzdin, Anton, Elena Kovalskaya-Alexandrova, Elena Gogvadze, and Eugene Sverdlov. 2006. "At Least 50% of Human-Specific HERV-K (HML-2) Long Terminal Repeats Serve In Vivo as Active Promoters for Host Nonrepetitive DNA Transcription." *Journal of Virology* 80 (21): 10752–62. https://doi.org/10.1128/jvi.00871-06.

Cáceres, Mario, and James W. Thomas. 2006. "The Gene of Retroviral Origin Syncytin 1 Is Specific to Hominoids and Is Inactive in Old World Monkeys." *Journal of Heredity* 97 (2): 100–106. https://doi.org/10.1093/jhered/esj011.

Carlton, Victoria E.H., Baruch Z. Harris, Erik G. Puffenberger, A. K. Batta, A. S. Knisely, Donna L. Robinson, Kevin A. Strauss, et al. 2003. "Complex Inheritance of Familial Hypercholanemia with Associated Mutations in TJP2 and BAAT." *Nature Genetics* 34 (1): 91–96. https://doi.org/10.1038/ng1147.

Cegolon, Luca, Cristiano Salata, Elisabete Weiderpass, Paolo Vineis, Giorgio Palù, and Giuseppe Mastrangelo. 2013. "Human Endogenous Retroviruses and Cancer Prevention: Evidence and Prospects." *BMC Cancer* 13 (1): 4. https://doi.org/10.1186/1471-2407-13-4.

Chambers, Stuart M., Christopher A. Fasano, Eirini P. Papapetrou, Mark Tomishima, Michel Sadelain, and Lorenz Studer. 2009. "Highly Efficient Neural Conversion of Human ES and IPS Cells by Dual Inhibition of SMAD Signaling." *Nature Biotechnology* 27 (3): 275–80. https://doi.org/10.1038/nbt.1529.

Chen, Jungang, Maryam Foroozesh, and Zhiqiang Qin. 2019. "Transactivation of Human Endogenous Retroviruses by Tumor Viruses and Their Functions in Virus-Associated Malignancies." *Oncogenesis*. Nature Publishing Group. https://doi.org/10.1038/s41389-018-0114-y.

Cheng, Albert W., Haoyi Wang, Hui Yang, Linyu Shi, Yarden Katz, Thorold W. Theunissen, Sudharshan Rangarajan, Chikdu S. Shivalila, Daniel B. Dadon, and Rudolf Jaenisch. 2013. "Multiplexed Activation of Endogenous Genes by CRISPR-on, an RNA-Guided Transcriptional Activator System." *Cell Research* 23 (10): 1163–71. https://doi.org/10.1038/cr.2013.122.

Choi, Myungwon, Hyun Chul Youn, Daegyeom Kim, Suji Lee, Sangil Suh, Joon Kyung Seong, Hyun Ghang Jeong, and Cheol E. Han. 2019. "Comparison of Neurodegenerative Types Using Different Brain MRI Analysis Metrics in Older Adults with Normal Cognition, Mild Cognitive Impairment, and Alzheimer's Dementia." *PLoS ONE* 14 (8). https://doi.org/10.1371/journal.pone.0220739.

Christensen, T. 2007. “Human Herpesviruses in MS .” *Int Ms J* 14 (2): 41–47. https://pubmed.ncbi.nlm.nih.gov/17686342/.

Chuong, Edward B. 2018. “The Placenta Goes Viral: Retroviruses Control Gene Expression in Pregnancy.” *PLOS Biology* 16 (10): e3000028. https://doi.org/10.1371/journal.pbio.3000028.

Chuong, Edward B., Nels C. Elde, and Cédric Feschotte. 2017. “Regulatory Activities of Transposable Elements: From Conflicts to Benefits.” *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg.2016.139.

Chuong, Edward B., M. A.Karim Rumi, Michael J. Soares, and Julie C. Baker. 2013. “Endogenous Retroviruses Function as Species-Specific Enhancer Elements in the Placenta.” *Nature Genetics* 45 (3): 325–29. https://doi.org/10.1038/ng.2553.

Cong, Le, F. Ann Ran, David Cox, Shuailiang Lin, Robert Barretto, Naomi Habib, Patrick D. Hsu, et al. 2013. “Multiplex Genome Engineering Using CRISPR/Cas Systems.” *Science* 339 (6121): 819–23. https://doi.org/10.1126/science.1231143.

Cordaux, Richard, and Mark A. Batzer. 2009. “The Impact of Retrotransposons on Human Genome Evolution.” *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg2640.

Dantec, Christelle Le, Marie-Michele Varin, Wesley H Brooks, Jacques-Olivier Pers, Pierre Youinou, and Yves Renaudineau. 2012. “Epigenetics and Sjogren’s Syndrome.” *Current Pharmaceutical Biotechnology* 13 (10): 2046–53. https://doi.org/10.2174/138920112802273326.

Deltcheva, Elitza, Krzysztof Chylinski, Cynthia M. Sharma, Karine Gonzales, Yanjie Chao, Zaid A. Pirzada, Maria R. Eckert, Jörg Vogel, and Emmanuelle Charpentier. 2011. “CRISPR RNA Maturation by Trans-Encoded Small RNA and Host Factor RNase III.” *Nature* 471 (7340): 602–7. https://doi.org/10.1038/nature09886.

Dembny, Paul, Andrew G. Newman, Manvendra Singh, Michael Hinz, Michal Szczepek, Christina Krüger, Robert Adalbert, et al. 2020. “Human Endogenous Retrovirus HERVK(HML-2) RNA Causes Neurodegeneration through Toll-like Receptors.” *JCI Insight* 5 (7). https://doi.org/10.1172/JCI.INSIGHT.131093.

Deniz, Özgen, Mamataz Ahmed, Christopher D. Todd, Ana Rio-Machin, Mark A. Dawson, and Miguel R. Branco. 2020. “Endogenous Retroviruses Are a Source of Enhancers with Oncogenic Potential in Acute Myeloid Leukaemia.” *Nature Communications* 11 (1): 1–14. https://doi.org/10.1038/s41467-020-17206-4.

Denne, Miriam, Marlies Sauter, Vivienne Armbruester, Jonathan D. Licht, Klaus Roemer, and Nikolaus Mueller-Lantzsch. 2007. “Physical and Functional Interactions of Human Endogenous Retrovirus Proteins Np9 and Rec with the Promyelocytic Leukemia Zinc Finger Protein.” *Journal of Virology* 81 (11): 5607–16. https://doi.org/10.1128/jvi.02771-06.

Dobson, R., and G. Giovannoni. 2019. “Multiple Sclerosis – a Review.” *European Journal of Neurology*. Blackwell Publishing Ltd. https://doi.org/10.1111/ene.13819.

Dolei, Antonina. 2018. “The Aliens inside Us: HERV-W Endogenous Retroviruses and Multiple Sclerosis.” *Multiple Sclerosis* 24 (1): 42–47. https://doi.org/10.1177/1352458517737370.

Domansky, Andrey N., Eugene P. Kopantzev, Eugene V. Snezhkov, Yuri B. Lebedev, Christine Leib-Mosch, and Eugene D. Sverdlov. 2000. "Solitary HERV-K LTRs Possess Bi-Directional Promoter Activity and Contain a Negative Regulatory Element in the U5 Region." *FEBS Letters* 472 (2–3): 191–95. https://doi.org/10.1016/S0014-5793(00)01460-5.

Douville, Renée, Jiankai Liu, Jeffrey Rothstein, and Avindra Nath. 2011. "Identification of Active Loci of a Human Endogenous Retrovirus in Neurons of Patients with Amyotrophic Lateral Sclerosis." *Annals of Neurology* 69 (1): 141–51. https://doi.org/10.1002/ana.22149.

Douville, Renée N., and Avindra Nath. 2014. "Human Endogenous Retroviruses and the Nervous System." In *Handbook of Clinical Neurology*, 123:465–85. Elsevier B.V. https://doi.org/10.1016/B978-0-444-53488-0.00022-5.

Douville, Renée N., and Avindra Nath. 2017. "Human Endogenous Retrovirus-K and TDP-43 Expression Bridges ALS and HIV Neuropathology." *Frontiers in Microbiology* 8 (OCT). https://doi.org/10.3389/fmicb.2017.01986.

Downey, Ronan F., Francis J. Sullivan, Feng Wang-Johanning, Stefan Ambs, Francis J. Giles, and Sharon A. Glynn. 2015. "Human Endogenous Retrovirus K and Cancer: Innocent Bystander or Tumorigenic Accomplice?" *International Journal of Cancer* 137 (6): 1249–57. https://doi.org/10.1002/ijc.29003.

Dunlap, Kathrin A., Massimo Palmarini, Mariana Varela, Robert C. Burghardt, Kanako Hayashi, Jennifer L. Farmer, and Thomas E. Spencer. 2006. "Endogenous Retroviruses Regulate Periimplantation Placental Growth and Differentiation." *Proceedings of the National Academy of Sciences of the United States of America* 103 (39): 14390–95. https://doi.org/10.1073/pnas.0603836103.

E, Fanales-Belasio, Raimondo M, Suligoi B, and Buttò S. 2010. "HIV Virology and Pathogenetic Mechanisms of Infection: A Brief Overview." *Annali Dell'Istituto Superiore Di Sanita* 46 (1). https://doi.org/10.4415/ANN_10_01_02.

Ecco, Gabriela, Michael Imbeault, and Didier Trono. 2017. "KRAB Zinc Finger Proteins." *Development (Cambridge)* 144 (15): 2719–29. https://doi.org/10.1242/dev.132605.

Ejtehadi, H. D., G. L. Freimanis, H. A. Ali, S. Bowman, A. Alavi, J. Axford, R. Callaghan, and P. N. Nelson. 2006. "The Potential Role of Human Endogenous Retrovirus K10 in the Pathogenesis of Rheumatoid Arthritis: A Preliminary Study." *Annals of the Rheumatic Diseases* 65 (5): 612–16. https://doi.org/10.1136/ard.2004.031146.

Elsässer, Simon J., Kyung Min Noh, Nichole Diaz, C. David Allis, and Laura A. Banaszynski. 2015. "Histone H3.3 Is Required for Endogenous Retroviral Element Silencing in Embryonic Stem Cells." *Nature* 522 (7555): 240–44. https://doi.org/10.1038/nature14345.

Emerson, R. O., and J. H. Thomas. 2011. "Gypsy and the Birth of the SCAN Domain." *Journal of Virology* 85 (22): 12043–52. https://doi.org/10.1128/jvi.00867-11.

Emerson, Ryan O., and James H. Thomas. 2009. "Adaptive Evolution in Zinc Finger Transcription Factors." Edited by Simon Myers. *PLoS Genetics* 5 (1): e1000325. https://doi.org/10.1371/journal.pgen.1000325.

Fagan, Anne M., Hong Zhang, Story Landis, Richard J. Smeyne, Inmaculada Silos-Santiago, and Mariano

Barbacid. 1996. “TrkA, but Not TrkC, Receptors Are Essential for Survival of Sympathetic Neurons in Vivo.” *Journal of Neuroscience* 16 (19): 6208–18. https://doi.org/10.1523/jneurosci.16-19-06208.1996.

Fasching, Liana, Adamandia Kapopoulou, Rohit Sachdeva, Rebecca Petri, Marie E. Jönsson, Christian Männe, Priscilla Turelli, et al. 2015. “TRIM28 Represses Transcription of Endogenous Retroviruses in Neural Progenitor Cells.” *Cell Reports* 10 (1): 20–28. https://doi.org/10.1016/j.celrep.2014.12.004.

Feschotte, Cleacuteldric, and Ellen J. Pritham. 2007. “DNA Transposons and the Evolution of Eukaryotic Genomes.” *Annual Review of Genetics*. Annu Rev Genet. https://doi.org/10.1146/annurev.genet.40.110405.090448.

Fotheringham, J Jacobson,S. 2005. “Human Herpesvirus 6 and Multiple Sclerosis: Potential Mechanisms for Virus-Induced Disease - PubMed.” *Herpes* 12 (1): 4–9. https://pubmed.ncbi.nlm.nih.gov/16026638/.

Frank, Oliver, Michelle Giehl, Chun Zheng, Rüdiger Hehlmann, Christine Leib-Mösch, and Wolfgang Seifarth. 2005. “Human Endogenous Retrovirus Expression Profiles in Samples from Brains of Patients with Schizophrenia and Bipolar Disorders.” *Journal of Virology* 79 (17): 10890–901. https://doi.org/10.1128/jvi.79.17.10890-10901.2005.

Frank, Oliver, Lorraine Jones-Brando, Christine Leib-Mösch, Robert Yolken, and Wolfgang Seifarth. 2006. “Altered Transcriptional Activity of Human Endogenous Retroviruses in Neuroepithelial Cells after Infection with *Toxoplasma Gondii*.” *The Journal of Infectious Diseases* 194 (10): 1447–49. https://doi.org/10.1086/508496.

Freimanis, G., P. Hooley, H. Davari Ejtehadi, H. A. Ali, A. Veitch, P. B. Rylance, A. Alawi, et al. 2010. “A Role for Human Endogenous Retrovirus-K (HML-2) in Rheumatoid Arthritis: Investigating Mechanisms of Pathogenesis.” *Clinical and Experimental Immunology* 160 (3): 340–47. https://doi.org/10.1111/j.1365-2249.2010.04110.x.

Frendo, Jean-Louis, Delphine Olivier, Valérie Cheynet, Jean-Luc Blond, Olivier Bouton, Michel Vidaud, Michèle Rabreau, Danièle Evain-Brion, and François Mallet. 2003a. “Direct Involvement of HERV-W Env Glycoprotein in Human Trophoblast Cell Fusion and Differentiation.” *Molecular and Cellular Biology* 23 (10): 3566–74. https://doi.org/10.1128/mcb.23.10.3566-3574.2003.

Friedman, Josh R., William J. Fredericks, David E. Jensen, David W. Speicher, Xiao Pei Huang, Eric G. Neilson, and Frank J. Rauscher. 1996. “KAP-1, a Novel Corepressor for the Highly Conserved KRAB Repression Domain.” *Genes and Development* 10 (16): 2067–78. https://doi.org/10.1101/gad.10.16.2067.

Frietze, Seth, Henriette O’Geen, Kimberly R. Blahnik, Victor X. Jin, and Peggy J. Farnham. 2010. “ZNF274 Recruits the Histone Methyltransferase SETDB1 to the 3′ Ends of ZNF Genes.” Edited by Pierre-Antoine Defossez. *PLoS ONE* 5 (12): e15082. https://doi.org/10.1371/journal.pone.0015082.

Fuchs, N. V., M. Kraft, C. Tondera, K.-M. Hanschmann, J. Lower, and R. Lower. 2011. “Expression of the Human Endogenous Retrovirus (HERV) Group HML-2/HERV-K Does Not Depend on Canonical Promoter Elements but Is Regulated by Transcription Factors Sp1 and Sp3.” *Journal of Virology* 85

(7): 3436–48. https://doi.org/10.1128/jvi.02539-10.

Fuentes, Daniel R., Tomek Swigut, and Joanna Wysocka. 2018. "Systematic Perturbation of Retroviral LTRs Reveals Widespread Long-Range Effects on Human Gene Regulation." *ELife* 7 (August). https://doi.org/10.7554/eLife.35989.

Gao, Lei, Keliang Wu, Zhenbo Liu, Xuelong Yao, Shenli Yuan, Wenrong Tao, Lizhi Yi, et al. 2018. "Chromatin Accessibility Landscape in Human Early Embryos and Its Association with Evolution." *Cell* 173 (1): 248-259.e15. https://doi.org/10.1016/j.cell.2018.02.028.

García-Montojo, Marta, Belén de la Hera, Jezabel Varadé, Ana de la Encarnación, Iris Camacho, María Domínguez-Mozo, Ana Arias-Leal, et al. 2014. "HERV-W Polymorphism in Chromosome X Is Associated with Multiple Sclerosis Risk and with Differential Expression of MSRV." *Retrovirology* 11 (1): 2. https://doi.org/10.1186/1742-4690-11-2.

Garcia-Etxebarria, Koldo, Maialen Sistiaga-Poveda, and Begona Jugo. 2014. "Endogenous Retroviruses in Domestic Animals." *Current Genomics* 15 (4): 256–65. https://doi.org/10.2174/1389202915666140520003503.

Garrison, Keith E., R. Brad Jones, Duncan A. Meiklejohn, Naveed Anwar, Lishomwa C. Ndhlovu, Joan M. Chapman, Ann L. Erickson, et al. 2007. "T Cell Responses to Human Endogenous Retroviruses in HIV-1 Infection." *PLoS Pathogens* 3 (11): 1617–27. https://doi.org/10.1371/journal.ppat.0030165.

Gasiunas, Giedrius, Rodolphe Barrangou, Philippe Horvath, and Virginijus Siksnys. 2012. "Cas9-CrRNA Ribonucleoprotein Complex Mediates Specific DNA Cleavage for Adaptive Immunity in Bacteria." *Proceedings of the National Academy of Sciences of the United States of America* 109 (39): E2579–86. https://doi.org/10.1073/pnas.1208507109.

Geis, Franziska K., and Stephen P. Goff. 2020. "Silencing and Transcriptional Regulation of Endogenous Retroviruses: An Overview." *Viruses*. MDPI AG. https://doi.org/10.3390/v12080884.

Gemmell, Patrick, Jotun Hein, and Aris Katzourakis. 2019. "The Exaptation of HERV-H: Evolutionary Analyses Reveal the Genomic Features of Highly Transcribed Elements." *Frontiers in Immunology* 10 (JULY): 1339. https://doi.org/10.3389/fimmu.2019.01339.

Gilbert, Luke A., Max A. Horlbeck, Britt Adamson, Jacqueline E. Villalta, Yuwen Chen, Evan H. Whitehead, Carla Guimaraes, et al. 2014. "Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation." *Cell* 159 (3): 647–61. https://doi.org/10.1016/j.cell.2014.09.029.

Gilbert, Luke A., Matthew H. Larson, Leonardo Morsut, Zairan Liu, Gloria A. Brar, Sandra E. Torres, Noam Stern-Ginossar, et al. 2013. "XCRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes." *Cell* 154 (2): 442. https://doi.org/10.1016/j.cell.2013.06.044.

Gogvadze, Elena, Elena Stukacheva, Anton Buzdin, and Eugene Sverdlov. 2009. "Human-Specific Modulation of Transcriptional Activity Provided by Endogenous Retroviral Insertions." *Journal of Virology* 83 (12): 6098–6105. https://doi.org/10.1128/jvi.00123-09.

Göke, Jonathan, Xinyi Lu, Yun Shen Chan, Huck Hui Ng, Lam Ha Ly, Friedrich Sachs, and Iwona Szczerbinska. 2015. "Dynamic Transcription of Distinct Classes of Endogenous Retroviral Elements Marks Specific Populations of Early Human Embryonic Cells." *Cell Stem Cell* 16 (2): 135–41.

https://doi.org/10.1016/j.stem.2015.01.005.

Göke, Jonathan, and Huck Hui Ng. 2016. " CTRL + INSERT : Retrotransposons and Their Contribution to Regulation and Innovation of the Transcriptome ." *EMBO Reports* 17 (8): 1131–44. https://doi.org/10.15252/embr.201642743.

Gonzalez-Cao, María, Paola Iduma, Niki Karachaliou, Mariacarmela Santarpia, Julià Blanco, and Rafael Rosell. 2016. "Human Endogenous Retroviruses and Cancer." *Cancer Biology and Medicine*. Cancer Biology and Medicine. https://doi.org/10.20892/j.issn.2095-3941.2016.0080.

Grandi, Nicole, and Enzo Tramontano. 2017. "Type W Human Endogenous Retrovirus (HERV-W) Integrations and Their Mobilization by L1 Machinery: Contribution to the Human Transcriptome and Impact on the Host Physiopathology." *Viruses*. MDPI AG. https://doi.org/10.3390/v9070162.

Grandi, Nicole, and Enzo Tramontano. 2018. "HERV Envelope Proteins: Physiological Role and Pathogenic Potential in Cancer and Autoimmunity." *Frontiers in Microbiology*. Frontiers Media S.A. https://doi.org/10.3389/fmicb.2018.00462.

Grassi, Daniela A., Marie E. Jönsson, Per Ludvik Brattås, and Johan Jakobsson. 2019. "TRIM28 and the Control of Transposable Elements in the Brain." *Brain Research*. Elsevier B.V. https://doi.org/10.1016/j.brainres.2018.02.043.

Greene, Warner C., and B. Matija Peterlin. 2002. "Charting HIV's Remarkable Voyage through the Cell: Basic Science as a Passport to Future Therapy." *Nature Medicine*. Nat Med. https://doi.org/10.1038/nm0702-673.

Greenwood, Alex D., Anna Stengel, Volker Erfle, Wolfgang Seifarth, and Christine Leib-Mösch. 2005. "The Distribution of Pol Containing Human Endogenous Retroviruses in Non-Human Primates." *Virology* 334 (2): 203–13. https://doi.org/10.1016/j.virol.2005.01.045.

Gregory, T. Ryan. 2005. "Synergy between Sequence and Size in Large-Scale Genomics." *Nature Reviews Genetics*. Nat Rev Genet. https://doi.org/10.1038/nrg1674.

Griffiths, D. J. 2001. "Endogenous Retroviruses in the Human Genome Sequence." *Genome Biology*. BioMed Central. https://doi.org/10.1186/gb-2001-2-6-reviews1017.

Gu, Bo, Tomek Swigut, Andrew Spencley, Matthew R. Bauer, Mingyu Chung, Tobias Meyer, and Joanna Wysocka. 2018. "Transcription-Coupled Changes in Nuclear Mobility of Mammalian Cis-Regulatory Elements." *Science* 359 (6379): 1050–55. https://doi.org/10.1126/science.aao3136.

Guo, Caiwei, Hyun Hwan Jeong, Yi Chen Hsieh, Hans Ulrich Klein, David A. Bennett, Philip L. De Jager, Zhandong Liu, and Joshua M. Shulman. 2018. "Tau Activates Transposable Elements in Alzheimer's Disease." *Cell Reports* 23 (10): 2874–80. https://doi.org/10.1016/j.celrep.2018.05.004.

Gutierrez-Arcelus, Maria, Stephen S. Rich, and Soumya Raychaudhuri. 2016. "Autoimmune Diseases-Connecting Risk Alleles with Molecular Traits of the Immune System." *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg.2015.33.

Hallböök, Finn. 1999. "Evolution of the Vertebrate Neurotrophin and Trk Receptor Gene Families." *Current Opinion in Neurobiology*. Current Biology Ltd. https://doi.org/10.1016/S0959-4388(99)00011-2.

Hammachi, Fella, Gillian M. Morrison, Alexei A. Sharov, Alessandra Livigni, Santosh Narayan, Eirini P.

Papapetrou, James O'Malley, et al. 2012. "Transcriptional Activation by Oct4 Is Sufficient for the Maintenance and Induction of Pluripotency." *Cell Reports* 1 (2): 99–109. https://doi.org/10.1016/j.celrep.2011.12.002.

Hardiman, Orla, Ammar Al-Chalabi, Adriano Chio, Emma M. Corr, Giancarlo Logroscino, Wim Robberecht, Pamela J. Shaw, Zachary Simmons, and Leonard H. Van Den Berg. 2017. "Amyotrophic Lateral Sclerosis." *Nature Reviews Disease Primers*. Nature Publishing Group. https://doi.org/10.1038/nrdp.2017.71.

Harris, Taylor C., Rijk de Rooij, and Ellen Kuhl. 2019. "The Shrinking Brain: Cerebral Atrophy Following Traumatic Brain Injury." *Annals of Biomedical Engineering* 47 (9): 1941–59. https://doi.org/10.1007/s10439-018-02148-2.

Hegyi, Hedi. 2013. "GABBR1 Has a HERV-W LTR in Its Regulatory Region - a Possible Implication for Schizophrenia." *Biology Direct*. BioMed Central. https://doi.org/10.1186/1745-6150-8-5.

Heinrichs, R. Walter, and Konstantine K. Zakzanis. 1998. "Neurocognitive Deficit in Schizophrenia: A Quantitative Review of the Evidence." *Neuropsychology* 12 (3): 426–45. https://doi.org/10.1037//0894-4105.12.3.426.

Herculano-Houzel, Suzana. 2009. "The Human Brain in Numbers: A Linearly Scaled-up Primate Brain." *Frontiers in Human Neuroscience* 3 (NOV): 31. https://doi.org/10.3389/neuro.09.031.2009.

Herzog, Marielle, Olivia Wendling, Florian Guillou, Pierre Chambon, Manuel Mark, Régine Losson, and Florence Cammas. 2011. "TIF1β Association with HP1 Is Essential for Post-Gastrulation Development, but Not for Sertoli Cell Functions during Spermatogenesis." *Developmental Biology* 350 (2): 548–58. https://doi.org/10.1016/j.ydbio.2010.12.014.

Hey, Jody. 2005. "The Ancestor's Tale A Pilgrimage to the Dawn of Evolution." *Journal of Clinical Investigation* 115 (7): 1680–1680. https://doi.org/10.1172/jci25761.

Hofman, Michel A. 1988. "Size and Shape of the Cerebral Cortex in Mammals: II. The Cortical Volume." *Brain, Behavior and Evolution* 32 (1): 17–26. https://doi.org/10.1159/000116529.

Hofman, Michel A.2014. "Evolution of the Human Brain: When Bigger Is Better." *Frontiers in Neuroanatomy* 8 (MAR): 15. https://doi.org/10.3389/fnana.2014.00015.

Hogstrom, Larson J., Lars T. Westlye, Kristine B. Walhovd, and Anders M. Fjell. 2013. "The Structure of the Cerebral Cortex across Adult Life: Age-Related Patterns of Surface Area, Thickness, and Gyrification." *Cerebral Cortex* 23 (11): 2521–30. https://doi.org/10.1093/cercor/bhs231.

Hohenadl, Christine, Herbert Germaier, Monika Walchner, Manuela Hagenhofer, Martin Herrmann, Michael Stürzl, Peter Kind, Rüdiger Hehlmann, Volker Erfle, and Christine Leib-Mösch. 1999. "Transcriptional Activation of Endogenous Retroviral Sequences in Human Epidermal Keratinocytes by UVB Irradiation." *Journal of Investigative Dermatology* 113 (4): 587–94. https://doi.org/10.1046/j.1523-1747.1999.00728.x.

Hohn, Oliver, Kirsten Hanke, and Norbert Bannert. 2013. "HERV-K(HML-2), the Best Preserved Family of HERVs: Endogenization, Expression, and Implications in Health and Disease." *Frontiers in Oncology*. Front Oncol. https://doi.org/10.3389/fonc.2013.00246.

Horvath, Philippe, and Rodolphe Barrangou. 2010. “CRISPR/Cas, the Immune System of Bacteria and Archaea.” *Science*. Science. https://doi.org/10.1126/science.1179555.

Hou, Zhonggang, Yan Zhang, Nicholas E. Propson, Sara E. Howden, Li Fang Chu, Erik J. Sontheimer, and James A. Thomson. 2013. “Efficient Genome Engineering in Human Pluripotent Stem Cells Using Cas9 from Neisseria Meningitidis.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (39): 15644–49. https://doi.org/10.1073/pnas.1313587110.

Howard, G., R. Eiges, F. Gaudet, R. Jaenisch, and A. Eden. 2008. “Activation and Transposition of Endogenous Retroviral Elements in Hypomethylation Induced Tumors in Mice.” *Oncogene* 27 (3): 404–8. https://doi.org/10.1038/sj.onc.1210631.

Hu, Wei Shau, and Stephen H. Hughes. 2012. “HIV-1 Reverse Transcription.” *Cold Spring Harbor Perspectives in Medicine* 2 (10). https://doi.org/10.1101/cshperspect.a006882.

Huang, E. J., and L. F. Reichardt. 2001. “Neurotrophins: Roles in Neuronal Development and Function.” *Annual Review of Neuroscience*. Annu Rev Neurosci. https://doi.org/10.1146/annurev.neuro.24.1.677.

Huang, Eric J., and Louis F. Reichardt. 2003. “Trk Receptors: Roles in Neuronal Signal Transduction.” *Annual Review of Biochemistry*. Annu Rev Biochem. https://doi.org/10.1146/annurev.biochem.72.121801.161629.

Huang, Kevin, and Guoping Fan. 2011. “Epigenetic Modifications in Distinction: Histone versus DNA Methylation in ESCs.” *Cell Stem Cell*. Cell Press. https://doi.org/10.1016/j.stem.2011.05.007.

Huang, W., T. Chen, Z. Meng, Y. Gan, X. Wang, F. Xu, Y. Gu, et al. 2013. “The Viral Oncogene Np9 Acts as a Critical Molecular Switch for Co-Activating β-Catenin, ERK, Akt and Notch1 and Promoting the Growth of Human Leukemia Stem/Progenitor Cells.” *Leukemia* 27 (7): 1469–78. https://doi.org/10.1038/leu.2013.8.

Hughes, Jennifer F., and John M. Coffin. 2004. “Human Endogenous Retrovirus K Solo-LTR Formation and Insertional Polymorphisms: Implications for Human and Viral Evolution.” *Proceedings of the National Academy of Sciences of the United States of America* 101 (6): 1668–72. https://doi.org/10.1073/pnas.0307885100.

Hurst, Tara P., and Gkikas Magiorkinis. 2017. “Epigenetic Control of Human Endogenous Retrovirus Expression: Focus on Regulation of Long-Terminal Repeats (LTRs).” *Viruses*. MDPI AG. https://doi.org/10.3390/v9060130.

Hutchins, Andrew Paul, and Duanqing Pei. 2015. “Transposable Elements at the Center of the Crossroads between Embryogenesis, Embryonic Stem Cells, Reprogramming, and Long Non-Coding RNAs.” *Science Bulletin*. Science in China Press. https://doi.org/10.1007/s11434-015-0905-x.

Hutnick, Leah K., Peyman Golshani, Masakasu Namihira, Zhigang Xue, Anna Matynia, X. William Yang, Alcino J. Silva, Felix E. Schweizer, and Guoping Fan. 2009. “DNA Hypomethylation Restricted to the Murine Forebrain Induces Cortical Degeneration and Impairs Postnatal Neuronal Maturation.” *Human Molecular Genetics* 18 (15): 2875–88. https://doi.org/10.1093/hmg/ddp222.

Hutnick, Leah K., Xinhua Huang, Tao Chuan Loo, Zhicheng Ma, and Guoping Fan. 2010. “Repression of Retrotransposal Elements in Mouse Embryonic Stem Cells Is Primarily Mediated by a DNA

Methylation-Independent Mechanism." *Journal of Biological Chemistry* 285 (27): 21082–91. https://doi.org/10.1074/jbc.M110.125674.

Ito, Jumpei, Izumi Kimura, Andrew Soper, Alexandre Coudray, Yoshio Koyanagi, Hirofumi Nakaoka, Ituro Inoue, Priscilla Turelli, Didier Trono, and Kei Sato. 2020. "Endogenous Retroviruses Drive KRAB Zinc-Finger Protein Family Expression for Tumor Suppression."*ScienceAdvances*6 (43): eabc3020. https://doi.org/10.1126/sciadv.abc3020.

Jeon, Gye Sun, Yu Mi Shim, Do Yeon Lee, Jun Soon Kim, Min Jin Kang, So Hyun Ahn, Je Young Shin, Dongho Geum, Yoon Ho Hong, and Jung Joon Sung. 2019. "Pathological Modification of TDP-43 in Amyotrophic Lateral Sclerosis with SOD1 Mutations." *Molecular Neurobiology* 56 (3): 2007–21. https://doi.org/10.1007/s12035-018-1218-2.

Jinek, Martin, Krzysztof Chylinski, Ines Fonfara, Michael Hauer, Jennifer A. Doudna, and Emmanuelle Charpentier. 2012. "A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity." *Science* 337 (6096): 816–21. https://doi.org/10.1126/science.1225829.

Johansson, E. M., D. Bouchet, R. Tamouza, P. Ellul, A. S. Morr, E. Avignone, R. Germi, M. Leboyer, H. Perron, and L. Groc. 2020. "Human Endogenous Retroviral Protein Triggers Deficit in Glutamate Synapse Maturation and Behaviors Associated with Psychosis." *Science Advances* 6 (29): 708–25. https://doi.org/10.1126/sciadv.abc0708.

Johnston, James B., Claudia Silva, Janet Holden, Kenneth G. Warren, Arthur W. Clark, and Christopher Power. 2001. "Monocyte Activation and Differentiation Augment Human Endogenous Retrovirus Expression: Implications for Inflammatory Brain Diseases." *Annals of Neurology* 50 (4): 434–42. https://doi.org/10.1002/ana.1131.

Jones, R. Brad, Keith E. Garrison, Shariq Mujib, Vesna Mihajlovic, Nasra Aidarus, Diana V. Hunter, Eric Martin, et al. 2012. "HERV-K-Specific T Cells Eliminate Diverse HIV-1/2 and SIV Primary Isolates." *Journal of Clinical Investigation* 122 (12): 4473–89. https://doi.org/10.1172/JCI64560.

Kaas, Jon H. 2013. "The Evolution of Brains from Early Mammals to Humans." *Wiley Interdisciplinary Reviews: Cognitive Science*. Wiley Interdiscip Rev Cogn Sci. https://doi.org/10.1002/wcs.1206.

Kantrowitz, Joshua, and Daniel C. Javitt. 2012. "Glutamatergic Transmission in Schizophrenia: From Basic Research to Clinical Practice." *Current Opinion in Psychiatry*. NIH Public Access. https://doi.org/10.1097/YCO.0b013e32835035b2.

Karn, Jonathan, and C. Martin Stoltzfus. 2012. "Transcriptional and Posttranscriptional Regulation of HIV-1 Gene Expression." *Cold Spring Harbor Perspectives in Medicine* 2 (2). https://doi.org/10.1101/cshperspect.a006916.

Katsumata, Kazuaki, Hitoshi Ikeda, Masayuki Sato, Akihiro Ishizu, You Kawarada, Hiroaki Kato, Akemi Wakisaka, Takao Koike, and Takashi Yoshiki. 1999. "Cytokine Regulation of Env Gene Expression of Human Endogenous Retrovirus-R in Human Vascular Endothelial Cells." *Clinical Immunology* 93 (1): 75–80. https://doi.org/10.1006/clim.1999.4762.

Kauffman, George. 2005. "Nature Encyclopedia of the Human Genome. Edited by David Cooper." *ChemBioChem* 6 (2): 445–46. https://doi.org/10.1002/cbic.200400183.

Kearns, Nicola A., Ryan M.J. Genga, Metewo S. Enuameh, Manuel Garber, Scot A. Wolfe, and René Maehr. 2014. “Cas9 Effector-Mediated Regulation of Transcription and Differentiation in Human Pluripotent Stem Cells.” *Development (Cambridge)* 141 (1): 219–23. https://doi.org/10.1242/dev.103341.

Kearns, Nicola A., Hannah Pham, Barbara Tabak, Ryan M. Genga, Noah J. Silverstein, Manuel Garber, and René Maehr. 2015. “Functional Annotation of Native Enhancers with a Cas9-Histone Demethylase Fusion.” *Nature Methods* 12 (5): 401–3. https://doi.org/10.1038/nmeth.3325.

Kelley, David, and John Rinn. 2012. “Transposable Elements Reveal a Stem Cell-Specific Class of Long Noncoding RNAs.” *Genome Biology* 13 (11): R107. https://doi.org/10.1186/gb-2012-13-11-r107.

Koch, Cody A., and Jeffrey L. Platt. 2007. “T Cell Recognition and Immunity in the Fetus and Mother.” *Cellular Immunology* 248 (1): 12–17. https://doi.org/10.1016/j.cellimm.2007.05.006.

Korsching, S. 1993. “The Neurotrophic Factor Concept: A Reexamination.” *Journal of Neuroscience*. Society for Neuroscience. https://doi.org/10.1523/jneurosci.13-07-02739.1993.

Kriks, Sonja, Jae Won Shim, Jinghua Piao, Yosif M. Ganat, Dustin R. Wakeman, Zhong Xie, Luis Carrillo-Reid, et al. 2011. “Dopamine Neurons Derived from Human ES Cells Efficiently Engraft in Animal Models of Parkinson’s Disease.” *Nature* 480 (7378): 547–51. https://doi.org/10.1038/nature10648.

Kunarso, Galih, Na Yu Chia, Justin Jeyakani, Catalina Hwang, Xinyi Lu, Yun Shen Chan, Huck Hui Ng, and Guillaume Bourque. 2010. “Transposable Elements Have Rewired the Core Regulatory Network of Human Embryonic Stem Cells.” *Nature Genetics* 42 (7): 631–34. https://doi.org/10.1038/ng.600.

Küry, Patrick, Avindra Nath, Alain Créange, Antonina Dolei, Patrice Marche, Julian Gold, Gavin Giovannoni, Hans Peter Hartung, and Hervé Perron. 2018. “Human Endogenous Retroviruses in Neurological Diseases.” *Trends in Molecular Medicine*. Elsevier Ltd. https://doi.org/10.1016/j.molmed.2018.02.007.

Kwun, Hyun Jin, Hae Jin Han, Woo Jung Lee, Hee Soo Kim, and Kyung Lib Jang. 2002. “Transactivation of the Human Endogenous Retrovirus K Long Terminal Repeat by Herpes Simplex Virus Type 1 Immediate Early Protein 0.” *Virus Research* 86 (1–2): 93–100. https://doi.org/10.1016/S0168-1702(02)00058-8.

Lagemaat, Louie N. van de, Patrik Medstrand, and Dixie L. Mager. 2006. “Multiple Effects Govern Endogenous Retrovirus Survival Patterns in Human Gene Introns.” *Genome Biology* 7 (9): R86. https://doi.org/10.1186/gb-2006-7-9-r86.

Lander, Eric S., Lauren M. Linton, Bruce Birren, Chad Nusbaum, Michael C. Zody, Jennifer Baldwin, Keri Devon, et al. 2001. “Initial Sequencing and Analysis of the Human Genome.” *Nature* 409 (6822): 860–921. https://doi.org/10.1038/35057062.

Large, Thomas H., Sarah C. Bodary, Dennis O. Clegg, Gisela Weskamp, Uwe Otten, and Louis F. Reichardt. 1986. “Nerve Growth Factor Gene Expression in the Developing Rat Brain.” *Science* 234 (4774): 352–55. https://doi.org/10.1126/science.3764415.

Laska, M. J., T. Brudek, K. K. Nissen, T. Christensen, A. Moller-Larsen, T. Petersen, and B. A. Nexo. 2012. “Expression of HERV-Fc1, a Human Endogenous Retrovirus, Is Increased in Patients with Active

Multiple Sclerosis." *Journal of Virology* 86 (7): 3713–22. https://doi.org/10.1128/jvi.06723-11.

Lättekivi, Freddy, Sulev Kõks, Maris Keermann, Ene Reimann, Ele Prans, Kristi Abram, Helgi Silm, Gea Kõks, and Külli Kingo. 2018. "Transcriptional Landscape of Human Endogenous Retroviruses (HERVs) and Other Repetitive Elements in Psoriatic Skin." *Scientific Reports* 8 (1). https://doi.org/10.1038/s41598-018-22734-7.

Lechner, Martin, Veit Höhn, Barbara Brauner, Irmtraud Dunger, Gisela Fobo, Goar Frishman, Corinna Montrone, Gabi Kastenmüller, Brigitte Waegele, and Andreas Ruepp. 2012. "CIDeR: Multifactorial Interaction Networks in Human D

Lemaitre, Herve, Aaron L. Goldman, Fabio Sambataro, Beth A. Verchinski, Andreas Meyer-Lindenberg, Daniel R. Weinberger, and Venkata S. Mattay. 2012. "Normal Age-Related Brain Morphometric Changes: Nonuniformity across Cortical Thickness, Surface Area and Gray Matter Volume?" *Neurobiology of Aging* 33 (3): 617.e1-617.e9. https://doi.org/10.1016/j.neurobiolaging.2010.07.013.

Levet, Sandrine, B. Charvet, A. Bertin, A. Deschaumes, H. Perron, and D. Hober. 2019. "Human Endogenous Retroviruses and Type 1 Diabetes." *Current Diabetes Reports*. Springer. https://doi.org/10.1007/s11892-019-1256-9.

Li, F., C. Nellaker, S. Sabunciyan, R. H. Yolken, L. Jones-Brando, A.-S. Johansson, B. Owe-Larsson, and H. Karlsson. 2014. "Transcriptional Derepression of the ERVWE1 Locus Following Influenza A Virus Infection." *Journal of Virology* 88 (8): 4328–37. https://doi.org/10.1128/jvi.03628-13.

Li J M Fan S, W Horsfall Anderson A C,Rigby S,Larsson E,Venables P J. 1996. "The Expression of Human Endogenous Retrovirus-3 in Fetal Cardiac Tissue and Antibodies in Congenital Heart Block - PubMed." *Clin Exp Immunol* . 104 (3): 388–93. https://pubmed.ncbi.nlm.nih.gov/9099920/.

Li, W. H., Z. Gu, H. Wang, and A. Nekrutenko. 2001. "Evolutionary Analyses of the Human Genome." *Nature* 409 (6822): 847–49. https://doi.org/10.1038/35057039.

Li, Wenxue, Myoung Hwa Lee, Lisa Henderson, Richa Tyagi, Muzna Bachani, Joseph Steiner, Emilie Campanac, et al. 2015. "Human Endogenous Retrovirus-K Contributes to Motor Neuron Disease." *Science Translational Medicine* 7 (307). https://doi.org/10.1126/scitranslmed.aac8201.

Lin, Ding Yen, Chi Chen Huang, Ya Ting Hsieh, Hsin Chuan Lin, Ping Chieh Pao, Jen Hui Tsou, Chien Ying Lai, et al. 2013. "Analysis of the Interaction between Zinc Finger Protein 179 (Znf179) and Promyelocytic Leukemia Zinc Finger (Plzf)." *Journal of Biomedical Science* 20 (1): 98. https://doi.org/10.1186/1423-0127-20-98.

Liu, Minmin, Hitoshi Ohtani, Wanding Zhou, Andreas Due Ørskov, Jessica Charlet, Yang W. Zhang, Hui Shen, et al. 2016. "Vitamin C Increases Viral Mimicry Induced by 5-Aza-2'-Deoxycytidine." *Proceedings of the National Academy of Sciences of the United States of America* 113 (37): 10238–44. https://doi.org/10.1073/pnas.1612262113.

Liu, Xiaoyu, Chenfei Wang, Wenqiang Liu, Jingyi Li, Chong Li, Xiaochen Kou, Jiayu Chen, et al. 2016. "Distinct Features of H3K4me3 and H3K27me3 Chromatin Domains in Pre-Implantation Embryos." *Nature* 537 (7621): 558–62. https://doi.org/10.1038/nature19362.

Livak, Kenneth J., and Thomas D. Schmittgen. 2001. "Analysis of Relative Gene Expression Data Using

Real-Time Quantitative PCR and the 2-ΔΔCT Method." *Methods* 25 (4): 402–8. https://doi.org/10.1006/meth.2001.1262.

Lu, Xinyi, Friedrich Sachs, Lee Ann Ramsay, Pierre Étienne Jacques, Jonathan Göke, Guillaume Bourque, and Huck Hui Ng. 2014. "The Retrovirus HERVH Is a Long Noncoding RNA Required for Human Embryonic Stem Cell Identity." *Nature Structural and Molecular Biology* 21 (4): 423–25. https://doi.org/10.1038/nsmb.2799.

Lynch, Vincent J., Robert D. Leclerc, Gemma May, and Günter P. Wagner. 2011. "Transposon-Mediated Rewiring of Gene Regulatory Networks Contributed to the Evolution of Pregnancy in Mammals." *Nature Genetics* 43 (11): 1154–59. https://doi.org/10.1038/ng.917.

Macfarlane, Catriona M., and Richard M. Badge. 2015. "Genome-Wide Amplification of Proviral Sequences Reveals New Polymorphic HERV-K(HML-2) Proviruses in Humans and Chimpanzees That Are Absent from Genome Assemblies." *Retrovirology* 12 (1): 35. https://doi.org/10.1186/s12977-015-0162-8.

Maeder, Morgan L., Samantha J. Linder, Vincent M. Cascio, Yanfang Fu, Quan H. Ho, and J. Keith Joung. 2013. "CRISPR RNA-Guided Activation of Endogenous Human Genes." *Nature Methods* 10 (10): 977–79. https://doi.org/10.1038/nmeth.2598.

Mager, Dixie L., and J. Douglas Freeman. 1995. "HERV-H Endogenous Retroviruses: Presence in the New World Branch but Amplification in the Old World Primate Lineage." *Virology* 213 (2): 395–404. https://doi.org/10.1006/viro.1995.0012.

Mager DL., and Medstrand P. 2003. "Retroviral Repeat Sequences. In: Cooper D, editor. Nature encyclopedia of the human genome". D. Cooper ed. London, United Kingdom: *Nature Publishing Group*. pp. 57-63.

Maksakova, I. A., D. L. Mager, and D. Reiss. 2008. "Endogenous Retroviruses - Keeping Active Endogenous Retroviral-like Elements in Check: The Epigenetic Perspective." *Cellular and Molecular Life Sciences*. Cell Mol Life Sci. https://doi.org/10.1007/s00018-008-8494-3.

Mallet, François, Olivier Bouton, Sarah Prudhomme, Valérie Cheynet, Guy Oriol, Bertrand Bonnaud, Gérard Lucotte, Laurent Duret, and Bernard Mandrand. 2004. "The Endogenous Retroviral Locus ERVWE1 Is a Bona Fide Gene Involved in Hominoid Placental Physiology." *Proceedings of the National Academy of Sciences of the United States of America* 101 (6): 1731–36. https://doi.org/10.1073/pnas.0305763101.

Mameli, Giuseppe, Luciana Poddighe, Alessandra Mei, Elena Uleri, Stefano Sotgiu, Caterina Serra, Roberto Manetti, and Antonina Dolei. 2012. "Expression and Activation by Epstein Barr Virus of Human Endogenous Retroviruses-W in Blood Cells and Astrocytes: Inference for Multiple Sclerosis." *PLoS ONE* 7 (9). https://doi.org/10.1371/journal.pone.0044991.

Manghera, Mamneet, and Renée N. Douville. 2013. "Endogenous Retrovirus-K Promoter: A Landing Strip for Inflammatory Transcription Factors?" *Retrovirology*. BioMed Central. https://doi.org/10.1186/1742-4690-10-16.

Margolin, J. F., J. R. Friedman, W. K.H. Meyer, H. Vissing, H. J. Thiesen -, and F. J. Rauscher. 1994.

"Kruppel-Associated Boxes Are Potent Transcriptional Repression Domains." *Proceedings of the National Academy of Sciences of the United States of America* 91 (10): 4509–13. https://doi.org/10.1073/pnas.91.10.4509.

Maroof, Asif M., Sotirios Keros, Jennifer A. Tyson, Shui Wang Ying, Yosif M. Ganat, Florian T. Merkle, Becky Liu, et al. 2013. "Directed Differentiation and Functional Maturation of Cortical Interneurons from Human Embryonic Stem Cells." *Cell Stem Cell* 12 (5): 559–72. https://doi.org/10.1016/j.stem.2013.04.008.

Martens, Joost H.A., Roderick J. O'Sullivan, Ulrich Braunschweig, Susanne Opravil, Martin Radolf, Peter Steinlein, and Thomas Jenuwein. 2005. "The Profile of Repeat-Associated Histone Lysine Methylation States in the Mouse Epigenome." *EMBO Journal* 24 (4): 800–812. https://doi.org/10.1038/sj.emboj.7600545.

Mayer, J., M. Sauter, A. Racz, D. Scherer, N. Mueller-Lantzsch, and E. Meese. 1999. "An Almost-Intact Human Endogenous Retrovirus K on Human Chromosome 7 [1]." *Nature Genetics*. Nat Genet. https://doi.org/10.1038/6766.

Mayer, Jens, Christian Harz, Laura Sanchez, Gavin C. Pereira, Esther Maldener, Sara R. Heras, Lyle W. Ostrow, et al. 2018. "Transcriptional Profiling of HERV-K(HML-2) in Amyotrophic Lateral Sclerosis and Potential Implications for Expression of HML-2 Proteins." *Molecular Neurodegeneration* 13 (1). https://doi.org/10.1186/s13024-018-0275-3.

McCLINTOCK, B. 1950. "The Origin and Behavior of Mutable Loci in Maize." *Proceedings of the National Academy of Sciences of the United States of America* 36 (6): 344–55. https://doi.org/10.1073/pnas.36.6.344.

McDonel, Patrick, Ita Costello, and Brian Hendrich. 2009. "Keeping Things Quiet: Roles of NuRD and Sin3 Co-Repressor Complexes during Mammalian Development." *International Journal of Biochemistry and Cell Biology*. https://doi.org/10.1016/j.biocel.2008.07.022.

Mi, Sha, Xinhua Lee, Xiang-ping Li, Geertruida M. Veldman, Heather Finnerty, Lisa Racie, Edward LaVallie, et al. 2000. "Syncytin Is a Captive Retroviral Envelope Protein Involved in Human Placental Morphogenesis." *Nature* 403 (6771): 785–89. https://doi.org/10.1038/35001608.

Mikkelsen, Tarjei S., Manching Ku, David B. Jaffe, Biju Issac, Erez Lieberman, Georgia Giannoukos, Pablo Alvarez, et al. 2007. "Genome-Wide Maps of Chromatin State in Pluripotent and Lineage-Committed Cells." *Nature* 448 (7153): 553–60. https://doi.org/10.1038/nature06008.

Morgen, T H. 1922. "Croonian Lecture:—On the Mechanism of Heredity." *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character* 94 (659): 162–97. https://doi.org/10.1098/rspb.1922.0053.

Moyes, David L., Alex Martin, Stephen Sawcer, Nigel Temperton, Jane Worthington, David J. Griffiths, and Patrick J. Venables. 2005. "The Distribution of the Endogenous Retroviruses HERV-K113 and HERV-K115 in Health and Disease." *Genomics* 86 (3): 337–41. https://doi.org/10.1016/j.ygeno.2005.06.004.

Murphy, Frederick A., Claude M. Fauquet, David H. L. Bishop, Said A. Ghabrial, Audrey W. Jarvis, Giovanni P. Martelli, Mike A. Wilen, Tilton, and Doms 2012Mayo, and Max D. Summers. 1995.

"Introduction to the Universal System of Virus Taxonomy." In *Virus Taxonomy*, 1–13. Springer Vienna. https://doi.org/10.1007/978-3-7091-6607-9_1.

Nazir, Faisal Hayat, Bruno Becker, Ann Brinkmalm, Kina Höglund, Åsa Sandelius, Petra Bergström, Tugce Munise Satir, et al. 2018. "Expression and Secretion of Synaptic Proteins during Stem Cell Differentiation to Cortical Neurons." *Neurochemistry International* 121 (December): 38–49. https://doi.org/10.1016/j.neuint.2018.10.014.

Nelson, P. N., P. R. Carnegie, J. Martin, H. Davari Ejtehadi, P. Hooley, D. Roden, S. Rowland-Jones, P. Warren, J. Astley, and P. G. Murray. 2003. "Demystified . . . Human Endogenous Retroviruses." *Journal of Clinical Pathology - Molecular Pathology*. Mol Pathol. https://doi.org/10.1136/mp.56.1.11

Nelson, P. N., A. M.L. Lever, S. Smith, R. Pitman, P. Murray, S. A. Perera, O. M.R. Westwood, F. C. Hay, H. D. Ejtehadi, and J. C. Booth. 1999. "Molecular Investigations Implicate Human Endogenous Retroviruses as Mediators of Anti-Retroviral Antibodies in Autoimmune Rheumatic Disease." *Immunological Investigations* 28 (4): 277–89. https://doi.org/10.3109/08820139909060862.

Neumann, Manuela, Deepak M. Sampathu, Linda K. Kwong, Adam C. Truax, Matthew C. Micsenyi, Thomas T. Chou, Jennifer Bruce, et al. 2006. "Ubiquitinated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis." *Science* 314 (5796): 130–33. https://doi.org/10.1126/science.1134108.

Nevalainen, Tapio, Arttu Autio, Binisha Hamal Mishra, Saara Marttila, Marja Jylhä, and Mikko Hurme. 2018. "Aging-Associated Patterns in the Expression of Human Endogenous Retroviruses." *PLoS ONE* 13 (12). https://doi.org/10.1371/journal.pone.0207407.

Nikoletopoulou, Vassiliki, Heiko Lickert, José Maria Frade, Chantal Rencurel, Patrizia Giallonardo, Lixin Zhang, Miriam Bibel, and Yves Alain Barde. 2010. "Neurotrophin Receptors TrkA and TrkC Cause Neuronal Death WhereasTrkB Does Not." *Nature* 467 (7311): 59–63. https://doi.org/10.1038/nature09336.

Ohnuki, Mari, Koji Tanabe, Kenta Sutou, Ito Teramoto, Yuka Sawamura, Megumi Narita, Michiko Nakamura, et al. 2014. "Dynamic Regulation of Human Endogenous Retroviruses Mediates Factor-Induced Reprogramming and Differentiation Potential." *Proceedings of the National Academy of Sciences of the United States of America* 111 (34): 12426–31. https://doi.org/10.1073/pnas.1413299111.

Pace, John K., and Cédric Feschotte. 2007. "The Evolutionary History of Human DNA Transposons: Evidence for Intense Activity in the Primate Lineage." *Genome Research* 17 (4): 422–32. https://doi.org/10.1101/gr.5826307.

Pal, Sangita, and Jessica K. Tyler. 2016. "Epigenetics and Aging." *Science Advances*. American Association for the Advancement of Science. https://doi.org/10.1126/sciadv.1600584.

Pengsong Li, Xiaofen Fu, Lei Zhang., and Shizhong Li. 2018. "CRISPR/Cas-based screening of a gene activation library in Saccharomyces cerevisiae identifies a crucial role of OLE1 in thermotolerance". *Microbial Biotechnol* 12(6):1154-1163.https://doi.org/10.1111/1751-7915.13333

Perl, Andras, David Fernandez, Tiffany Telarico, and Paul E. Phillips. 2010. "Endogenous Retroviral Pathogenesis in Lupus." *Current Opinion in Rheumatology*. NIH Public Access. https://doi.org/10.1097/BOR.0b013e32833c6297.

Perron, Hervé, Hei-Lanne Dougier-Reynaud, Christina Lomparski, Iuliana Popa, Reza Firouzi, Jean-Baptiste Bertrand, Suzana Marusic, et al. 2013. "Human Endogenous Retrovirus Protein Activates Innate Immunity and Promotes Experimental Allergic Encephalomyelitis in Mice." Edited by Pablo Villoslada. *PLoS ONE* 8 (12): e80128. https://doi.org/10.1371/journal.pone.0080128.

Perzova, Raisa, Elliot Graziano, Swathi Sanghi, Caitlin Welch, Patricia Benz, Lynn Abbott, Danielle Lalone, et al. 2013. "Increased Seroreactivity to HERV-K10 Peptides in Patients with HTLV Myelopathy." *Virology Journal* 10 (1): 360. https://doi.org/10.1186/1743-422X-10-360.

Pokrishevsky, Edward, Leslie I. Grad, Masoud Yousefi, Jing Wang, Ian R. Mackenzie, and Neil R. Cashman. 2012. "Aberrant Localization of FUS and TDP43 Is Associated with Misfolding of SOD1 in Amyotrophic Lateral Sclerosis." Edited by Mark R. Cookson. *PLoS ONE* 7 (4): e35050. https://doi.org/10.1371/journal.pone.0035050.

Popovic, Mikulas, M. G. Sarngadharan, Elizabeth Read, and Robert C. Gallo. 1984. "Detection, Isolation, and Continuous Production of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and Pre-AIDS." *Science* 224 (4648): 497–500. https://doi.org/10.1126/science.6200935.

Qi, Yuchen, Xin Jun Zhang, Nicolas Renier, Zhuhao Wu, Talia Atkin, Ziyi Sun, M. Zeeshan Ozair, 2017. "Combined Small-Molecule Inhibition Accelerates the Derivation of Functional Cortical Neurons from Human Pluripotent Stem Cells." *Nature Biotechnology* 35 (2): 154–63. https://doi.org/10.1038/nbt.3777.

Quiroga, Juan A., Inmaculada Castillo, Javier Bartolomé, and Vicente Carreño. 2007. "Serum Immunoglobulin G Antibodies to the GOR Autoepitope Are Present in Patients with Occult Hepatitis C Virus (HCV) Infection despite Lack of HCV-Specific Antibodies." *Clinical and Vaccine Immunology* 14 (10): 1302–6. https://doi.org/10.1128/CVI.00128-07.

Qureshi, Irfan A., and Mark F. Mehler. 2012. "Emerging Roles of Non-Coding RNAs in Brain Evolution, Development, Plasticity and Disease." *Nature Reviews Neuroscience*. Nat Rev Neurosci. https://doi.org/10.1038/nrn3234.

Richardson, Sandra R., Aurélien J. Doucet, Huira C. Kopera, John B. Moldovan, José Luis Garcia-Pérez, and John V. Moran. 2015. "The Influence of LINE-1 and SINE Retrotransposons on Mammalian Genomes." In *Mobile DNA III*, 3:1165–1208. American Society of Microbiology. https://doi.org/10.1128/microbiolspec.mdna3-0061-2014.

Robbez-Masson, Luisa, and Helen M. Rowe. 2015. "Retrotransposons Shape Species-Specific Embryonic Stem Cell Gene Expression." *Retrovirology*. BioMed Central Ltd. https://doi.org/10.1186/s12977-015-0173-5.

Römer, Christine, Manvendra Singh, Laurence D. Hurst, and Zsuzsanna Izsvák. 2017. "How to Tame an Endogenous Retrovirus: HERVH and the Evolution of Human Pluripotency." *Current Opinion in Virology*. Elsevier B.V. https://doi.org/10.1016/j.coviro.2017.07.001.

Romito, Antonio, and Gilda Cobellis. 2016. “Pluripotent Stem Cells: Current Understanding and Future Directions.” *Stem Cells International*. Hindawi Publishing Corporation. https://doi.org/10.1155/2016/9451492.

Rous, Peyton. 1911. “A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells.” *Journal of Experimental Medicine* 13 (4): 397–411. https://doi.org/10.1084/jem.13.4.397.

Rowe, Helen M., Johan Jakobsson, Daniel Mesnard, Jacques Rougemont, Séverine Reynard, Tugce Aktas, Pierre V. Maillard, et al. 2010. “KAP1 Controls Endogenous Retroviruses in Embryonic Stem Cells.” *Nature* 463 (7278): 237–40. https://doi.org/10.1038/nature08674.

Rowe, Helen M., Adamandia Kapopoulou, Andrea Corsinotti, Liana Fasching, Todd S. Macfarlan, Yara Tarabay, Stéphane Viville, Johan Jakobsson, Samuel L. Pfaff, and Didier Trono. 2013. “TRIM28 Repression of Retrotransposon-Based Enhancers Is Necessary to Preserve Transcriptional Dynamics in Embryonic Stem Cells.” *Genome Research* 23 (3): 452–61. https://doi.org/10.1101/gr.147678.112.

Rowland, Lewis P., and Neil A. Shneider. 2001. “Amyotrophic Lateral Sclerosis.” *New England Journal of Medicine* 344 (22): 1688–1700. https://doi.org/10.1056/NEJM200105313442207.

Rucheton, M., H. Graafland, H. Fanton, L. Ursule, P. Ferrier, and C. J. Larsen. 1985. “Presence of Circulating Antibodies against Gag-Gene MuLV Proteins in Patients with Autoimmune Connective Tissue Disorders.” *Virology* 144 (2): 468–80. https://doi.org/10.1016/0042-6822(85)90287-9.

Sakai, Kazuhisa, Hidemi Shimizu, Tatsuro Koike, Shigeki Furuya, and Masahiko Watanabe. 2003. “Neutral Amino Acid Transporter ASCT1 Is Preferentially Expressed in L-Ser-Synthetic/Storing Glial Cells in the Mouse Brain with Transient Expression in Developing Capillaries.” *Journal of Neuroscience* 23 (2): 550–60. https://doi.org/10.1523/jneurosci.23-02-00550.2003.

Sankowski, Roman, Joshua J. Strohl, Tomás S. Huerta, Elham Nasiri, Andrea N. Mazzarello, Cristina D’Abramo, Kai Fan Cheng, et al. 2019. “Endogenous Retroviruses Are Associated with Hippocampus-Based Memory Impairment.” *Proceedings of the National Academy of Sciences of the United States of America* 116 (51): 25982–90. https://doi.org/10.1073/pnas.1822164116.

Santoni, Federico A., Jessica Guerra, and Jeremy Luban. 2012. “HERV-H RNA Is Abundant in Human Embryonic Stem Cells and a Precise Marker for Pluripotency.” *Retrovirology* 9 (December). https://doi.org/10.1186/1742-4690-9-111.

Schultz, David C., Kasirajan Ayyanathan, Dmitri Negorev, Gerd G. Maul, and Frank J. Rauscher. 2002. “SETDB1: A Novel KAP-1-Associated Histone H3, Lysine 9-Specific Methyltransferase That Contributes to HP1-Mediated Silencing of Euchromatic Genes by KRAB Zinc-Finger Proteins.” *Genes and Development* 16 (8): 919–32. https://doi.org/10.1101/gad.973302.

SenGupta, D., R. Tandon, R. G. S. Vieira, L. C. Ndhlovu, R. Lown-Hecht, C. E. Ormsby, L. Loh, et al. 2011. “Strong Human Endogenous Retrovirus-Specific T Cell Responses Are Associated with Control of HIV-1 in Chronic Infection.” *Journal of Virology* 85 (14): 6977–85. https://doi.org/10.1128/jvi.00179-11.

Sha, Mi, Xinhua Lee, Xiang ping Li, Geertrulda M. Veldman, Heather Finnerty, Lisa Racie, Edward LaVallie, et al. 2000. “Syncytin Is a Captive Retroviral Envelope Protein Involved in Human Placental

Morphogenesis." *Nature* 403 (6771): 785–89. https://doi.org/10.1038/35001608.

Shen, Ting, Yuyi You, Chitra Joseph, Mehdi Mirzaei, Alexander Klistorner, Stuart L. Graham, and Vivek Gupta. 2018. "BDNF Polymorphism: A Review of Its Diagnostic and Clinical Relevance in Neurodegenerative Disorders." *Aging and Disease*. International Society on Aging and Disease. https://doi.org/10.14336/AD.2017.0717.

Sher, A., E. Y. Denkers, and R. T. Gazzinelli. 1995. "Induction and Regulation of Host Cell-Mediated Immunity by Toxoplasma Gondii." *Ciba Foundation Symposium*. Ciba Found Symp. https://doi.org/10.1002/9780470514849.ch7.

Shi, Lihua, Zhe Zhang, Angela M. Yu, Wei Wang, Zhi Wei, Ehtisham Akhter, Kelly Maurer, et al. 2014. "The SLE Transcriptome Exhibits Evidence of Chronic Endotoxin Exposure and Has Widespread Dysregulation of Non-Coding and Coding RNAs." Edited by Ludmila Prokunina-Olsson. *PLoS ONE* 9 (5): e93846. https://doi.org/10.1371/journal.pone.0093846.

Slokar, Gorjan, and Gregor Hasler. 2016. "Human Endogenous Retroviruses as Pathogenic Factors in the Development of Schizophrenia." *Frontiers in Psychiatry*. Frontiers Media S.A. https://doi.org/10.3389/fpsyt.2015.00183.

Sripathy, Smitha P., Jessica Stevens, and David C. Schultz. 2006. "The KAP1 Corepressor Functions To Coordinate the Assembly of De Novo HP1-Demarcated Microenvironments of Heterochromatin Required for KRAB Zinc Finger Protein-Mediated Transcriptional Repression." *Molecular and Cellular Biology* 26 (22): 8623–38. https://doi.org/10.1128/mcb.00487-06.

Stengel, Anna, Christian Roos, Gerhard Hunsmann, Wolfgang Seifarth, Christine Leib-Mösch, and Alex D. Greenwood. 2006. "Expression Profiles of Endogenous Retroviruses in Old World Monkeys." *Journal of Virology* 80 (9): 4415–21. https://doi.org/10.1128/jvi.80.9.4415-4421.2006.

Stoye, Jonathan P. 2012. "Studies of Endogenous Retroviruses Reveal a Continuing Evolutionary Saga." *Nature Reviews Microbiology*. Nat Rev Microbiol. https://doi.org/10.1038/nrmicro2783.

Stoye, Jonathan P., and Christoph Moroni. 1983. "Endogenous Retrovirus Expression in Stimulated Murine Lymphocytes: Identification of a New Locus Controlling Mitogen Induction of a Defective Virus." *Journal of Experimental Medicine* 157 (5): 1660–74. https://doi.org/10.1084/jem.157.5.1660.

Subramanian, Ravi P., Julia H. Wildschutte, Crystal Russo, and John M. Coffin. 2011. "Identification, Characterization, and Comparative Genomic Distribution of the HERV-K (HML-2) Group of Human Endogenous Retroviruses." *Retrovirology* 8: 90. https://doi.org/10.1186/1742-4690-8-90.

Suntsova, Maria, Elena V. Gogvadze, Sergey Salozhin, Nurshat Gaifullin, Fedor Eroshkin, Sergey E. Dmitriev, Natalia Martynova, et al. 2013. "Human-Specific Endogenous Retroviral Insert Serves as an Enhancer for the Schizophrenia-Linked Gene PRODH." *Proceedings of the National Academy of Sciences of the United States of America* 110 (48): 19472–77. https://doi.org/10.1073/pnas.1318172110.

Sutkowski, Natalie, Bernard Conrad, David A. Thorley-Lawson, and Brigitte T. Huber. 2001. "Epstein-Barr Virus Transactivates the Human Endogenous Retrovirus HERV-K18 That Encodes a Superantigen." *Immunity* 15 (4): 579–89. https://doi.org/10.1016/S1074-7613(01)00210-2.

Sverdlov, Eugene D. 1998. “Perpetually Mobile Footprints of Ancient Infections in Human Genome.” *FEBS Letters*. Elsevier B.V. https://doi.org/10.1016/S0014-5793(98)00478-5.

Tandon, R., D. SenGupta, L. C. Ndhlovu, R. G. S. Vieira, R. B. Jones, V. A. York, V. A. Vieira, et al. 2011. “Identification of Human Endogenous Retrovirus-Specific T Cell Responses in Vertically HIV-1-Infected Subjects.” *Journal of Virology* 85 (21): 11526–31. https://doi.org/10.1128/jvi.05418-11.

Tavakolian, Shaian, Hossein Goudarzi, and Ebrahim Faghihloo. 2019. “Evaluating the Expression Level of HERV-K Env, Np9, Rec and Gag in Breast Tissue.” *Infectious Agents and Cancer* 14 (1). https://doi.org/10.1186/s13027-019-0260-7.

Tchieu, Jason, Bastian Zimmer, Faranak Fattahi, Sadaf Amin, Nadja Zeltner, Shuibing Chen, and Lorenz Studer. 2017. “A Modular Platform for Differentiation of Human PSCs into All Major Ectodermal Lineages.” *Cell Stem Cell* 21 (3): 399-410.e7. https://doi.org/10.1016/j.stem.2017.08.015.c

Thomas, James H., and Sean Schneider. 2011. “Coevolution of Retroelements and Tandem Zinc Finger Genes.” *Genome Research* 21 (11): 1800–1812. https://doi.org/10.1101/gr.121749.111.

Tie, Christopher HC, Liane Fernandes, Lucia Conde, Luisa Robbez-Masson, Rebecca P Sumner, Tom Peacock, Maria Teresa Rodriguez-Plata, et al. 2018. “ KAP 1 Regulates Endogenous Retroviruses in Adult Human Cells and Contributes to Innate Immune Control .” *EMBO Reports* 19 (10). https://doi.org/10.15252/embr.201745000.

Ting, C. N., M. P. Rosenberg, C. M. Snow, L. C. Samuelson, and M. H. Meisler. 1992. “Endogenous Retroviral Sequences Are Required for Tissue-Specific Expression of a Human Salivary Amylase Gene.” *Genes and Development* 6 (8): 1457–65. https://doi.org/10.1101/gad.6.8.1457.

Toufaily, Chirine, Sebastien Landry, Christine Leib-Mosch, Eric Rassart, and Benoit Barbeau. 2011. “Activation of LTRs from Different Human Endogenous Retrovirus (HERV) Families by the HTLV-1 Tax Protein and T-Cell Activators.” *Viruses* 3 (11): 2146–59. https://doi.org/10.3390/v3112146.

Tovo, Pier Angelo, Ivana Rabbone, Davide Tinti, Ilaria Galliano, Michela Trada, Valentina Daprà, Franco Cerutti, and Massimiliano Bergallo. 2020. “Enhanced Expression of Human Endogenous Retroviruses in New-Onset Type 1 Diabetes: Potential Pathogenetic and Therapeutic Implications.” *Autoimmunity* 53 (5): 283–88. https://doi.org/10.1080/08916934.2020.1777281.

Trapp, Bruce D., and Klaus Armin Nave. 2008. “Multiple Sclerosis: An Immune or Neurodegenerative Disorder?” *Annual Review of Neuroscience*. Annu Rev Neurosci. https://doi.org/10.1146/annurev.neuro.30.051606.094313.

Tugnet, Nicola, Paul Rylance, Denise Roden, Malgorzata Trela, and Paul Nelson. 2013. “Human Endogenous Retroviruses (HERVs) and Autoimmune Rheumatic Disease: Is There a Link?” *The Open Rheumatology Journal* 7 (1): 13–21. https://doi.org/10.2174/1874312901307010013.

Turner, Ryan C., Brandon P. Lucke-Wold, Matthew J. Robson, John M. Lee, and Julian E. Bailes. 2016. “Alzheimer’s Disease and Chronic Traumatic Encephalopathy: Distinct but Possibly Overlapping Disease Entities.” *Brain Injury*. Taylor and Francis Ltd. https://doi.org/10.1080/02699052.2016.1193631.

Vincendeau, Michelle, Ingmar Göttesdorfer, Julia M.H. Schreml, Armand Ngounou G. Wetie, Jens Mayer,

Alex D. Greenwood, Markus Helfer, et al. 2015. “Modulation of Human Endogenous Retrovirus (HERV) Transcription during Persistent and de Novo HIV-1 Infection.” *Retrovirology* 12 (1): 1–17. https://doi.org/10.1186/s12977-015-0156-6.

Wang-Johanning, Feng, Andra R. Frost, Bixi Jian, Lidia Epp, Danielle W. Lu, and Gary L. Johanning. 2003. “Quantitation of HERV-K Env Gene Expression and Splicing in Human Breast Cancer.” *Oncogene* 22 (10): 1528–35. https://doi.org/10.1038/sj.onc.1206241.

Wang, Guangchuan, Ryan D. Chow, Zhigang Bai, Lvyun Zhu, Youssef Errami, Xiaoyun Dai, Matthew B. Dong, et al. 2019. “Multiplexed Activation of Endogenous Genes by CRISPRa Elicits Potent Antitumor Immunity.” *Nature Immunology* 20 (11): 1494–1505. https://doi.org/10.1038/s41590-019-0500-4.

Wang, Tongguang, Marie Medynets, Kory R. Johnson, Tara T. Doucet-O’Hare, Brianna DiSanza, Wenxue Li, Yadi Xu, et al. 2020. “Regulation of Stem Cell Function and Neuronal Differentiation by HERV-K via MTOR Pathway.” *Proceedings of the National Academy of Sciences of the United States of America* 117 (30): 17842–53. https://doi.org/10.1073/pnas.2002427117.

Waterston, Robert H., Kerstin Lindblad-Toh, Ewan Birney, Jane Rogers, Josep F. Abril, Pankaj Agarwal, Richa Agarwala, et al. 2002. “Initial Sequencing and Comparative Analysis of the Mouse Genome.” *Nature* 420 (6915): 520–62. https://doi.org/10.1038/nature01262.

Weinshenker, Brian G., and Robert Nelson. 1990. “The Second Canadian Conference on Multiple Sclerosis.” *Canadian Journal of Neurological Sciences / Journal Canadien Des Sciences Neurologiques* 17 (1): 53–60. https://doi.org/10.1017/S0317167100030031.

Wilen, Craig B., John C. Tilton, and Robert W. Doms. 2012. “HIV: Cell Binding and Entry.” *Cold Spring Harbor Perspectives in Medicine* 2 (8). https://doi.org/10.1101/cshperspect.a006866.

Wolf, Gernot, Alberto de Iaco, Ming An Sun, Melania Bruno, Matthew Tinkham, Don Hoang, Apratim Mitra, Sherry Ralls, Didier Trono, and Todd S. Macfarlan. 2020. “Krab-Zinc Finger Protein Gene Expansion in Response to Active Retrotransposons in the Murine Lineage.” *ELife* 9 (June): 1–22. https://doi.org/10.7554/eLife.56337.

Wu, Zhouwei, Xingyu Mei, Di Zhao, Yue Sun, Jun Song, Weihua Pan, and Weimin Shi. 2015. “DNA Methylation Modulates HERV-E Expression in CD4+ T Cells from Systemic Lupus Erythematosus Patients.” *Journal of Dermatological Science* 77 (2): 110–16. https://doi.org/10.1016/j.jdermsci.2014.12.004.

Xie, Hui, and Wing Ho Yung. 2012. “Chronic Intermittent Hypoxia-Induced Deficits in Synaptic Plasticity and Neurocognitive Functions: A Role for Brain-Derived Neurotrophic Factor.” *Acta Pharmacologica Sinica*. Nature Publishing Group. https://doi.org/10.1038/aps.2011.184.

Yang, Bin Xia, Chadi A. El Farran, Hong Chao Guo, Tao Yu, Hai Tong Fang, Hao Fei Wang, Sharon Schlesinger, et al. 2015. “Systematic Identification of Factors for Provirus Silencing in Embryonic Stem Cells.” *Cell* 163 (1): 230–45. https://doi.org/10.1016/j.cell.2015.08.037.

Yang, Liuliu, Yuling Han, Benjamin E. Nilsson-Payant, Vikas Gupta, Pengfei Wang, Xiaohua Duan, Xuming Tang, et al. 2020. “A Human Pluripotent Stem Cell-Based Platform to Study SARS-CoV-2

Tropism and Model Virus Infection in Human Cells and Organoids." *Cell Stem Cell* 27 (1): 125-136.e7. https://doi.org/10.1016/j.stem.2020.06.015.

Yang, Peng, Yixuan Wang, and Todd S. Macfarlan. 2017. "The Role of KRAB-ZFPs in Transposable Element Repression and Mammalian Evolution." *Trends in Genetics*. Elsevier Ltd. https://doi.org/10.1016/j.tig.2017.08.006.

Yao, Y., J. Schröder, C. Nellåker, C. Bottmer, S. Bachmann, R. H. Yolken, and H. Karlsson. 2008. "Elevated Levels of Human Endogenous Retrovirus-W Transcripts in Blood Cells from Patients with First Episode Schizophrenia." *Genes, Brain and Behavior* 7 (1): 103–12. https://doi.org/10.1111/j.1601-183X.2007.00334.x.

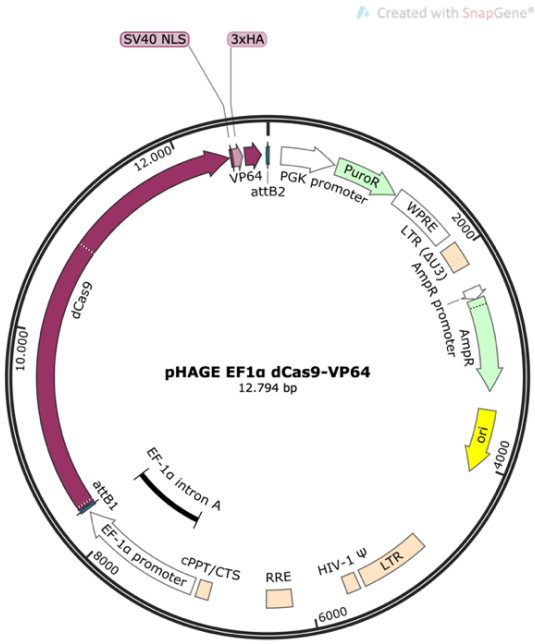
Zhou, Ting, Lei Tan, Gustav Y. Cederquist, Yujie Fan, Brigham J. Hartley, Suranjit Mukherjee, Mark Tomishima, et al. 2017. "High-Content Screening in HPSC-Neural Progenitors Identifies Drug Candidates That Inhibit Zika Virus Infection in Fetal-like Organoids and Adult Brain." *Cell Stem Cell* 21 (2): 274-283.e5. https://doi.org/10.1016/j.stem.2017.06.017.

Zufiría, Mónica, Francisco Javier Gil-Bea, Roberto Fernández-Torrón, Juan José Poza, Jose Luis Muñoz-Blanco, Ricard Rojas-García, Javier Riancho, and Adolfo López de Munain. 2016. "ALS: A Bucket of Genes, Environment, Metabolism and Unknown Ingredients." *Progress in Neurobiology*. Elsevier Ltd. https://doi.org/10.1016/j.pneurobio.2016.05.004.

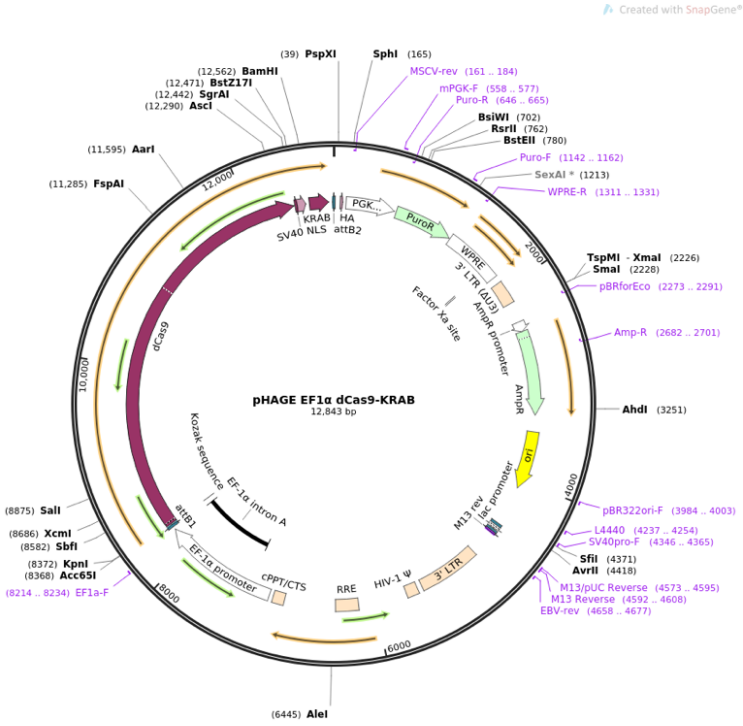
8 Appendix

8.1 Plasmid maps

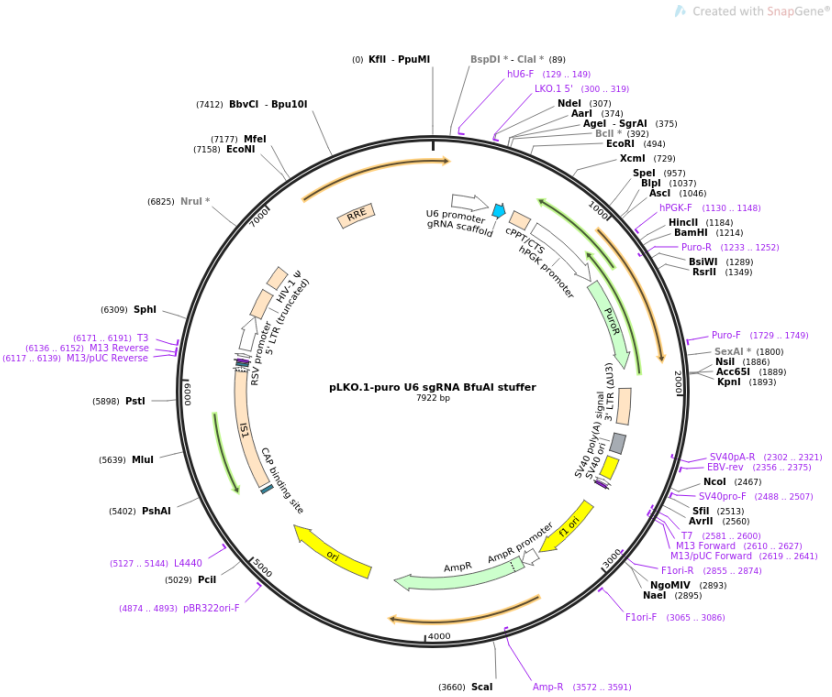
pHAGE EF1 alpha dCas9-VP64



pHAGE EF1 alpha dCas9-KRAB



pLKO.1-puro U6 sgRNA BfuA1 stuffer



8.2 Publications and Conferences

8.2.1 *Research articles in peer reviewed journals*

- **Nair et al**, 2021: *Activation of HERV-K(HML-2) limits cortical neuronal differentiation by modulating Neurotropic Tyrosine Receptor Kinase 3 (NTRK3)*- Manuscript submitted.
- Melanie Weber, **Vidya Padmanabhan Nair**, Tanja Bauer, Martin Sprinzl, Ulrike Protzer, Michelle Vincendeau, 2021: *Increased HERV-K(HML-2) levels correlate with clinical parameters indicating liver cirrhosis*- Manuscript submitted.
- **Nair et al,** 2021: *An inducible CRISPR platform in human stem cells identifies novel cellular processes connected to HERV-H*- Manuscript in preparation

8.2.2 *Presentations at Scientific Conferences*

- **2018 - Retreat of the Institute of Virology 2018 (June 18-19, Herrsching, Germany)**
  Oral presentation: Analyzing the role of human endogenous retrovirus K (HERV-K(HML-2) in cortical neuronal differentiation
- **2019 - Retreat of the Institute of Virology 2019 (June 18-19, Herrsching, Germany)**
  Oral presentation: Analyzing the role of human endogenous retrovirus K (HERV-K(HML-2) in cortical neuronal differentiation
- **2019 - 7th Annual German Stem Cell Network (GSCN) conference (September 23-25, Berlin, Germany)**
  Poster presentation: CRISPR-mediated regulation of human endogenous retroviruses
- **2020 - Interact 2020- International scientific conference (February 27-28, Munich, Germany)**
  Oral presentation: Analysing the role of HERV-K(HML-2) in neuronal differentiation and brain development
- **2020- ISSCR 2020 Virtual- The global stem cell event (June 23-27, Boston, USA)**
  Poster presentation: An inducible CRISPR platform in human stem cells identifies novel cellular processes connected to HERV-H

Acknowledgement

I would like to thank a bunch of amazing people in my life who made this four-year journey of doctoral studies memorable, enjoyable and exciting. First of all, a heartfelt thankyou to Prof Dr Ulrike Protzer, who gave me this wonderful opportunity to fulfil my PhD dissertation at the Institute of Virology. A special thanks to Prof Dr Ruth Brack-Werner who has been extremely supportive, encouraging and welcoming.

This project would not have been the same without my supervisor, Dr Michelle Vincendeau. I cannot thank her enough for the amazing person she is and the ever-optimistic boss she is. Thank you, Michelle, for this great project, for believing in me, for motivating me and guiding me in the best way one could expect. You are the best.

A special mention to Dr Kamyar Hadian and his team at the Institute of Toxicology for the constant support and assists. I would also like to thank Ms Tanja Orschmann for being so kind and helping me out with reagents in critical times of need. Not to forget my lab mates and Ms Regina Geiger for making everything easier.

Special thanks to my dear friend, Amelie Bauer for being there for me always, for making me feel at home, and helping me out when my German language skills failed me. A happy mention to Lisa Pleninger, a dear friend who has inspired me with her intelligence and dedication towards Science. It is hard to find someone with similar thought process. I am glad for our friendship. A huge hug to Team JGM, my home away from home. They have truly made my Munich days one of the best times of my life so far.

Special thanks go to my family. My father who taught me to dream. My mother who taught me to focus. My sister who taught me to laugh and let go. Thank you for trusting in me. Thank you for letting me pursue my journey.

My greatest gratitude goes to my best friend and now my husband Anoop Kodakkal, who has been there for me at all times. Thank you for all the great conversations. Thank you for being my pillar of strength since always. Thank you for making me who I am now. Thank you for everything.

I would extend my note of gratitude to the known knowns that makes me appreciate Science, the known unknowns that makes me stay intuitive and creative, and the unknown unknowns that makes me stay grounded and accept the greatness of life. Thank you, Science, for making my mind sharp and Thank you God for giving me Science to understand you.