Re-activation of Human Endogenous Retrovirus-K in Neuroinflammatory Disease

by

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DEDICATION

I dedicate this thesis to

My family, amazing friends, and proactive lab team without whom none of my successes would be possible;

Individuals affected by ALS who continue to motivate me to gain a better understanding of this devastating disease;

Caffeine and Sugar for keeping me awake during long sleepless nights of thesis writing;

and, **God** for giving me the strength to pursue my passion and success in my endeavours.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CTXLP	Conotoxin-like Protein
DNA	Deoxyribonucleic Acid
Env	Envelope
ERV	Endogenous Retrovirus
ERVK	Endogenous Retrovirus K
ERVW	Endogenous Retrovirus W
Gag	Group-specific Antigen
HAND	HIV-associated Neurocognitive Disorder
HIV	Human Immunodeficiency Virus
HML	Human MMTV-like
HSV	Herpes Simplex Virus
HTLV1	Human T Lymphotropic Virus
IFNγ	Interferon gamma
IN	Integrase
INSL4	Insulin 4
IRF1	Interferon Regulatory Factor 1
ISRE	Interferon Stimulated Response Element
LIGHT	homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes
LTR	Long Terminal Repeat
MMTV	Murine Mammary Tumor Virus
MS	Multiple Sclerosis
NF-κB	Nuclear Factor – kappa B
PAMP	Pathogen Associated Molecular Pattern

Pol	Polymerase
Pro	Protease
PRR	Pattern Recognition Receptor
RIG-I	Retinoic Acid Inducible Gene I
RNA	Ribonucleic Acid
RRM	RNA Recognition Motif
RT	Reverse Transcriptase
RTC	Reverse Transcription Complex
SCZ	Schizophrenia
SG	Stress Granule
SOD1	Superoxide Dismutase 1
TAR	Trans-activating Response
TARDBP	TAR DNA Binding Protein
TNFα	Tumor Necrosis Factor alpha
XRV	Exogenous Retrovirus

1. INTRODUCTION

1.1 | Introduction to endogenous retroviruses

Retroviruses belong to the family *Retroviridae*, which comprises a diverse range of single-stranded RNA viruses capable of reversing the flow of genetic information from RNA to DNA[1]. The enzyme Reverse Transcriptase (RT), which generates a DNA copy of the RNA genome[1], imparts this unique characteristic to retroviruses (**Figure 1**). Another remarkable feature of retroviruses is their ability to insert this DNA copy of the viral genome into genomic material of the host cell – a process mediated by the viral enzyme Integrase[1] (**Figure 1**). This integrated copy of the retrovirus is called a provirus[1]. Thus, exogenous retroviruses have the capacity to permanently incorporate themselves into the genome of the host cells they infect.

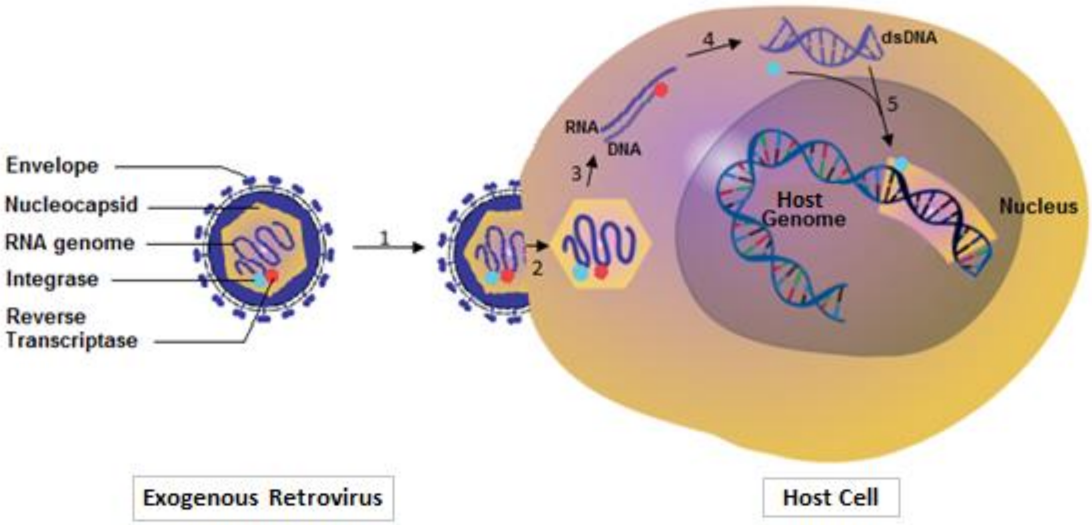

Figure 1. Integration process of an exogenous retrovirus into the genome of the host cell. The exogenous retrovirus binds to the host cell (1) and releases the nucleocapsid containing the viral RNA genome and enzymes into the cytoplasm of the infected cell (2). Un-coating of the nucleocapsid releases viral RNA, Reverse Transcriptase (red), and Integrase (light blue) into the cytoplasm. Reverse Transcriptase synthesizes a DNA copy of the viral RNA (3). This single-stranded DNA is converted to a double-stranded DNA (dsDNA) (4), and inserted into the genome of the infected cell via Integrase (5). Artwork by M. Manghera.

Retroviruses usually infect only somatic cells, and are thus only transmitted horizontally. Nonetheless, some retroviruses are able to infect germ line cells (eggs and sperm) and incorporate their viral genes into the genomic material of the infected gametes[1]. During fertilization, the fusion of infected, yet viable, gametes results in the vertical transmission of these integrated retroviral elements to progeny. These proviruses are inherited by the subsequent generations in a Mendelian fashion[2]. Thus, exogenous retroviruses (XRVs) that become permanent residents of the host genome are called endogenous retroviruses (ERVs).

Endogenous retroviruses have been identified in a diverse range of ancient and modern vertebrate host species; and, humans are no exception. Ancient exogenous retroviruses permanently entered the human lineage through infection of germ line cells of our ancestors dating as far back as 55 million years ago[3]. These human endogenous retroviruses now constitute over 8% of our genetic material, whereas vertebrate genes comprise merely 1.5% of our DNA[4]. Our genome harbours approximately 203,000 copies of ERVs, with human endogenous retrovirus-K (ERVK; former HERV-K[5]) being the most recently integrated and the best preserved[3,6]. It is estimated that ERVK alone contributes 6400 solitary Long Terminal Repeats (LTRs; viral promoters) and 550 proviruses – a total of about 7000 retroviral elements – to our genetic material[3]. ERVs are named according to the type of transfer RNA (tRNA) used to prime reverse transcription; since ERVK uses lysine tRNA (abbreviated as K) for this process, it is designated as "K"[6]. ERVK is most closely related to a murine betaretrovirus – mouse mammary tumor virus (MMTV); hence, the ERVK group is further divided into ten human MMTV-like (HML) families, designated as HML-1 to HML-10[3]. The ERVK HML-2 family is thought be the youngest, as it comprises retroviruses that may have endogenized into the human genome as recently as 200,000 to 2 million years ago[3,7]. Clearly, humans are holobiontic organisms, with a genetic inheritance comprising dynamic interactions between the human and retroviral genomes.

1.2 | Role of Human Endogenous Retroviruses in health and disease

Over evolutionary time, the majority of ERVs have been silenced through accumulation of point mutations and deletions, as well as through epigenetic mechanisms such as DNA methylation and cytosine deamination, providing a natural defense against these intragenomic parasites[8–11]. Nonetheless, some ERVs remain transcriptionally active and have become crucial for a variety of biological processes within us, resulting in a symbiotic relationship between these endogenous retroviruses and their human hosts. For instance, the *env* (envelope) genes of ERVW encode syncytin proteins, which are crucial for the differentiation of syncytiotrophoblast in chorionic villi, and thus aid in normal development of the human placenta[12]. The syncytin proteins also have immunosuppressive properties, allowing the embryo to escape immune rejection and thus survive during pregnancy[12]. More recently, ERVK expression has been shown to upregulate a specific viral restriction pathway in early stages of human pre-implantation embryos. As a result, it has been postulated that ERVK expression during early embryogenesis may protect human embryos against infections from either infectious ERV particles or exogenous viruses sensitive to restriction by innate immune pathways[13]. In addition, ERVK elements serve as alternative gene promoters and enhancers, and play a key role in regulating the expression of a wide variety of human genes[4,14,15], such as *INSL4* (encodes insulin-like protein 4)[16,17]. Some ERV sequences are also known to silence human gene transcription, as they produce mRNA complementary to cellular gene transcripts, thus down-regulating the expression of select human genes through RNA interference[18]. Hence, the symbiogenesis between endogenous retroviruses and the human genome has contributed enormously towards shaping human evolution and physiology.

Given the huge retroviral presence in our genome and their ability to express viral RNA and proteins, ERVs may be deleterious and play an important role in human diseases. The ERVK (HML-2) loci are of particular relevance in this context, as they comprise the most recently integrated and most intact proviruses present within the human genome. More than 90 ERVK proviruses belonging to the HML-2 family have full length open reading frames encoding functional retroviral proteins[3]. The enhanced expression of these ERVK loci has often been associated with inflammatory diseases including neurological disorders[19], rheumatic diseases[20,21], multiple types of cancers[22], and infections[23,24,25]. There is also evidence that some ERVK proviruses are capable of producing mature virus particles[23,26-27]. Accordingly, ERVK virions have been detected in the blood of patients with human immunodeficiency virus-1 (HIV-1) infection[23], breast cancer[23], and lymphoma[26]. In addition, structurally intact ERVK virus particles have been shown to be produced in cancer cell lines derived from teratocarcinomas[28], breast cancer[29], and melanomas[27].

Yet, the infectivity of these ERVK particles has not been clearly demonstrated. Due to a non-functional envelope protein, ERVK virions are generally thought to be non-infectious[27,28,30]. However, a recent study depicted that ERVK particles derived from teratocarcinoma and breast cancer cell lines, as well as from peripheral blood lymphocytes obtained from lymphoma patients, are able to package a synthetic ERVK HML-2 genetic probe and transmit it to other cells[29]. This probe was also shown to be reverse transcribed in target cells and form episomes (integrase-mediated circularization of proviral DNA), but it did not integrate into the genetic material of the host cells[29]. This may be attributed to a lack of unique sequences in the synthetic probe that are crucial for integration. Nevertheless, these findings challenge the prevailing notion that human endogenous retroviruses, such as ERVK, are non-infectious and lack transmission capacity. Although there is no documented evidence of a causal relationship between ERVK activity and disease, transient cellular transmission of ERVK sequences through viral particles may activate typical anti-retroviral immune responses against infected host cells, leading to inflammation and subsequent cellular damage. Overall, not only do ERVs confer biological benefits to their human hosts, but retroviral activity stemming from particular ERV loci may also play crucial roles in the pathophysiology of their associated inflammatory diseases.

1.3 | Human Endogenous Retrovirus-K and neuroinflammatory diseases

Evidence of enhanced ERVK activity in a variety of neuroinflammatory diseases has accumulated over the recent years. Patients with neurological disorders, including Amyotrophic Lateral Sclerosis (ALS), Schizophrenia (SCZ), Multiple Sclerosis (MS), and HIV-associated neurocognitive disorder (HAND) exhibit augmented ERVK RNA and protein levels in their post-mortem brain tissue, blood, and/or cerebrospinal fluid[31,32,33,34,35,36]. As with other ERVK-associated inflammatory diseases, enhanced ERVK expression has not been conclusively demonstrated to be a causative agent of the aforementioned neurological disorders.

Nonetheless, ERVK re-activation has the potential to influence the pathogenesis of the associated neurodegenerative diseases through a variety of mechanisms, which have been extensively reviewed in our **Publication 1.** Most importantly, ERVK RNA and protein detection by the host immune system may trigger chronic inflammation in the central nervous system (CNS), leading to extensive neuronal damage. Indeed, exacerbated immune signaling and chronic production of inflammatory mediators known as cytokines are common hallmarks of ERVK-associated neurodegenerative diseases[37,38,39,40]. However, the ability of the human immune system to detect ERVK RNA and proteins has been scarcely studied to date. Few studies have depicted antibodies against ERVK gag and envelope proteins in the sera of individuals with ERVK-associated cancers and HIV infection[22,41–44]. In patients with breast cancer, ERVK-specific cytotoxic T lymphocytes, which are effector cells of the adaptive immune system and are responsible for killing pathogen-infected host cells, have also been detected[41]. Thus, the literature provides some evidence of immune recognition of ERVK proteins, but this area of ERVK research clearly needs further exploration in order to establish any relationships between ERVK expression, chronic immune activation, and the resulting inflammatory pathology in neurodegenerative disorders.

1.3.1 | Enhanced expression of ERVK in Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is the most common type of motor neuron disease. It is characterized by gradual degeneration of both upper (in the brain) and lower (in the spinal cord) motor neurons; this leads to progressive deterioration of associated muscle tissues, causing paralysis and ultimately death[45]. ALS neuropathology and the resulting muscle atrophy progresses in several stages. The earliest symptoms include fasciculations, muscle spasticity, muscle weakness affecting the limbs, slurred speech, and difficulty swallowing[46]. As neurodegeneration progresses throughout the CNS, muscle weakness and atrophy spreads to other parts of the body[46]. At this point, patients experience difficulty moving and speaking, as well as exhibit exaggerated or abnormal reflexes[46]. In the final stages of ALS, deterioration of respiratory muscles leads to respiratory failure, culminating in death[46].

Currently, this devastating and incurable disease strikes six to eight individuals per 100,000 population[47,48]. This means that approximately 3000 Canadians are currently living with ALS[49]. It is estimated that two to three Canadians die from ALS per day (ALS Canada). According to the World Health Organization, neurodegenerative diseases, like ALS, are predicted to surpass cancer as the second leading cause of death in Canada by 2040 (ALS Canada). Thus, new and more efficient diagnostic and therapeutic techniques are required in order to decrease the local and global incidence of neurodegenerative diseases, including ALS.

ALS is a complex disorder involving multiple pathophysiological mechanisms that culminate in neurodegeneration. Notably, several genetic defects that affect protein function and metabolism have been identified as causative factors in ALS. Mutations in *SOD1* gene were the first to be associated with this disease. *SOD1* encodes the antioxidant enzyme Copper, Zinc – Superoxide Dismutase, mutation of which leads to an accumulation of oxygen free radicals inside cells and increased oxidative damage[50]. Recently, mutations in other genes, particularly *TARDBP*, have been implicated in ALS. The *TARDBP* gene encodes TAR DNA binding protein-43 (TDP-43), which is a DNA and RNA binding protein involved in transcriptional regulation of many genes, as well as in RNA splicing[50]. Mutated forms of TDP-43 form cytosolic aggregates in neurons which are a hallmark of ALS[47]. Although mutant TDP-43 has the propensity to abrogate a wide array of cellular functions, it has been shown to exaggerate the neuroinflammatory response and lead to neuronal death[51,52]. Despite these advances, the exact pathological mechanisms involved in the onset and progression of ALS still largely remain obscure.

Recently, the retroviral enzyme reverse transcriptase (RT) was identified in the serum and cerebrospinal fluid of ALS patients at levels equivalent to those found in individuals infected with HIV-1[36,53]. In addition, the RT activity was also enhanced in the first degree relatives of these ALS patients, suggesting that this retroviral enzyme may originate from active proviruses present within the human genome[35]. Accordingly, the source of this enzyme was demonstrated to be active ERVK loci in the cortical neurons of these ALS patients[31], suggesting a role for this endogenous retrovirus in the pathophysiology of ALS.

A viral etiology of ALS has long been suspected, as some individuals infected with the exogenous retrovirus HIV-1 also develop HIV-associated neurocognitive disorder (HAND) – an ALS-like syndrome exhibiting motor neuron pathology[54–56]. However, HIV-1 is not known to infect neurons[57]; thus, neuronal damage in this case must result from indirect pathological effects. This includes the release of pro-inflammatory mediators called cytokines by HIV-1 infected astrocytes, microglia, or infiltrating immune cells in the CNS, which can have cytotoxic effects on neurons[58]. Interestingly HIV-1 infection has also been associated with enhanced ERVK expression in PBMCs and autopsy brain tissue obtained from infected individuals, as well as in astrocytic and T-lymphoma cell lines[34,59,60]. Thus, HIV-induced ERVK expression in neurons may alternatively serve as the link between this retroviral infection and the development of HAND. However, the role that ERVK re-activation plays in the neuropathology of ALS and HAND is yet obscure.

Re-activation of ERVK may contribute to neurodegeneration through several mechanisms. ERVK transcripts and proteins may activate host anti-retroviral immune responses against the ERVK-expressing neurons, leading to inflammation, neuronal injury, and loss. This antiviral response may be mediated by innate immune sensors called pattern recognition receptors (PRRs) present in neurons and other resident cells of the CNS, particularly astrocytes and microglia. These PRRs detect a variety of pathogen associated molecular patterns (PAMPS), including viral proteins, RNA, and DNA. Retinoic acid inducible gene – I (RIG-I), which is known to detect HIV RNA[61], is one of the key putative cytosolic ERVK sensors expressed in the cells of the CNS (The Human Protein Atlas). Interaction between viral ligands and their respective PRRs stimulates downstream signalling pathways, which drives both pro-inflammatory and anti-viral responses. The activation of key transcription factors, such as nuclear factor-kappa B (NF-κB), promotes the production of antiviral proteins (viral restriction factors) and pro-inflammatory cytokines. As an undesirable side effect, these inflammatory mediators provoke cytotoxic responses in the surrounding tissue. Interestingly, astrocytic and microglial activation has been demonstrated to actively participate in ALS and HAND pathogenesis through release of toxic mediators, including cytokines such as Tumor Necrosis Factor α (TNFα)[62,63,39]. TNFα has been determined to be a major contributor to inflammation and neuronal loss in ALS as a result of excessive NF-κB activation[64]. We have preliminary evidence that astrocytes have the capacity to mount an innate anti-viral immune response against ERVK virions purified from a teratocarcinoma cell line (Raizman and Douville, unpublished). Thus, recognition of ERVK proteins and mRNA may activate astrocytes and microglia, which may trigger inflammatory responses to eliminate ERVK-expressing neurons, thereby contributing to neurodegeneration observed in ALS and ALS-like syndromes.

Similar to other retroviruses such as Human Immunodeficiency Virus-1 (HIV-1), ERVK may exploit the inflammatory proteins produced during an anti-retroviral innate immune response, particularly NF-κB and IRF1[65]. Since these proteins have the potential to induce ERVK transcription (which will be discussed later), anti-ERVK immune response may culminate in a positive feedback loop favouring further ERVK expression and chronic inflammation. Thus, the inflammatory response initiated to restrict neuronal ERVK activity may actually be detrimental instead of being protective, leading to the progressive neurodegeneration.

In addition, bioinformatics analysis has revealed that some ERVK proviruses may encode a neurotoxic protein homologous to omega-conotoxins (Fineblit, Jonasson, Ferguson-Parry, and Douville, unpublished). These toxins are known to inhibit voltage-gated calcium channels in neural and associated muscle tissues, which hinders neuro-muscular communication[66,67]. Similarly, the ERVK conotoxin-like protein (CTXLP) may inhibit communication between neurons and associated cells, resulting in typical ALS symptoms, such as paralysis and muscle wasting. Omega-conotoxins have also been demonstrated to cause neuronal death in animal models[58]. Thus, ERVK CTXLP may also directly cause neurodegeneration.

Hence, ERVK re-activation may serve as a novel marker of ALS and define the pathophysiology of neuronal loss in this disease, as well as in HAND. However, the mode of ERVK re-activation during neuroinflammation in general, and in ALS and HAND, remains poorly understood. Through exploration into the ERVK proviral promoter and binding sites for cellular proteins[68], as well as select inflammatory signalling pathways and protein functions deregulated in ALS and HAND, the sections hereafter will highlight the putative mechanisms by which ERVK activity may be augmented in these neurodegenerative conditions.

1.4 | Transcriptional regulation of Human Endogenous Retrovirus-K

1.4.1 | ERVK provirus: genomic structure and gene expression

The structure of an ERVK provirus resembles that of a typical betaretrovirus. A full-length ERVK provirus is approximately 9.5 Kb in size and consists of four overlapping retroviral genes –*gag* (group specific antigen), *pro* (protease), *pol* (polymerase), and *env* (envelope) –

flanked on each side by a Long Terminal Repeat (LTR)[3] (**Figure 2A**). The *gag*, *pro*, *pol*, and *env* genes encode the retroviral capsid proteins, the enzyme protease (PR), retroviral polymerase (with reverse transcriptase (RT) and integrase (IN) subunits), and the viral envelope proteins, respectively [6].

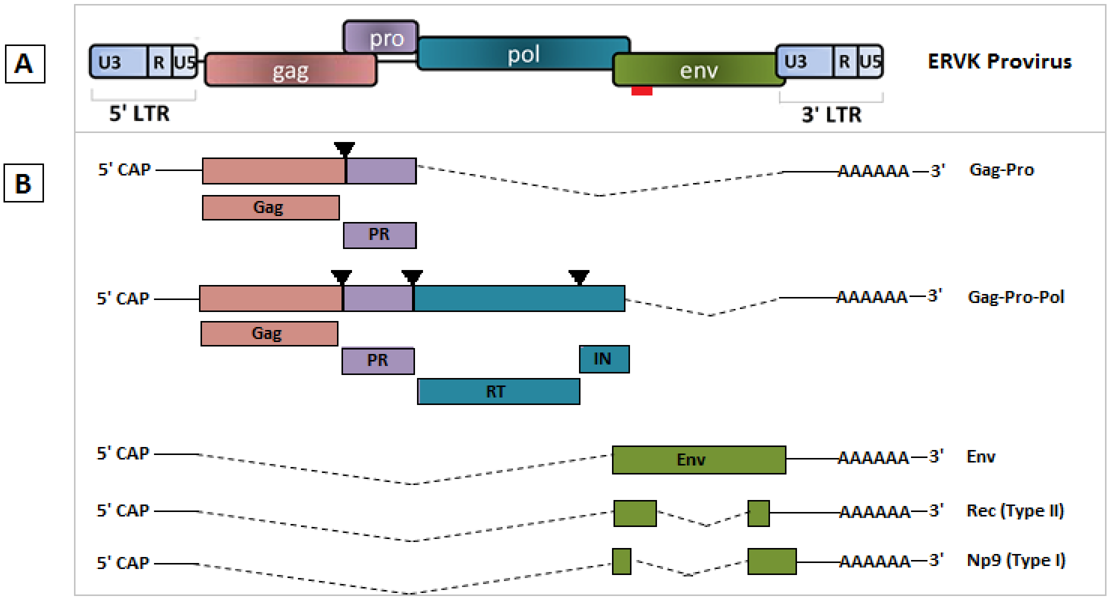

FIGURE 2. Structure of an intact ERVK provirus within the human genome and the ERVK transcripts produced. (A) The four ERVK genes – *gag* (group specific antigen), *pro* (protease; PR), *pol* (polymerase; with Reverse Transcriptase (RT) and Integrase (IN) activities), and *env* (envelope) – are flanked by Long Terminal Repeats (LTRs) on each side. Each LTR has one U3, R, and U5 region in a 5′ to 3′ direction. **(B)** ERVK genes are expressed as gag-pro and gag-pro-pol polypeptides, which are cleaved by protease (black arrowheads) to yield individual proteins. The envelope transcript can be alternatively spliced to yield Rec (in Type II ERVK) and Np9 (in Type I ERVK) accessory proteins. The red horizontal bar in *env* marks the 292 bp deletion in Type I ERVK. Adapted from Douville & Nath, Clinical Handbook of Neurology,2014. Artwork by M. Manghera.

The transcriptional activity of ERVK proviruses is regulated by their Long Terminal Repeats (LTRs), which serve as the viral promoters. A promoter is a region of DNA located upstream of a particular gene from which the transcription of that gene is initiated. Each LTR consists of U3, R, and U5 regions in a 5′ to 3′ direction[68] (**Figure 2A**). U3 region is perhaps the most important as it contains all the sequences – TATA independent promoter, enhancer elements, and transcription factor binding sites – necessary for initiation of transcription of the downstream ERVK genes. The 5′ LTR modulates the sense transcription of ERVK by interacting with certain viral proteins and human transcription factors[68]. The role of 3′ LTR in the transcriptional regulation of ERVK remains obscure; however, it may modulate the antisense transcription of ERVK as observed for other retroviruses including HIV-1 and HTLV-1[69,70].

Retroviral *gag, pro, and pol* genes are initially transcribed and translated into gag-pro and gag-pro-pol precursor polypeptides, which are cleaved by the viral protease into individual peptides to form each mature protein, including the RT enzyme (**Figure 2B**). This protease-mediated processing of the gag-pro-pol polyprotein has been extensively described for exogenous retroviruses such as HIV-1, HTLV-1, and MMTV (Mouse Mammary Tumor Virus)[71–75], but remains poorly studied for endogenous retroviruses including ERVK. The exogenous retroviral gag-pro-pol polyprotein is known to be sequentially cleaved (**Figure 3**) to yield intermediate proteins, and finally a heterodimeric RT protein comprised of two subunits: a larger catalytic isoform with DNA polymerase activity and a ribonuclease H (RNase H) domain responsible for degrading the viral RNA genome template as viral DNA is synthesized, and a smaller isoform which serves a structural role[72,76,77]. In contrast, little work has been done to understand the synthesis and structure of ERVK RT, despite the fact that the augmented levels of this protein have been implicated in a variety of inflammatory and neurological diseases. For the first time, we have shown ERVK gag-pro-pol polyprotein cleavage leading to the production of intermediate RT-containing proteins, and ultimately the two distinct ERVK RT subunits, in human cell line models of neuroinflammatory disease (**Publication 2**). Thus, the ERVK gag-pro-pol polyprotein is likely processed by the viral protease in a similar fashion as that seen with other retroviruses (**Figure 3**), culminating in the production of an active heterodimeric ERVK RT enzyme under select conditions.

Finally, ERVK *env* is transcribed in a different reading frame than the gag-pro-pol polyprotein to yield a full length envelope protein. The envelope transcript can be alternatively spliced to yield an accessory protein called Rec in ERVK HML-2 proviruses termed Type II[3] (**Figure 2B**). Type I proviruses have a 292 base pair deletion in the *env* gene; as a result, the accessory protein Np9 is produced instead of Rec[3] (**Figure 2B**). Rec is responsible for transporting ERVK mRNAs from the nucleus into the cytoplasm[3]. Np9 has no known physiological function in ERVK replication. However, Rec and Np9 may serve as ERVK oncoproteins as they have been implicated in tumor development; they have been demonstrated to increase the levels of c-Myc protein, leading to enhanced cell growth and reduced apoptosis[78].

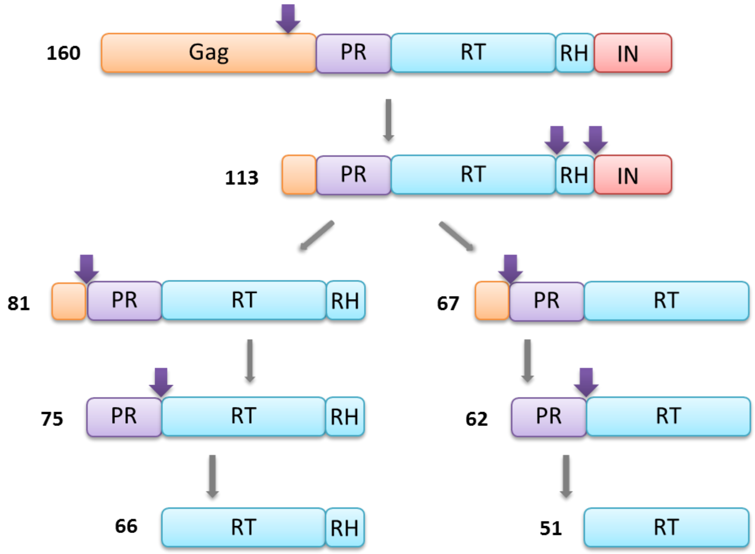

FIGURE 3. Protease-mediated sequential cleavage of the retroviral gag-pro-pol polyprotein to produce mature RT isoforms. Here, HIV-1 is used as the model to illustrate the sizes of each intermediate protein and the final RT subunits produced: 66 KDa RT with RNase H and 51 KDa RT without RNase H. ERVK polyprotein cleavage is predicted to proceed in a similar fashion, leading to the generation of a heterodimeric RT. However, the sizes of the ERVK intermediate cleavage products and RT subunits may be different in comparison to that of HIV-1. Adapted from[72].

1.4.2 |Transcriptional silencing of ERVK

The transcription of the majority of ERVs, including ERVK, has been silenced over evolutionary time through accumulation of deleterious point mutations and deletions. In addition, several layers of epigenetic control restrict ERVK expression in various cell types and tissues. Cellular proteins, particularly APOBEC3G, partake in nucleotide deamination of ERVK sequences prior to their integration into host genome, introducing G to A and C to T mutations to prevent the binding of transcriptional activators recognizing that region of the ERVK promoter[68]. Methylation of CpG dinucleotides in the U3 region of the ERVK 5′ LTR may also repress transcription of ERVK genes by preventing the binding of crucial transcription factors to the methylated sites[68]. The methylated CpG dinucleotides can further be spontaneously deaminated, which is a major source of abundant G to A and C to T mutations in many ERVK LTRs and renders them incapable of transcription[68]. Thus, epigenetic factors play a major role in restricting ERVK transcription in human cells.

Other than epigenetic mechanisms, transcription factors are also crucial for regulating the activity of the ERVK promoter. Although there is accumulating evidence of ERVK transcriptional activators, human and viral transcription factors that repress ERVK gene expression largely remain unidentified. To date, a single report depicts that HIV-1 Tat (Trans-activating) protein is able to repress the transcription of several ERVK HML-2 proviruses[60], although it was previously shown to induce global ERVK transcription in the context of HIV infection[59,79]. The use of different types of cells in these studies may account for disparate results. Such a cell-type dependent inductive and repressive transcriptional activity of Tat has also been described for the HIV promoter. For instance, Tat is able to stimulate HIV transcription in macrophages, but represses it in monocytes[80]. Genetic variations within the HIV LTRs have been shown to further alter the course of viral transcription by modulating the interactions of Tat with the HIV promoter. Thus, whether Tat is an ERVK transcriptional repressor or activator is currently unclear; in reality, it may act as a bifunctional transcription regulator whose activity is dependent on both the ERVK promoter and the cellular context. In addition, only a single transcriptional corepressor called tripartite containing motif 28 (TRIM28) is known to silence the transcription of MMERVK10C (a beta-like ERV similar to ERVK HML-2) in neural progenitor cells derived from transgenic mice[81]. This is achieved via TRIM28-mediated repressive histone modifications of MMERVK10C followed by DNA methylation[81]. However, the influence of TRIM28 on ERVK transcription has not been determined in human cells.

Several human transcription factors are known to restrict the expression of retroviral genes. These include the TAR-DNA binding protein-43 (TDP-43), which is a nuclear RNA/DNA binding protein involved in RNA metabolism and transcriptional regulation of a variety of genes [50,82]. TDP-43 was originally described to repress HIV-1 transcription by interacting with a TAR-DNA element present within the HIV-1 LTR; this likely displaces essential transcriptional machinery required to initiate transcription from the viral promoter, thereby inhibiting its gene expression[83]. However, the influence of TDP-43 on the transcription of other retroviruses including ERVK remains unexplored. Through bioinformatics analysis of ERVK (HML-2) 5′ LTRs, we have identified a putative conserved TAR-RNA encoding element within the ERVK promoter (**Figure 4**). Additional conserved putative TDP-43 binding sites are also present throughout the ERVK LTR, three of which lie within the predicted TAR-like encoding motif (**Figure 4**). Therefore, endogenous TDP-43 may be able to restrict ERVK gene expression in a manner similar to that identified in HIV-1. On the contrary, a recent report depicts that TDP-43 is capable of slightly enhancing HIV-1 transcription in T cells[84]. Likewise, whether TDP-43 will act as a repressor or an activator of ERVK remains to be determined empirically; in fact, it may differentially modulate ERVK transcription in a promoter and cell-type specific manner.

Clearly, there is a lack of knowledge regarding cellular and viral proteins capable of inhibiting ERVK transcription. Only a few studies document the repressive effect of retroviral and cellular proteins on ERVK transcription. TDP-43 is potentially a novel cellular ERVK transcriptional repressor – an area of research which undoubtedly warrants further investigation.

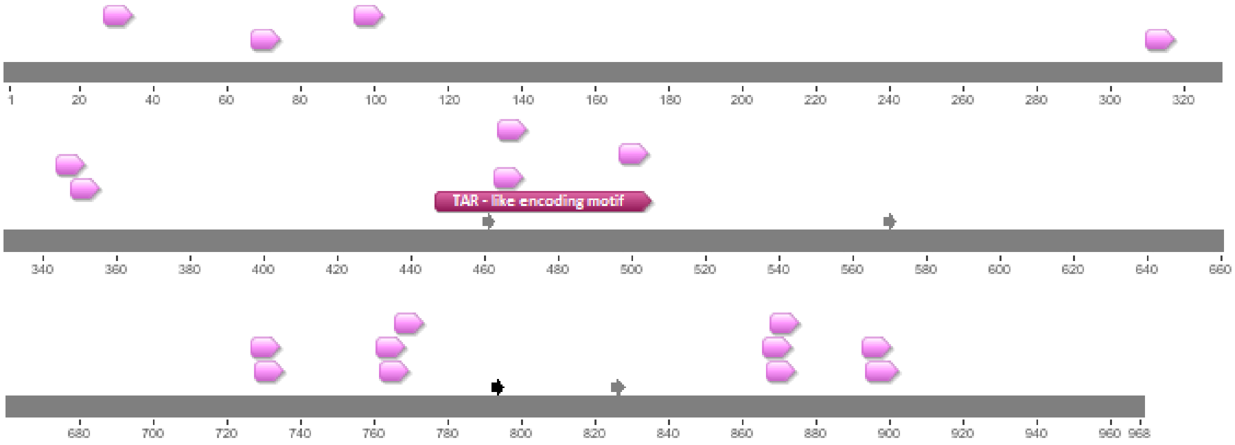

Figure 4. Conserved putative TDP-43 binding sites (pink) and TAR-RNA encoding motif within the consensus ERVK HML-2 5′LTR. The sequences of the TDP-43 DNA binding sites were adapted from[83], and used to identify multiple putative conserved TDP-43 binding sites within the 5′ LTRs of five prototypic ERVK (HML-2) proviruses. Here, binding sites are shown on the consensus ERVK 5′ LTR sequence constructed from the alignment of these prototypic promoters. The sequence of the HIV-1 TAR-RNA encoding element was obtained from GenBank (accession number AM076891.1) and used to identify a conserved potential TAR-RNA encoding motif spanning the nucleotides 448 to 505 within the ERVK 5′ LTRs. Black and gray arrows indicate conventional and alternative transcription start sites, respectively. All alignments and annotations were performed in Geneious (Kearse et al. 2012).

1.4.3 |Known Transcriptional activators of the ERVK LTR

Unlike identifying transcriptional repressors of ERVK, a greater research focus has been placed on elucidating transcriptional activators of this endogenous retrovirus. To date, several human transcription factors have been experimentally shown to induce ERVK expression in human cells through their interactions with the ERVK promoter. These include Specificity protein 1 and 3 (Sp1, Sp3)[85], Yin Yang 1 (YY1)[86], Microphthalmia-associated transcription factor-M (MITF-M)[87], Octamer binding transcription factor-4 (Oct4)[13], and hormonal receptors for progesterone[88], estrogen[88], and androgen[89]. In addition, exogenous viral proteins are also known to induce ERVK transcription. For instance, HIV-1 Tat and HTLV-1 Tax proteins are able to trans-activate the ERVK promoter[68].Tat interacts with and enhances the binding of NF-κB and NFAT-1 (nuclear factor of activated T-cells 1) transcription factors to the ERVK LTR, which correlates with an increased ERVK *gag* transcription[68]. Similarly, Tax has been postulated to increase the affinity of transcription factors including Sp1 and NF-κB to their DNA binding sites on the ERVK LTR[68]. However, majority of the transcription factors shown to activate ERVK LTR are not specific to inflammatory conditions, which exhibit most notable increases in the expression of this endogenous retrovirus. Thus, the transcriptional regulation of ERVK is yet to be fully elucidated, especially in the context of neuroinflammation.

1.4.4 |Pro-inflammatory transcription factors: Novel ERVK transcriptional inducers

By utilizing extensive bioinformatics analyses, we have recently identified conserved putative binding sites for a plethora of other human transcription factors in the ERVK (HML-2) 5′ LTRs, in addition to those aforementioned[68]. The ERVK promoter is laden with potential binding sites for transcription factors involved in inflammatory signaling cascades. A striking feature of the ERVK promoter is the presence of two conserved Interferon-Stimulated Response Elements (ISREs), which bind the pro-inflammatory transcription factors Nuclear Factor-kappa B (NF-κB) and Interferon Regulatory Factor 1 (IRF1) (**Figure 5**). In addition, the 5′ LTR harbors many other conserved putative NF-κB binding sites (**Figure 5**). NF-κB and IRF1 are known to induce LTR-dependent transcription of other retroviruses, notably HIV-1. IRF1 has been shown to interact with NF-κB, and is in fact required for full NF-κB mediated activation of the HIV-1 LTR[65].

Accordingly, overlapping binding sites for NF-κB and IRF1 have been identified at the HIV-1 promoter[65]. The ERVK 5′ LTR also contains overlapping binding sites for these pro-inflammatory transcription factors (**Figure 5**). Thus, increased NF-κB and IRF1 activity may synergistically augment ERVK transcription in the context of inflammation, including in neurological diseases.

Overall, it is clear that the transcriptional signals which normally limit, as well as the signals that enhance ERVK expression in the associated inflammatory diseases, are not well understood. Through *in-silico* analyses of the ERVK promoter, we have determined that TDP-43, NF-κB, and IRF1 may be novel transcriptional regulators of ERVK. In the succeeding sections, a closer look at perturbed activity of these cellular transcription factors will aim to highlight potential mechanisms which may lead to ERVK re-activation in neuroinflammatory diseases, with a major focus on ALS.

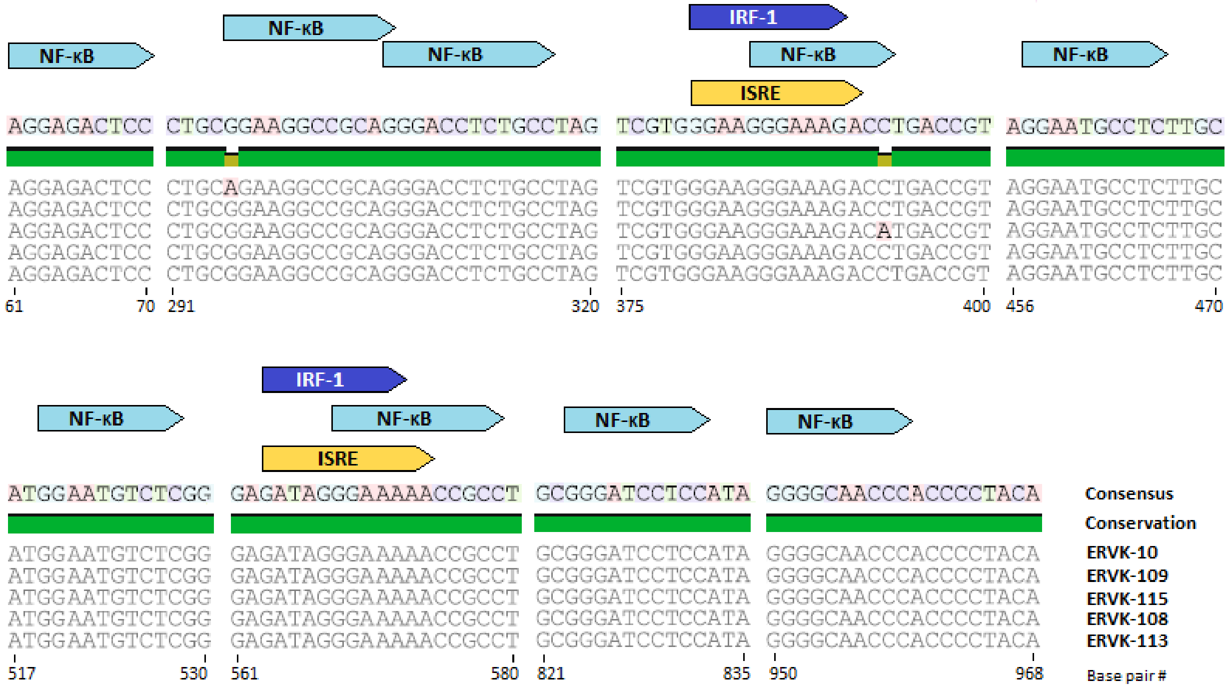

FIGURE 5. In silico examination of the conserved transcription factor binding sites and response elements within five prototypic human endogenous retrovirus-K (ERVK) 5′-LTRs using ALGGEN-PROMO software (Messeguer et al. 2002). This excerpt highlights two Interferon Stimulated Response elements (ISRE) that bind IRF1 and NF-κB, as well as other NF-κB binding sites scattered throughout the LTR. The ERVK LTR consensus sequence was constructed using individual ERVK LTRs in the following order (GenBank accession numbers in brackets): ERVK-10 (M12854.1), ERVK-109 (AF164615.1), ERVK-115 (AY037929.1), ERVK-108 (AF074086.2) and ERVK-113 (JF742069.1). Sequence alignment and annotations were performed using Geneious software (Kearse et al. 2012). Adapted from[68].

1.5 | Putative cellular pathways involved in ERVK re-activation in ALS

1.5.1 | Pro-inflammatory cytokines in ALS pathology

Immune activation and inflammation of the central nervous system (CNS) is a pathological hallmark of ERVK-associated neurodegenerative diseases including ALS. Neuroinflammation in this disease is characterized by the activation of resident innate immune cells in the CNS – microglia and astrocytes – which is accompanied by progressive degeneration of surrounding neurons[90–92]. T lymphocytes, effector cells of the adaptive immune system, have been observed to accumulate at sites of neurodegeneration in ALS[91]. Interestingly, there is also evidence of an anti-viral immune response in ALS. For instance, antibodies against ERVK (HML-2) gag proteins have been detected in the sera obtained from ALS patients[56]. In addition, the majority of infiltrating T cells in the CNS of ALS patients are CD8+ cytotoxic T lymphocytes, which are responsible for destroying virus-infected host cells[91]. Increased numbers of natural killer T cells, which also kill virus-infected host cells, have also been observed in the spinal cord of ALS patients[93]. Together, these findings are suggestive of a putative immunological reaction to endogenous viral PAMPs, potentially ERVK protein and/or nucleic acids accumulation.

Parallel to immune cell activation and infiltration, significantly higher levels of pro-inflammatory mediators called cytokines have been reported in the cerebrospinal fluid (CSF) and sera of ALS patients as compared to healthy controls[94–96]. These include cytokines belonging to the Tumor Necrosis Factor superfamily, LIGHT (homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) and Tumor Necrosis Factor alpha (TNFα), as well as Interferon gamma (IFNγ)[94,97]. Reactive microglia, astrocytes, and T cells are the major sources of these pro-inflammatory cytokines in the ALS brain[98,99,91].

There is growing recognition that TNFα, LIGHT, and IFNγ play critical roles in ALS neuropathology, as these cytokines are neurotoxic and have been associated with enhanced neuronal death. TNFα is a potent activator of the canonical nuclear factor kappa B (NF-κB) signaling pathway, culminating in the activation of p65 and p50 isoforms of this pro-inflammatory transcription factor[100]. TNFα-induced NF-κB has been shown to cause motor neuron death *in vitro*[64]. In addition, IFNγ has been demonstrated to synergize with TNFα to induce NF-κB, and enhance motor neuron death[101]. In line with this finding, anti-IFNγ therapy is protective and delays motor neuron damage in ALS mouse models[102]. Recently, elevated LIGHT signaling has been shown to selectively contribute to motor neuron death in ALS spinal cords[97,103]. IFNγ secreted by astrocytes is a key player in this process, as it leads to enhanced LIGHT production in spinal motor neurons[97,103]. Similar to TNFα, LIGHT is also a potent activator of the canonical, as well as the non-canonical, NF-κB pathways, leading to the activation of an alternate p52 NF-κB isoform[100]. Additionally, TNFα and IFNγ are known to synergistically activate interferon regulatory factor 1 (IRF1) expression[95]. But, the role of IRF1 activation in ALS pathology remains unexplored. Overall, the sum of these augmented cytokine signaling pathways likely results in excessive activation of NF-κB and IRF1 in the brain; these transcription factors may subsequently interact with the ERVK promoter and significantly enhance ERVK gene expression in CNS cells (**Figure 6**).

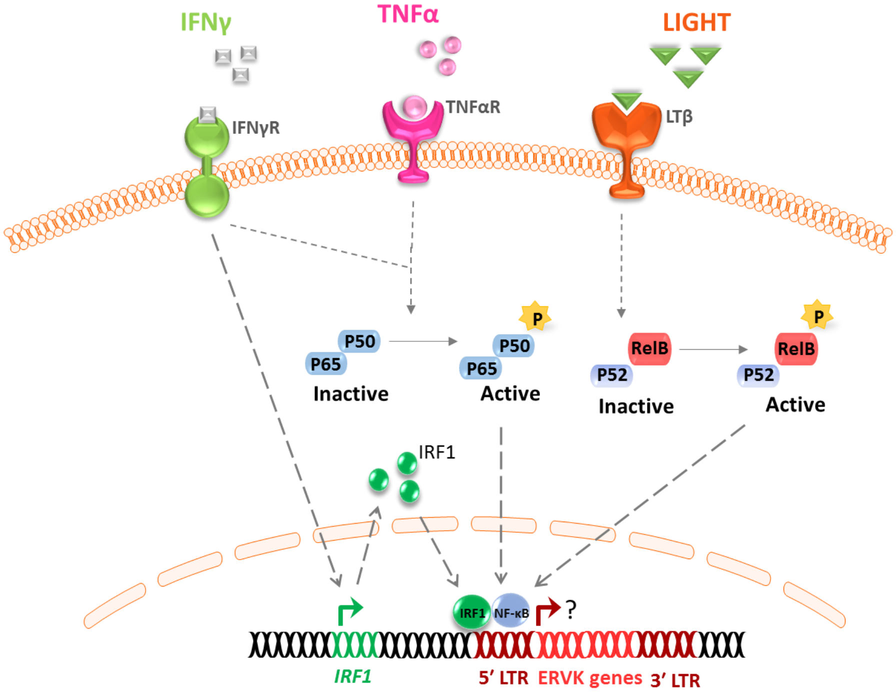

FIGURE 6. Exacerbated pro-inflammatory cytokine signaling may re-activate ERVK in ALS by facilitating the binding of active NF-κB and IRF1 transcription factors with the ERVK 5′ LTR. Artwork by M. Manghera.

In support of this theory, pro-inflammatory cytokines have previously been shown to trigger endogenous retrovirus expression in several ERVK-associated inflammatory diseases. For instance, TNFα treatment has been demonstrated to enhance ERVK (HML-2) *gag* transcription in synoviocytes obtained from patients with rheumatoid arthritis[20]. TNFα and IFNγ are able to enhance ERVW expression in peripheral blood mononuclear cells (PBMCs) obtained from patients with Multiple Sclerosis[104]. TNFα has been shown to trigger ERVW syncytin protein expression by enhancing the binding of NF-κB subunit p65 to the ERVW promoter in a human astrocytic cell line[105]. Nonetheless, how pro-inflammatory cytokines trigger ERVK expression in human cells, particularly in the CNS, remains to be studied.

1.5.2 | Deregulation of TDP-43 function in ALS

TDP-43 is a nuclear RNA/DNA binding protein involved in RNA metabolism and transcriptional regulation of a variety of genes[50,82]. This protein consists of several domains exhibiting distinct functions (**Figure 7A**). These include a nuclear localization signal (NLS) at the N terminus, two RNA-recognition motifs (RRM1 and RRM2) that interact with both DNA and RNA, and a C-terminal glycine-rich domain responsible for regulating alternative splicing and transcriptional repression of several genes[106,107].

Aggregation of TDP-43 mutants is a striking feature of several neurological disorders, including ALS[50]. Over 40 mutations have been reported in TDP-43, majority of which occur in the carboxyl terminus of this protein (**Figure 7A**)[50]. These include the two most frequent TDP-43 mutants associated with ALS – A382T and G348C[108]. The single nucleotide changes that may affect the RNA/DNA binding function of TDP-43 include the missense mutations N267S and K263E in RRM2 domain, and the mutation D169G in RRM1 domain[50,82,108]. The significance of these mutations in ALS pathology remains unknown; however, these variants may affect the interaction of TDP-43 with its target genes, such as ERVK, and alter their expression. In addition, truncated C-terminal fragments of TDP-43 often dominate in the aggregates formed within the neurons of ALS patients (**Figure 7B**)[109]. The loss of NLS redirects these TDP-43 fragments from the nucleus to the cytosol, abolishing the nuclear functions of this protein. It has been demonstrated that these fragments can also drive aggregation of normal TDP-43, and thus

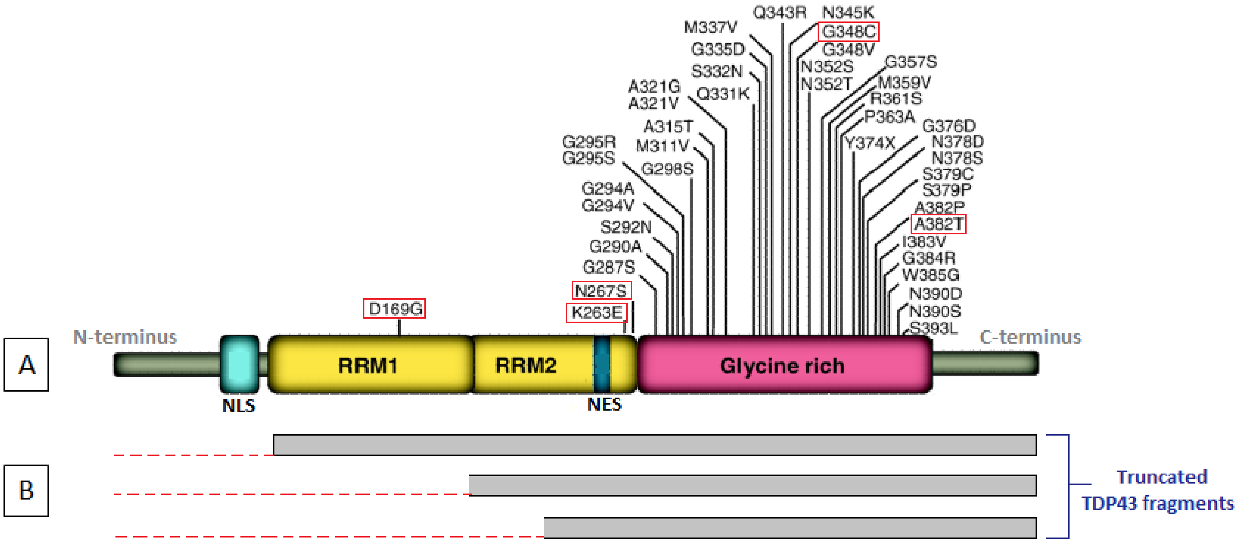

Figure 7. (A) Structure of the TDP-43 protein including mutations that affect the function of each domain. Mutations in the RRM1, RRM2, and glycine rich domains that may play a role in ERVK re-activation in ALS are boxed in red. Adapted from [110]. **(B) Truncated forms of TDP-43 known to form aggregates within neurons.** The dotted red line indicates the missing domains. These fragments are mislocalized to the cytosol, and may abolish nuclear functions of TDP-43. Adapted from [109].

reduce the levels of nuclear TDP-43[109]. If TDP-43 serves as an ERVK repressor, the loss of nuclear TDP-43 function may relieve TDP-43-dependent inhibition of ERVK expression, thus augmenting ERVK RNA and protein levels in ALS.

In contrast, a strong correlation has been found between the overexpression of wild-type TDP-43 and high levels of ERVK *pol* transcripts in neurons of ALS patients[31], suggesting that TDP-43 may induce ERVK transcription. This effect may be mediated through enhanced interaction of TDP-43 with its putative binding sites on the ERVK promoter (**Figure 4**). Overexpression of TDP-43 has also been associated with increased levels of active NF-κB in neurons, astrocytes, and microglia from ALS spinal cord tissue[111]. Interestingly, TDP-43 has been shown to directly interact with p65 through its N terminus and RRM1 domain[111]. This interaction mediates activation of p65, causing its translocation to the nucleus[111]. Thus, TDP-43 variants that lead to constitutive p65 activity may be responsible for inducing ERVK expression in ALS. In addition, overexpression of wild type and ALS-associated TDP-43 mutants in glial cells causes hyperactive innate immune responses against bacterial lipopolysaccharides (LPS), significantly increasing TNFα production and microglia-mediated neurotoxicity[111]. Similarly, in the presence of TDP-43 mutants, detection of ERVK-associated molecular patterns by innate immune sensors may aggravate the inflammatory response and lead to neuronal damage in ALS. Although substantial progress has been made in elucidating the role of TDP-43 dysfunction in ALS, the underlying mechanisms by which it mediates neurodegeneration still remain unclear.

Recently, it was demonstrated that the 25 KDa C-terminal fragment of TDP-43, known as TDP-25, leads to increased levels of this protein in the nucleus and the cytosol of neurons from transgenic TDP-25 homozygous mice[112]. TDP-25 aggregation is a consistent feature of ALS[112]. In this study, TDP-25 accumulation associated with severe memory deficits and poor motor performance as compared to TDP-25 heterozygous mice. Interestingly, increased TDP-25 levels were found to reduce the function of autophagic and proteasomal clearance pathways – dysfunction of both of these pathways has been implicated in ALS[67]. Autophagy and the proteasome system are responsible for clearing protein aggregates and pathogens, and

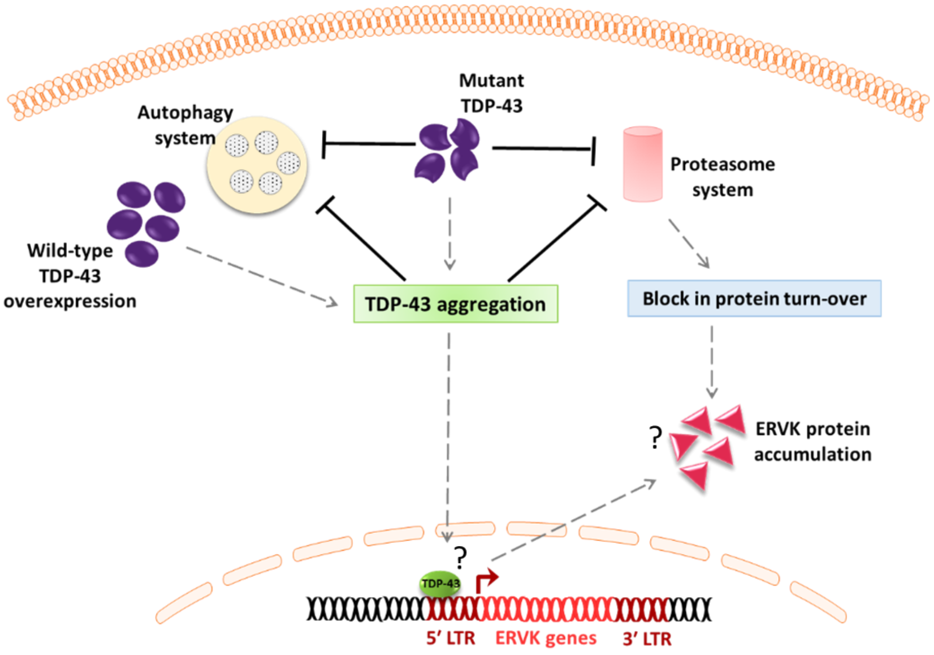

FIGURE 8. TDP-43 aggregation, proteasome inhibition, or dysfunction of the autophagy system may lead to ERVK transcriptional re-activation and protein accumulation in ALS. Artwork by M.Manghera.

thus may also clear ERVK expression in human cells[48]. However, in the presence of high TDP-25 levels, a blockade in protein turnover may lead to ERVK proteinopathy in ALS (**Figure 8**). Thus, not only do TDP-43 variants have the potential to alter ERVK gene transcription, but they may also inhibit ERVK protein turnover in the CNS, contributing towards enhanced ERVK activity in this disease.

1.5.3 | The sum of deregulated pathways likely determines the ultimate pathological outcome

Overall, elevated pro-inflammatory cytokine signaling and simultaneous TDP-43 dysfunction may cooperate to considerably enhance ERVK expression in ALS. Elevated levels of pro-inflammatory cytokines may contribute to enhanced levels of inflammatory transcription factors NF-κB and IRF1, which may synergize to promote ERVK re-activation in CNS cells. Simultaneously, TDP-43 overexpression may enhance NF-κB binding to the ERVK promoter. TDP-43 may itself bind to the ERVK promoter and stimulate ERVK transcription. Alternatively, TDP-43 mutations may relieve inhibition of ERVK expression, and thus enhance ERVK protein levels. In addition, TDP-43 variants may reduce ERVK protein turnover in cells, thus leading to ERVK protein aggregation. Together, the sum of these multiple events likely determines the level of ERVK proteinopathy in the CNS.

1.6 | Publication 1

Endogenous retrovirus-K and nervous system diseases

Mamneet Manghera, Jennifer Ferguson, and Renée Douville

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(Invited review)

The goal of this article was to write a comprehensive review of ERVK-associated neuroinflammatory diseases, with the aim to reconcile the pathologic contribution of ERVK by providing evidence of altered molecular regulation of this endogenous retrovirus, detailed examples of ERVK-mediated pathological processes, and altered inter-individual differences in ERVK genotypes in disparate neurologic diseases.

Endogenous retrovirus-K and nervous system diseases

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Abstract

A new appreciation of the microbiome is changing the way we perceive human health and disease. The holobiontic nature of humans is even etched into our DNA in the form of viral symbionts. Empirical evidence for the presence of endogenous retroviruses (ERVs) in the human genome and their activity in homeostatic and pathological states has accumulated; however, no causal relationship with human disease has been established to date. In this review, we will focus on the role of endogenous retrovirus-K in neurological disease. Specifically, we will attempt to reconcile the pathological contribution of ERVK in disparate neurological diseases by providing evidence as to inter-individual differences in ERVK genotypes, addressing the molecular regulation of ERVK, and providing detailed examples of ERVK-mediated processes in nervous system diseases.

Keywords

Endogenous retrovirus; Human endogenous retrovirus-K; Polymorphism; Transcription factor; Inflammation; Amyotrophic Lateral Sclerosis; Schizophrenia; Bipolar disorder; Multiple Sclerosis; Human Immunodeficiency Virus; Prion Disease; Anti-virals

Introduction

The DNA provirus hypothesis – where viral DNA integrates into a host genome – was proposed by Nobel laureate Howard M. Temin in the 1960s [1]. Indeed, over 8% of human DNA is the result of retrovirus integrations scattered throughout the genome. Among the 31 lineages of endogenous retroviruses (ERVs) within the human genome (which are spread among several *Retroviridae* subfamilies), the betaretrovirus ERVK (alias Human Endogenous Retrovirus-K, HERV-K) is the most recently endogenated ERV. The ERVK (HML-2) clade is estimated to have been active as recently as 250,000 years ago [2], and is considered the most transcriptionally active ERV. Several insertions in the human genome are relatively intact, permitting the expression of viral RNA and proteins. Full-length ERVK elements retain a classical retroviral genome structure, with core genes *gag* (group-specific antigen), *pr* (protease), *pol* (polymerase) and *env* (envelope) flanked by long terminal repeats (LTRs) [3]. Regulatory proteins within ERVK have also been described [4,5]. There is even evidence of ERVK virion production in HIV infection [6] and lymphoma [7]. Unlike canonical retroviruses, Dube *et al.* have recently proposed that ERVK virions can contain either infectious viral RNA or viral DNA genomes [8], thus changing how ERVK expression and replication should be viewed in the context of health and disease pathology.

Genotypic inter-individual differences in ERVK

There are approximately one thousand ERVK (HML-2) integrations in humans, based on the human reference genome. Of these, all are considered replication-defective, with only 24 fixed loci retaining the capacity to encode viral proteins from at least one of their genes [3,9]. However, evidence suggests that this is a fraction of the entire ERVK presence within individual human genomes [2,10,11].

Polymorphic ERVK insertions (unfixed proviruses) have been identified in several cohort studies [2,3,9,12,13], with considerable variation between ethnic groups, as well as distinct inter-individual profiles. These studies indicate that people carry a distinctive ERVK signature based on individual genotypes. For a given loci, ERV polymorphism can occur as integration of a full-length ERV (with varying degrees of coding capacity), a solitary LTR, or an unoccupied pre-integration site [3,9,14]. Recently, Belshaw's group has performed Next Generation Sequencing on individual human genomes revealing that several unfixed ERVK (HML-2) loci are absent from the human reference genome annotation [2]. Moreover, the frequency of unfixed ERVK (HML-2) loci varied dramatically in the populations tested [2], further supporting the idea that specific ERVK signatures may be associated with inter-individual differences in ERVK expression, pathology and disease states.

Sequence variation, resulting in ERV alleles, may also alter the function of viral proteins. For example, the ERVK-18 envelope protein is a superantigen that is encoded by three distinct alleles which can alter the amino acid sequence of the protein [15], with predicted but uncharacterized biological effects. Among these three ERVK-18 *env* alleles, the K18.3 form is the minor allele with a frequency of 10.8% within the Caucasian population [15]. The ERVK-18 *env* polymorphism has been shown to be a risk factor for Multiple Sclerosis (MS); homozygous carriers of the K18.3 allele had a significantly increased risk of this disease, suggesting that ERVK-18 may influence the genetic susceptibility to MS [16,17]. ERVK-18 has also been associated with enhanced risk of Type 2 diabetes (T2D) in individuals with schizophrenia (SCZ) [18], with a risk haplotype comprised of two single nucleotide polymorphisms (SNPs) in the *env* region (rs558648 and rs1090799). These results remain controversial, as several cohort studies disagree over whether ERVK-18 polymorphisms are risk factors in T2D and SCZ [18,19].

Phenotypic variation in the expression of ERVK

Current research indicates that not all ERVs remain silent passengers within our genomes; re-activation of ERVK is associated with many inflammatory diseases, such as cancers [7], HIV infection [4], Rheumatoid Arthritis [20], Systemic Lupus Erythematosus [20] as well as neurological conditions including Multiple Sclerosis (MS) [21], Schizophrenia (SCZ) [22], Bipolar disorder (BD) [22], Amyotrophic Lateral Sclerosis (ALS) [23] and Creutzfeldt-Jakob disease (CJD) [24]. While there is ubiquitous ERV expression in many tissues, regardless of health or disease, it has been shown that individuals largely exhibit distinct ERV expression signatures [22]. A difficulty in understanding these individual profiles and their association with disease states is a lack of appreciation for the biological control of ERVs.

At the molecular level, there is limited experimental evidence to indicate the cellular state or signals that are required to control the expression of ERVK. Accumulating evidence points to the importance of epigenetic mechanisms in the control of transposable elements including ERVs, and has been reviewed elsewhere [25]. The transcription of ERVK is under the control of viral promoters called Long Terminal Repeats (LTRs), which flank either side of the provirus. To date, only transcription factors Sp1 [26], Sp3 [26], YY1[27], MITF-M[28] and steroid hormone receptors [29,30] have been experimentally shown to induce ERVK activity in human cells. Our group has recently focused on examining the role of pro-inflammatory transcription factors in the induction of ERVK expression. Using bioinformatics, we have revealed that the ERVK promoter contains multiple conserved putative binding sites for pro-inflammatory transcription factors, including Nuclear Factor Kappa B (NF-κB) and Interferon Response Factors (IRFs) [31]. Specifically, the viral promoter harbors two conserved Interferon Stimulated Response Elements (ISREs) (**Figure 1**); thus, inflammatory stimuli may modulate ERVK transcription. We have also generated substantial experimental evidence using human neuron and astrocyte *in vitro* models to support this claim (unpublished results). Thus, ERVK can exploit anti-viral immune responses and perhaps certain disease backgrounds, as select transcription factors can promote ERVK expression.

Additional evidence supports the importance of innate immune signaling in ERVK re-activation, as select anti-viral and pro-inflammatory cytokines can enhance ERVK expression. Cytokines, notably Tumor Necrosis Factor α (TNFα) and Interferon γ (IFNγ), play critical roles in the pathology of many neurodegenerative diseases including ALS [32,33], SCZ [34], MS [35], and CJD [36]. TNFα and IFNγ are potent activators of NF-κB and IRF1, respectively, and may thus enhance ERVK transcription in these neuroinflammatory diseases (**Figure 1**). We have recently generated evidence in human neuron and astrocyte *in vitro* models to support this claim (unpublished results). TNFα has previously been demonstrated to augment ERVK expression in rheumatoid arthritis – another inflammatory disease [37]. TNFα-mediated induction of ERVW env expression, following the binding of NF-κB with the ERVW promoter, has also been documented [35]. In addition, ERVK-18 expression can be enhanced upon IFNα treatment of peripheral blood lymphocytes [15]. Exogenous IFNα drives IRF9 activation and its translocation to the nucleus where it binds to ISREs in target promoters (**Figure 1**). These results are consistent with our observation that ISREs in the ERVK LTR serve as key promoter elements. The ERVK *env* may also confer a self-regulating capacity, as an immunosuppressive domain in the transmembrane (TM) protein alters cytokine release through its immunomodulatory effects [38]. Although recombinant ERVK transmembrane protein and ERVK virions induced substantial IL-10 secretion in peripheral blood mononuclear cells (PBMCs), reproducible inter-individual differences in the IL-10 response were observed [38]. Moreover, notable enhancement of pro-inflammatory cytokine expression and impairment of genes involved in innate immunity [38], further suggests that the ERVK TM protein will alter the regulation of ERVK, as well as host genes. Additionally, ERVK encoded dUTPase can activate NF-κB and promote pro-inflammatory cytokine secretion[39]. Additional ERV proteins are suspected to influence protein-protein interactions in humans [40]. Considering that signalling pathways are finely tuned based on the activity of interacting proteins, the genetic background of the host will play a significant role in ERVK expression and immunomodulation. These findings suggest that ongoing signaling cascades in neuro-inflammatory disease may trigger ERVK re-activation, thus promoting the expression of viral RNA and proteins which may further modulate the pathological status.

Putative protective and pathological roles of ERVK in neurological disease

Amyotrophic Lateral Sclerosis (ALS)

Retroviruses, such as Human Immunodeficiency Virus (HIV) and Human T-cell Leukemia virus (HTLV), have been associated with an increased incidence of ALS-like syndromes [41,42]. Currently, a single study has demonstrated a direct association between ERVK and ALS [23], despite evidence for retroviral pathology stemming from the repeated measurement of reverse transcriptase (RT: the retroviral enzyme that transcribes viral RNA into DNA) activity in this disease [43-45]. Elevated levels of ERVK *pol* transcripts (derived from select HML-2 and HML-3 loci) are detectable in post-mortem brain tissues of patients with ALS, as compared to tissues from Parkinson's disease, systemic disease and accidental death [23]. Not only was ERVK RNA expressed in ALS, immunohistological analysis revealed the presence of RT protein in the cortical neurons of patients with ALS [23]. Clusters of neurons in the prefrontal and motor cortex of patients with ALS exhibited the strongest RT expression, coinciding with the affected brain areas in this disease. An earlier report demonstrated that over half of ALS patients examined showed serum IgG reactivity against ERVK (HML-2) gag protein [46]. Patients with reactive anti-HML-2 gag antibodies exhibited a 10-fold reduction of viral RNA in PBMCs, suggesting an effective and ongoing immune response against ERVK in these patients with ALS [46]. As discussed by Alfahad and Nath [47], these studies open new avenues of investigation into the treatment of ALS.

Schizophrenia (SCZ) and bipolar disorder (BD)

Several studies have documented aberrant expression of ERVs in patients with schizophrenia [22,48-53], and to a lesser extent, in patients with bipolar disorder [22,48]. ERVW gene expression has been discovered in blood samples [49-51], in cerebrospinal fluid (CSF) [52], and in post-mortem brain tissue [52,53] of patients with SCZ, and has been reviewed extensively elsewhere [54]. Specifically, only ERVK10 (HML-2) RNA was significantly over-expressed in both SCZ and BD compared with healthy post-mortem brain tissue [22]. The ERVK HML-7 clade is also significantly over-represented in SCZ compared to BD samples (but not in SCZ compared to healthy controls), and under-represented in samples from patients with BD compared to healthy-brain samples [22]. A study by Diem *et al.* further demonstrated that ERVK transcription was not affected by treatment with valproic acid (VPA; a medication used to treat SCZ) or any of the other medications tested, indicating that previous findings of an association between ERVK transcription and SCZ cannot be explained by patient treatment with any of the four medications analyzed in this study [55]. To date, this represents limited and loci-specific alterations in ERVK expression in these neuropsychiatric diseases.

It has been postulated that it may not be mutations in genes associated with SCZ that result in a disease state, but rather mutations in the regulatory regions of these genes [56]. ERV LTRs are known to have promoter, enhancer and regulatory functions [57]. Approximately 50% of all human-specific ERVK (hsERVK – HML-2) elements show promoter activity in human tissues [58]. Epigenetic silencing of ERVs by DNA methylation is a known phenomenon, and is thought to be a part of the anti-retroviral defense system [25]. Therefore the silencing or down-regulation of genes with ERV sequences in their regulatory regions may be the consequence of the host's attempts to stop the expression of these endogenous viruses.

Recently, a full-length almost intact ERVK (HML-2) sequence that displays strong enhancer activity, was identified near the *PRODH* gene [59]. Mutations in *PRODH*, which encodes a mitochondrial enzyme, have been found to be associated with neuropsychiatric disorders, including SCZ [60]. Given this link between *PRODH* and schizophrenia, Suntsova *et al.* attempted to characterize this ERVK locus (referred to as hsERV$_{PRODH}$) and its potential enhancer activity for *PRODH* [59]*.* They showed that the enhancer activity of hsERV$_{PRODH}$ is regulated by methylation and it acts synergistically with the *PRODH* internal CpG island to activate the *PRODH* promoter. Transcriptional analysis showed that *PRODH* displays the highest expression level in the hippocampus, where hsERV$_{PRODH}$ is hypo-methylated [59]. The hippocampus is known to be one of the structures of the brain that is most affected in SCZ [61]; if hyper-methylation of hsERV$_{PRODH}$ occurred, aberrant expression of *PRODH* in the hippocampus would likely result.

Similarly, an ERVW LTR is located in the regulatory region of the GABA receptor B1 gene (GABBR1) [56], a gene located in region associated with risk for SCZ [62]. It is speculated that hyper-methylation of this ERVW LTR may down-regulate GABBR1 in brains of patients with SCZ [56], thus accounting for its altered expression pattern [63,64]. As a result, Hegyi *et al.* propose that the over-expression of ERVs at the onset of disease leads to their subsequent silencing by hyper-methylation, which may pathologically contribute to diseases such as SCZ [56]. This hypothesis also offers an explanation as to why ERVW transcripts are readily found in the CSF of patients with recent-onset SCZ, but rarely in chronic patients [49,52,65]. It could be that the activation of ERVs occurs early in the etiopathology of schizophrenia or during highly symptomatic periods of disease, resulting in the up-regulation of some genes for which ERV elements act as promoters or enhancers. This may be followed by hyper-methylation of ERV sequences as a defense mechanism, leading to down-regulation of ERV-regulated genes.

Multiple Sclerosis (MS)

Among ERVs associated with MS, ERVW has been the most extensively studied. Many studies have reported significant up-regulation of ERVW RNA in brain samples from MS patients [35,66]. ERVW env protein is highly expressed within astrocytes and microglia in MS plaques, and correlates with the extent of inflammation and active demyelination [35,66]. Augmented ERVW expression has also been observed in the CSF and blood of MS patients [67,68]. A recent study has also shown enhanced ERVW DNA copy number in the PBMCs of women with MS; this phenomenon correlated with disease severity scores [68]. In contrast, other studies depict a lack of association between enhanced ERVW expression and MS. Using high-throughput amplicon sequencing, Schmitt *et al.* reported a lack of significant difference in ERVW transcripts between MS and control brain tissue samples, despite clear evidence of inter-individual variability [69]. Similarly, enhanced ERVW expression in the CSF and blood of MS patients could not be detected in several studies [70,71]. Thus, a definitive association between ERVW activation and MS neuropathology remains to be established.

Nonetheless, other human endogenous retroviruses, including ERVK, have been reported to be up-regulated in MS. Elevated levels of ERVK RNA have been found in the brain tissue from MS patients [72]. As mentioned above, the ERVK-18.3 *env* allele has been determined to be a risk factor for MS. Interestingly, ERVK-18 env superantigen can be transactivated by Epstein Barr Virus (EBV) latent membrane protein LMP-2A [73,74], and EBV infection is considered to be one of the major risk factors for MS [75]. Similarly, ERVW env protein also displays superantigenic properties, and can be transactivated by EBV infection of astrocytes *in vitro* [76]. Together, these superantigens may promote the non-specific activation of T lymphocytes in the CNS, leading to extensive demyelination and neuronal injury [77]. Thus, ERV-derived superantigens may contribute to MS immuno-pathogenesis, particularly in the context of EBV infection.

Activation of the host immune system has been implicated as the ultimate effector in MS pathogenesis. Re-activation of human endogenous retroviruses in the CNS may play an important role in this process, as the immune system may mount an anti-viral response against ERV elements in order to eliminate ERV-expressing cells. Anti-retroviral defense mechanisms can be mediated by a variety of innate immune sensors including Pattern Recognition Receptors (PRRs) that detect retroviral RNA and proteins. PRRs, including Tripartite motif containing 5 (TRIM5) and Toll like receptor 4 (TLR4), are known to recognize retroviral capsid and envelope proteins, respectively; engagement of these sensors with their viral ligands activates signaling cascades that stimulate innate immunity [78,79]. The role of TRIM5 in detection of gag proteins encoded by MS-associated ERVs has not yet been studied. Nonetheless, single nucleotide polymorphisms (SNPs) in *TRIM5* gene (as well as SNPs in other viral restriction factors) have been associated with the risk of MS [80]. However, the functional outcomes of these SNPs remain unclear.

Another mechanism by which ERV proteins may trigger MS immunopathology is through molecular mimicry. Recently, ERVW env proteins were predicted to share several T and B cell epitope regions with myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) [81]. This suggests that ERVW env over-expression in the CNS may break tolerance towards host MOG and MBP, generating an autoimmune response against these myelin proteins, which can explain extensive demyelination typically observed in MS. However, the cross-reactivity between ERVW env and myelin protein epitopes, and the resulting autoimmune reaction, needs to be validated experimentally. In addition, whether antigen mimicry is also employed by other MS-associated ERVs, such as ERVK, remains to be explored.

HIV infection

ERVK activity is well-documented in HIV infection [6,82] (reviewed in [4]), including the nervous system (Douville and Nath, unpublished) [83]. Recently, Bhat *et al.* have provided evidence that enhanced ERVK (HML-2) env protein expression in the brains of HIV infected individuals may confer neuroprotective effects [83]. This is based on the observation that neuroblastoma cells transfected with an ERVK env expressing construct were protected from injury by staurosporine and the HIV-1 Vpr protein, as compared to the control vector alone. Moreover, the protection from HIV-1 Vpr toxicity was recapitulated in vpr/RAG1$^{-/-}$ mice which were adoptively transferred with neural stem cells expressing ERV-K Env into the striatum; these animals exhibited a significant reduction in TNFα expression as compared with controls.

Exaptation of ERVK Env may provide neurons a degree of protection in the context of chronic neurodegenerative diseases.

Moreover, cellular cytotoxic responses and antibodies produced against ERVK can prove to be detrimental to HIV-infected cells [84,85]. During HIV infection, ERVK env peptides can be a target for cytotoxic T cells [84]. NK cells may also destroy HIV-infected cells via an antibody-dependent cytotoxic mechanism, based on *in vitro* assays [85]. Additionally, it was observed that either the HIV strain or the host were important factors in determining the extent of ERVK env induction in HIV-infected cells [85,86], and thus may alter the degree of CNS tissue injury in HIV-associated neurocognitive disorder (HAND).

In addition, other ERVK proteins may promote changes in dendritic spine morphology in pyramidal neurons. The ERVK regulatory protein Rec has been shown to interact with the mRNA binding protein Staufen-1, causing its accumulation in the nucleus [5]. This interaction may alter Staufen-1-mediated mRNA trafficking and turnover, functions that are essential for regulation of neuronal synapses during long-term plasticity in learning and memory [87]. The interaction with Staufen-1 also favoured Rec-dependent viral RNA transport [5], and thus may enhance ERVK protein expression. Moreover, ERVK and HIV Gag proteins can both independently interact with Staufen-1 to enhance their respective production of virions [5,88], as well as ERVK Env expression within HIV-1 virions [89]. Together, these studies suggest that ERVK expression in the CNS may have both protective and pathological consequences.

Prion Disease

Prion diseases, such as Creutzfeldt-Jakob disease (CJD) in humans, are a group of rare but fatal neurodegenerative disorders. The causative agent of these diseases is believed to be an infectious misfolded cellular protein called a prion protein (PrP^{SC}), which is resistant to proteinase degradation and accumulates inside neurons, leading to neuronal toxicity and death [24,90]. The disease propagates upon transmission of PrP^{SC} to new cells, which further catalyzes the conversion of the normal cellular prion protein (Prp^{C}) into its abnormal form; however, the mechanisms behind this conversion have not been clearly elucidated.

Recently, augmented expression of several ERVs has been observed in the CSF of CJD patients [24]. Although the frequency of ERVK transcripts was higher in CJD CSF samples as compared to the controls, this result did not reach statistical significance. Nonetheless, the increased expression of ERVs in CJD suggests that endogenous retroviruses may contribute to the pathogenesis of this prion disease. For instance, ERV viral RNA molecules may elicit the transformation of PrP^{C} to PrP^{SC} [90]. In support of this hypothesis, small highly structured RNAs have been shown to interact with human recombinant PrP^{C} and stimulate its conversion to a proteinase resistant isoform [91]. Interestingly, RNA molecules derived from ERVK elements have extremely conserved complex secondary structures resembling that of the small highly structured RNAs used in these studies [91](Carr and Douville, unpublished). Highly structured RNAs derived from HIV-1 have also been shown to interact with the human recombinant PrP^{C} and impart proteinase resistance to it *in vitro* [92]. Thus, increased levels of ERVK RNA in the CSF of CJD patients have the potential to drive the transformation of the normal human prion protein to its infectious misfolded isoform.

In addition, ERVs may facilitate the spread of pathological prion agents intercellularly by recruiting prion proteins to virions as ERVs replicate. In fact, it was recently demonstrated that murine PrP^{SC} associates with gag and env proteins on Moloney Murine Leukemia Virus (MMLV) particles, and infection with MMLV strongly enhances the extracellular release of murine PrP^{SC}, thus augmenting the infectivity of this prion protein [90,93]. Similarly, human PrP^{SC} has also been shown to be recruited by HIV-1 virions [93]. Human endogenous retroviruses, ERVW and ERVK, which are capable of producing virions [7,21], may also be able to recruit PrP^{SC} either through interactions with surface gag and env proteins or with viral RNA, thereby transmitting prion proteins to new cells and facilitating the progression of human prion diseases.

Moreover, CJD is neuroinflammatory and marked by augmented levels of pro-inflammatory cytokines, including TNFα [36,94]. Mice models of CJD also exhibit increased TNFα, as well as NF-κB activity [95]. Recently, the toxic domain of human prion protein has been shown to activate NF-κB, and lead to TNFα production in a macrophage cell line [96]. Based on our prediction of NF-κB responsive elements in the ERVK promoter [31] (**Figure 1**), it is possible that PrP^{SC}-induced TNFα production and NF-κB activation may enhance ERVK transcription in CJD brains. This may culminate in a positive feedback loop favouring further neuroinflammation, ERVK re-activation, and prion infection.

Conclusion

Although ERVK has not been shown to be a causative agent of nervous system disease, its expression can clearly influence both protective and pathological aspects of motor neuron, neuropsychiatric and neurodegenerative diseases (**Table 1**). A common thread among ERVK-associated disease appears to be the presence of inflammatory signals; but how this retrovirus fits into the complex interplay between infection, immunity, autoimmunity and environmental exposures is yet to be fully elucidated. Activation of multiple ERVs may cooperatively stimulate a multitude of host anti-retroviral immune responses against ERV-expressing cells; and, ERVs may exploit this response, culminating in a positive feedback loop favouring further viral gene expression, excessive neuroinflammation, and subsequent neuronal injury and loss. Moreover, it is important to consider that specific ERVK loci can confer select pathological contributions. Bulk measurement of ERVs (without consideration of the individual integrations and their genomic context) may be an insufficient methodology to address their role in distinct neurological diseases. Examining an individual's unique complement of ERVs may prove to be a better predictor of disease risk, once further inroads are made in understanding the protective and pathological roles of each integrated provirus. It will be important for future studies to expand how we measure ERVK activity in the CNS; improved screening for ERVK expression in specific cell types, CNS regions and disease stages, as well as an expansion towards single-loci ERVK measurements will broaden our current knowledge in this area. Current studies are limited by the availability of commercial ERV-specific reagents for molecular biology and the expense of high-throughput screening techniques – a possible solution for our field would be the development of an ERV resource bank, as has been accomplished with the NIH AIDS Reagent Program.

Another benefit of ERV research in the context of nervous system disease is the possibility of improved therapeutics. For example, patients with schizophrenia and bipolar disorder are treated with a range of chemotherapeutics including antipsychotics and lithium. Lithium is protective against HIV neurotoxicity, and HIV patients treated with this medication show cognitive improvements [97]. Clozapine is an antipsychotic drug, which actually inhibits HIV replication *in vitro* [98]. Since both of these drugs interact with exogenous retroviruses, it is possible that they may have some effect on ERVs as well. In support of this notion, there is epidemiologic evidence that incidence of ALS is extremely rare among individuals with SCZ (much lower than predicted for the general population) [99]. Common medications for SCZ may convey prophylactic neuro-protection, inhibiting the development of ALS [99]. There is some evidence that medications routinely prescribed to schizophrenics may stop inflammation and support neuronal survival [100]. Abating inflammation may also decrease the expression of ERVK, which is up-regulated by inflammatory transcription factors [31]. With improved biomarkers for neurological disease risk, the use of currently vetted SCZ medications may be repurposed for the prevention or delay of ERVK-associated nervous system diseases.

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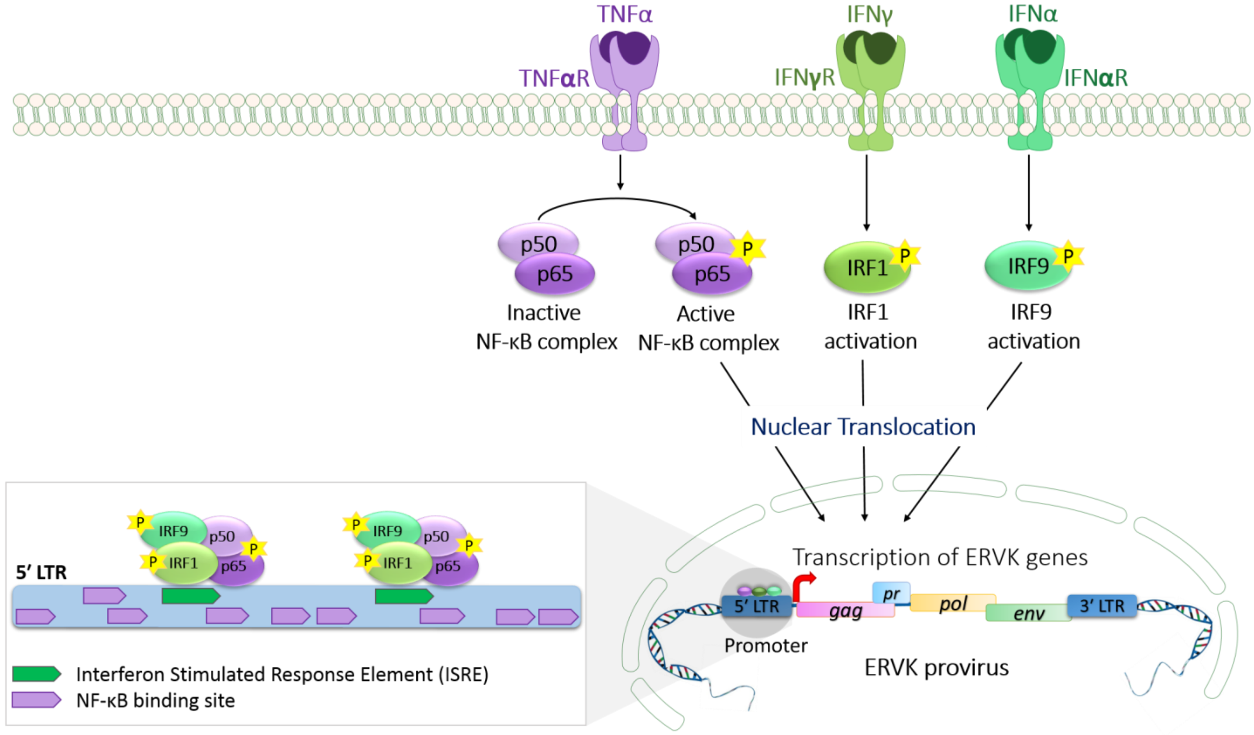

Figure 1. Pro-inflammatory signaling cascades and the associated transcription factors that may stimulate ERVK gene expression in multiple neurodegenerative diseases. TNFα, IFNγ and IFNα signaling leads to the phosphorylation (P) and activation of NF-κB (isoforms p50 and p65), IRF1, and IRF9, respectively. These pro-inflammatory transcription factors then translocate to the nucleus, where they bind their respective sites in the target promoters. The ERVK promoter (5′ LTR) contains multiple conserved putative NF-κB binding sites, as well as two Interferon Stimulated Response Elements (ISREs) that bind IRFs including IRF1 and IRF9 [31]. Binding of nuclear NF-κB, IRF1, and/or IRF9 to the ERVK promoter may induce the expression of downstream proviral genes – *gag* (group specific antigen), *pr* (protease), *pol* (polymerase), and *env* (envelope).

Table 1: Summary of potential mechanisms of ERVK protection and pathogenesis in neurodegenerative diseases

Neurological Disease	Putative mechanisms of protection or pathogenesis
Amyotrophic Lateral Sclerosis (ALS)	ERVK RNA and proteins may stimulate a pro-inflammatory immune response against ERVK-expressing neurons [23], leading to neuronal injury and loss. ERVK may also exploit this inflammatory response [31], thus establishing a cycle of ERVK re-activation and excessive inflammation.
Schizophrenia (SCZ) and Bipolar Disorder (BD)	ERVK LTR sequences and Env protein may act as regulatory elements for genes associated with SCZ and BD [56,59]. Upon detection of ERVK over-expression (brought on by infection or inflammation), methylation of ERVK sequences may decrease the subsequent expression of SCZ and BD associated genes (necessary for normal neurological function), leading to a diseased state.
Multiple Sclerosis (MS)	ERVK-encoded superantigens may exacerbate neuroinflammation [73,77], and thus lead to demyelination. Recognition of ERVK RNA and proteins by innate immune sensors may generate an anti-retroviral response against ERVK-expressing cells in the CNS [78,79], thus causing neuronal injury and loss. ERVK env may mimic myelin proteins, which may produce an autoimmune response and contribute to demyelination [81].
HIV infection	ERVK Env protein may confer protection against HIV-1 Vpr-induced toxicity [83]. Humoral and cytotoxic immune responses targeted at ERVK antigens may promote the killing of HIV-infected cells [84,85]. ERVK Rec protein may promote changes in neuronal dendritic spine morphology by interacting with Staufen-1 [5].
Creutzfeldt-Jakob Disease (CJD)	ERVK RNA may stimulate conversion of normal proteins to pathogenic prion proteins [91]. ERVK virions may recruit and facilitate intercellular transmission of prion agents [92]. ERVK RNA and proteins may exacerbate neuroinflammation.

2. HYPOTHESES AND OBJECTIVES

The first aim of this study was to elucidate the influence of augmented levels of pro-inflammatory cytokines on ERVK re-activation in CNS cells. **I hypothesized that pro-inflammatory cytokines will enhance ERVK transcription and protein levels by facilitating NF-κB and/or IRF1 interactions with the ERVK promoter in astrocytes and neurons.**

To test this premise, we derived the following experimental objectives:

1) Determine whether TNFα, LIGHT, and IFNγ enhance ERVK transcription and polyprotein/RT levels in a dose-dependent manner.
2) Evaluate the interactions of NF-κB and IRF1 at the ISREs on the ERVK promoter in untreated and cytokine-stimulated cells.
3) To validate the *in vitro* findings, determine if the brain tissue from ALS patients exhibits increased levels of NF-κB and IRF1 in ERVK positive cells.

Another key goal of this study was to evaluate the influence of wild-type and ALS-associated C-terminal truncated forms of TDP-43, as well as TDP-43 turnover, on ERVK transcription and protein levels. We also aimed to determine whether TDP-43 is able to interact with the ERVK promoter, and how truncated TDP-43 fragments alter this interaction. **I hypothesized that wild type and truncated TDP-43 fragments will modulate ERVK transcription and protein accumulation in astrocytes and neurons by altering TDP-43 binding to the ERVK promoter. I also hypothesized that decreased TDP-43 turnover will enhance ERVK transcription and ERVK proteinopathy in astrocytes and neurons.**

To test this premise, we derived the following experimental objectives:

1) Determine whether overexpression and aggregation of wild-type and truncated TDP-43 forms enhances ERVK transcription and polyprotein/RT levels.
2) Determine whether proteasomal inhibition, and thus decreased TDP-43 turnover, increases ERVK transcription and polyprotein/RT aggregation.
3) Evaluate the interactions of TDP-43 with the ERVK promoter under normal conditions, as well as during proteasomal blockade and in the presence of truncated TDP-43 fragments.

3. RESULTS

3.1 | Publication 2

ERVK Polyprotein Processing and Reverse Transcriptase Expression in Human Cell Line Models of Neurological Disease

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The goal of this study was to elucidate the influence of IFNγ on ERVK transcription, polyprotein cleavage, as well as RT expression and enzymatic activity in astrocytes and neurons. This is the first report to establish that inflammatory conditions, such as IFNγ exposure, markedly induce ERVK expression in CNS cells. We are the first to characterize sequential ERVK gag-pro-pol polyprotein cleavage, culminating in the production of active ERVK RT subunits in human astrocytic and neuronal cell lines, under inflammatory conditions. This study has also revealed distinct patterns of cytosolic, nuclear, and perinuclear ERVK polyprotein/RT localization in IFNγ-stimulated cells. Overall, we have established new *in-vitro* models of inducible ERVK expression, which will serve as useful tools in exploring ERVK biology in the context of neuroinflammatory diseases.

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Research Article

ERVK Polyprotein Processing and Reverse Transcriptase Expression in Human Cell Line Models of Neurological Disease

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Abstract: Enhanced expression of the reverse transcriptase (RT) protein encoded by human endogenous retrovirus-K (ERVK) is a promising biomarker for several inflammatory and neurological diseases. However, unlike RT enzymes encoded by exogenous retroviruses, little work has been done to identify ERVK RT isoforms, their expression patterns, and cellular localization. Using Western blot, we showcase the ERVK gag-pro-pol polyprotein processing leading to the production of several ERVK RT isoforms in human neuronal (ReNcell CX) and astrocytic (SVGA) models of neuroinflammatory disease. Since the pro-inflammatory cytokine IFNγ plays a key role in the pathology of several ERVK-associated neurological diseases, we sought to determine if IFNγ can drive ERVK RT expression. IFNγ signalling markedly enhanced ERVK polyprotein and RT expression in both human astrocytes and neurons. RT isoforms were expressed in a cell-type specific pattern and the RT-RNase H form was significantly increased with IFNγ treatment. Fluorescent imaging revealed distinct cytoplasmic, perinuclear and nuclear ERVK RT staining patterns upon IFNγ stimulation of astrocytes and neurons. These findings indicate that ERVK expression is inducible under inflammatory conditions such as IFNγ exposure—and thus, these newly established *in vitro* models may be useful in exploring ERVK biology in the context of neuroinflammatory disease.

Keywords: endogenous retrovirus; reverse transcriptase; astrocyte; neuron; neurological disease; inflammation; IFNγ

1. Introduction

Reverse transcriptase (RT) is the signature protein of retroviruses; however, for endogenous retrovirus-K (ERVK; alias HERV-K) there is limited knowledge regarding its RT isoforms, expression patterns and cellular localization in human health and disease. Despite evidence of enhanced ERV expression (ERVW, ERVH, ERVK, *etc.*) associated with several inflammatory and neurological diseases [1–7], few studies have sought to specifically examine ERVK polymerase (*pol*) gene and RT protein expression [8–11]. Elevated levels of ERVK RT have been observed in the cortical neurons of patients with Amyotrophic Lateral Sclerosis (ALS) [8]. This observation is consistent with the measurement of RT activity in the CSF and serum of individuals with ALS, at levels similar to those found in Human Immunodeficiency Virus (HIV) positive individuals [12]. ERVK RT is emerging as a promising prognostic biomarker in breast cancer [13]. Clearly, improved detection assays for ERVK RT expression are likely to be useful in other ERVK-associated diseases, including cancers [13], HIV infection [14,15], ALS [8], schizophrenia [16], rheumatic disease [17] and human prion disease [18]. Despite no known causal relationship between ERVK and human disease, pathological contributions of ERVK proteins continue to shape our understanding of complex disease processes [1,15,19–21].

ERVK (HML-2) encodes a reverse transcriptase enzyme with RNase H activity of approximately 65 kDa [22]. It is currently unclear if the active form of ERVK RT acts as a heterodimer—one monomer with an RNase H domain and the other without—as seen with other RT enzymes [23]. As with typical RT proteins, this enzyme contains a conserved LPQG motif and the catalytic YIDD motif [22]. The expression of ERVK RT is dependent on protease processing of the Gag-Pro-Pol polyprotein. Protease cleavage of the ERVK Gag precursor has recently been examined using recombinant constructs [24,25]; however, there is little known regarding the proteolytic processing of the entire Gag-Pro-Pol polyprotein *in situ*.

ERVK expression often occurs in diseases with inflammatory underpinnings. For example, ERVK is concomitantly expressed during HIV infection, both in the periphery and the central nervous system [15,26]. ERVK-specific T cells have been shown to secrete IFNγ in response to their cognate ligands [27,28]. Enhanced IFNγ levels in the brains of HIV-infected individuals [29], are believed to contribute to HIV-associated neuropathology [30]. Indeed, IFNγ has been shown to enhance HIV replication in astrocytes [31,32]. Therefore, we sought to stimulate human astrocyte and neuronal cell cultures with IFNγ, as a potential mechanism to drive ERVK RT expression.

2. Materials and Methods

2.1. Cell Culture and Cytokine Treatment

The SVGA cell line [33] (gifted by Dr. Avindra Nath, NIH) is derived from immortalized human foetal astrocytes, and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin (HyClone, South Logan, UT, USA). ReNcell CX cells [34] (Millipore, Temecula, CA, USA) are immortalized human neural progenitor cells (HNPCs), and were maintained in a proprietary ReNcell neural stem cell medium (Millipore) supplemented with 20 ng/mL human epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ, USA), 20 ng/mL human basic fibroblast growth factor (bFGF; Peprotech), and 1% Penicillin/Streptomycin. All cell lines were maintained in a humidified chamber containing 5% CO_2 at 37 °C.

SVGA cells were seeded into six-well plates and onto glass coverslips in twelve-well plates at a density of 300,000 cells/mL and 30,000 cells/mL, respectively, and grown for 24 h. To differentiate HNPCs into neurons, ReNcells were seeded in laminin (20 μg/mL; Millipore) coated six-well plates at a density of 50,000 cells/mL for 24 h. Adhered cells were rinsed with 1X PBS and allowed to differentiate in the presence of ReNcell medium lacking EGF and bFGF for two weeks. SVGAs and neurons were treated with 0.1, 0.5, 1, and 5 ng/mL doses of human IFNγ (PeproTech) for 24 h. Plated untreated cells were used as negative controls.

2.2. Quantitative Polymerase Chain Reaction (Q-PCR)

Total RNA was extracted and purified from cells using an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA). RNA concentration was measured with a NanoDrop spectrophotometer. The acceptable RNA purity was $A_{260}/A_{280} > 2.0$. The iScript Reverse Transcription kit (Bio-Rad) was used to synthesize cDNA from extracted RNA. CFX Connect Real Time System (Bio-Rad) was employed to perform Q-PCR in order to measure alterations in ERVK gag and pol transcripts using SYBR Green detection method. The primers used to amplify ERVK gag were F: 5' TCGGGAAACGAGCAAAGG 3' and R: 5' GAATTGGGAATGCCCCAGTT 3', and for ERVK pol were F: 5' TGATCCCMAAAGAYTGGCCTT 3' and R: 5' TTAAGCATTCCCTGAGGYAACA 3'. 18S rRNA was used as the endogenous control (Ambion kit #1718, Carlsbad, CA, USA). The data was analysed using the ΔΔCT (Livak) method. GraphPad Prism [51] was used to carry out statistical analyses including column statistics, One-way Anova Friedman test, and Dunn's post-test.

2.3. Reverse Transcriptase (RT) Assay

The activity of reverse transcriptase (RT) in protein fractions isolated from cells was measured using an EnzChek Reverse Transcriptase Assay Kit (Molecular Probes, Carlsbad, CA, USA), as per manufacturer's instructions. Soluble and insoluble protein fractions were prepared at a fixed protein concentration, and pooled at a 1:1 ratio to perform each reaction. MMLV RT standards (Bio-Rad) were also run over a 4-$\log_{10}$ dilution series, and used to construct the standard curve. RT activity was quantitated by measuring the end point fluorescence of each reaction using CFX Connect Real Time System (Bio-Rad) and compared to that of the standard curve. GraphPad Prism [51] was used to carry out statistical analyses including column statistics, One-way Anova Friedman test, and Dunn's post-test.

2.4. Western Blotting

Cells were lysed on ice with 50 µL of in-house lysis buffer (0.05 M Tris (pH 7.4), 0.15 M NaCl, 0.002 M EDTA, 10% glycerol and 1% NP-40 in ultra-pure water) to extract the soluble proteins, followed by extraction of insoluble proteins in 50 µL of RIPA buffer (10% 1X TBS, 1% SDS, 1%

NP-40 and 0.5% DOC in ultra-pure water). Both buffers were supplemented with 1x HALT protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). BCA assay (Thermo Scientific) was used to determine the protein content of each sample as per manufacturer's instructions. Cell lysates were prepared for SDS-PAGE and heated at 95 °C for 10 min. Proteins (15 μg per lane) were separated by SDS-PAGE using a 10% polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk solution for one hour and probed with mouse anti-human ERVK2 RT primary antibody (1:1000 dilution; Abnova, Jhongli City, Taiwan, ROC) overnight at 4 °C, followed by incubation at room temperature for 3 h. The membrane was then probed with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000 dilution; Bio-Rad) for 2 h at room temperature. β-actin was detected using mouse anti-human β-actin primary (1:5000 dilution; Thermo Scientific) and goat anti-mouse secondary antibodies, and was used as the loading control. The membrane was developed with 2 mL of Luminata Crescendo Western HRP substrate (Millipore) and imaged using Bio-Rad ChemiDoc XRS+ chemiluminescent imager. Image Lab software [52] was used to determine the molecular weight of each band, as well as their density relative to that of the negative control. The identity of each band was predicted based on the molecular weight of each ERVK protein [35] and informed by the gag-pro-pol processing pattern of HIV [36,37], including HIV protein post-translational modifications [38].

2.5. Fluorescent Imaging

Cells were fixed with methanol (Fisher Scientific, Fair Lawn, NJ, USA) for 1 min and rinsed with 1× PBS. Cells were permeabilised with 250 μL of PBS-T (PBS with 0.25% TritonX-100) and blocked with 250 μL of 3% BSA in TBS-T (TBS with 0.25% TritonX-100) for 30 min. Cells were incubated in primary antibodies (1:200 dilution) for one hour, followed by incubation in appropriate fluorophore-conjugated secondary antibodies (1:1500 dilution) for one hour. Mouse anti-human ERVK2 RT and rabbit anti-human α-tubulin (Abnova) were used as primary antibodies. Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) and Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes) were the secondary antibodies. Nuclei were counter-stained with DAPI (1:50,000 dilution; Molecular Probes). Controls were prepared by immunostaining without the primary antibodies. Coverslips with stained SVGAs were mounted onto slides using ProLong Gold anti-fade reagent (Molecular Probes). Confocal 2D images and 3-plane view images were acquired using an Olympus Fluoview FV1200 confocal microscope with the FV10-ASW4.0 software suite. Six-well plates with stained neurons were imaged using an EVOS FL Cell Imaging System (Life Technologies, Carlsbad, CA, USA).

3. Results and Discussion

Augmented IFNγ signalling is a hallmark of several neurological diseases including ALS [39] and HIV-associated neuropathology [30]. Both exogenous (HIV) and endogenous (ERVW) retrovirus expression can be enhanced by IFNγ stimulation [7,31,32,40]. IFNγ is a potent activator of pro-inflammatory transcription factors Interferon Response Factor 1 (IRF1) and Nuclear Factor-kappa B (NF-κB), and can enhance HIV gene expression through interaction of these transcription factors with the HIV promoter [31,32,41]. Similarly, we have recently shown that the ERVK promoter also harbours multiple conserved putative binding sites for IRF1 and NF-κB [42], suggesting that IFNγ signalling may also enhance ERVK transcription and protein levels. In support of this evidence, Figure 1A shows that indeed ERVK transcription is enhanced upon IFNγ treatment of human astrocytes, perhaps through increased binding of NF-κB and IRF1 with the ERVK promoter. The levels of the ERVK *gag-pol* transcript were assessed by Q-PCR using *gag* and *pol*-specific primers. IFNγ treatment significantly enhanced ERVK transcription in a dose-dependent manner (5 ng/mL IFNγ, $p < 0.05$). In order to determine whether this transcriptional increase in ERVK expression was correlated with evidence of functional viral proteins, we also assessed overall RT activity in this model. Basal RT activity was observed in untreated SVGA cells; however, upon IFNγ treatment RT activity substantially increased (Figure 1B; 5 ng/mL IFNγ, $p < 0.05$). This method is unable to identify the viral source of the RT activity; therefore, we employed an ERVK RT-specific antibody to address whether ERVK polyprotein processing occurred, producing active RT isoforms, under inflammatory conditions. Figure 1C demonstrates that IFNγ is capable of enhancing ERVK polyprotein (gag-pro-pol, 180 kDa) and RT (60 and 52 kDa forms) expression in astrocytes.

Similar to HIV polyprotein processing [36,37], multiple protease cleavage steps produce intermediate protein products, before each RT isoform is released from the polyprotein. Active RT enzymes are generally heterodimers comprised of a large catalytic RT isoform containing an RNase H domain and a smaller RT isoform without the RNase H domain, which plays a structural role [38,43,44]. Figure 1C shows the formation of two different sized ERVK RT isoforms. The short ERVK RT form of 52/54 kDa is expressed at basal levels in astrocytes, and dose-dependently increases with IFNγ treatment (Table 1), with an optimal stimulating dose of 0.5 ng/mL of IFNγ. Of note, ERVK RT bands appear as doublets, suggesting that they may be post-translationally modified, as seen with HIV-1 RT phosphorylation [38]. The 60 kDa ERVK RT-RNaseH isoform is expressed only upon IFNγ stimulation, suggesting that RT activity may optimally occur under inflammatory conditions, such as low-level chronic IFNγ exposure. Additionally, the appearance of active and structural ERVK RT isoforms leads us to propose that ERVK may be a cellular source of RT activity in inflammatory disease. Figure 1D demonstrates that the majority of the ERVK polyprotein (pro-pol form) is found in the insoluble fraction of the SVGA whole cell lysate, whereas the RT proteins were found within the soluble cytoplasmic fraction.

The anti-ERVK RT antibody was also used to perform fluorescent immunocytochemistry on SVGA cells. Based on the Western blot data from Figure 1, we expect that the ERVK RT staining pattern represents the sum of intracellular polyprotein and RT isoforms. IFNγ-mediated ERVK RT expression was observed in the cytoplasm, with a non-uniform distribution (Figure 2A). ERVK proteins may act similarly to HIV, whereby the large subunit of RT or the polyprotein can interact with β-actin [46]. RT-actin interactions are known to be a fundamental and dynamic process in reverse transcription and localization of reverse transcription complexes (RTCs) [47]. ERVK RT expression also accumulated around the nucleus (Figure 2 A,B), as observed with HIV-1 RTCs [48]. The formation of a perinuclear ring with a large RT protein aggregate proximal to the nucleus occurs concurrently with cellular swelling. Nuclear ERVK RT expression exhibited a speckled pattern (Figure 2B), and may reflect nuclear import of RTCs [48].

In ALS [8] and HIV infection [15], ERVK expression occurs in cortical neurons. We have employed ReNcell CX neural progenitor cell line [34] as a means to study ERVK expression in human neurons. Figure 3A demonstrates that these progenitor cells exhibit enhanced ERVK polyprotein expression; however, differentiation of these neural progenitor cells through growth factor deprivation substantially reduces ERVK pro-pol polyprotein levels (>10-fold decrease) and promotes the expression of RT (10-fold increase) in the soluble fraction. ERVK RT isoforms in ReNcell cultures exhibited increased mass as compared to SVGAs (RT-RH 68 kDa *versus* 60 kDa and RT 56/58 kDa *versus* 52/54 kDa, Figure 3 *versus* Figure 1, respectively), and may represent cell-type specific post-translational modification of RT [38]. For example, HIV RT is phosphorylated at several sites [49], suggesting that our data also may depict several phosphorylated forms of ERVK RT. The cell-type specific differences in RT isoform mass may be related to differential capacity for phosphorylation patterns in neurons *versus* astrocytes [50]. Treatment of these neuronal cultures with IFNγ also enhances the expression of the 180 kDa ERVK polyprotein and 56 kDa RT from that of basal levels (2.6 fold and 2.4 fold, respectively) (Figure 3B). Figure 3C shows that within the differentiated ReNcell culture, IFNγ treatment promotes ERVK RT expression in neuronal cells, but not glial cells. Basal ERVK RT expression was evident in untreated neurons; however, ERVK RT expression was markedly enhanced in the cell body of IFNγ-treated neurons. This model of enhanced ERVK RT in the neuronal cell body is consistent with the immunoreactive staining pattern observed in the cortical brain tissue of patients with ALS [8].

For the first time, we show that the pro-inflammatory cytokine IFNγ can enhance ERVK polyprotein expression and promote its cleavage into heterodimeric RT isoforms. These working *in vitro* models of RT expression in astrocytes and neurons will permit the further examination of ERVK biology in the context of inflammatory neurological disease.

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Author Contributions

Conceived and designed the experiments: M.M. and R.D. Performed the experiments: M.M. and J.F. Analyzed the data: M.M., J.F. and R.D. Contributed reagents/materials/analysis tools: R.D. Wrote the paper: M.M., J.F. and R.D.

Conflicts of Interest

The authors declare no conflict of interest.

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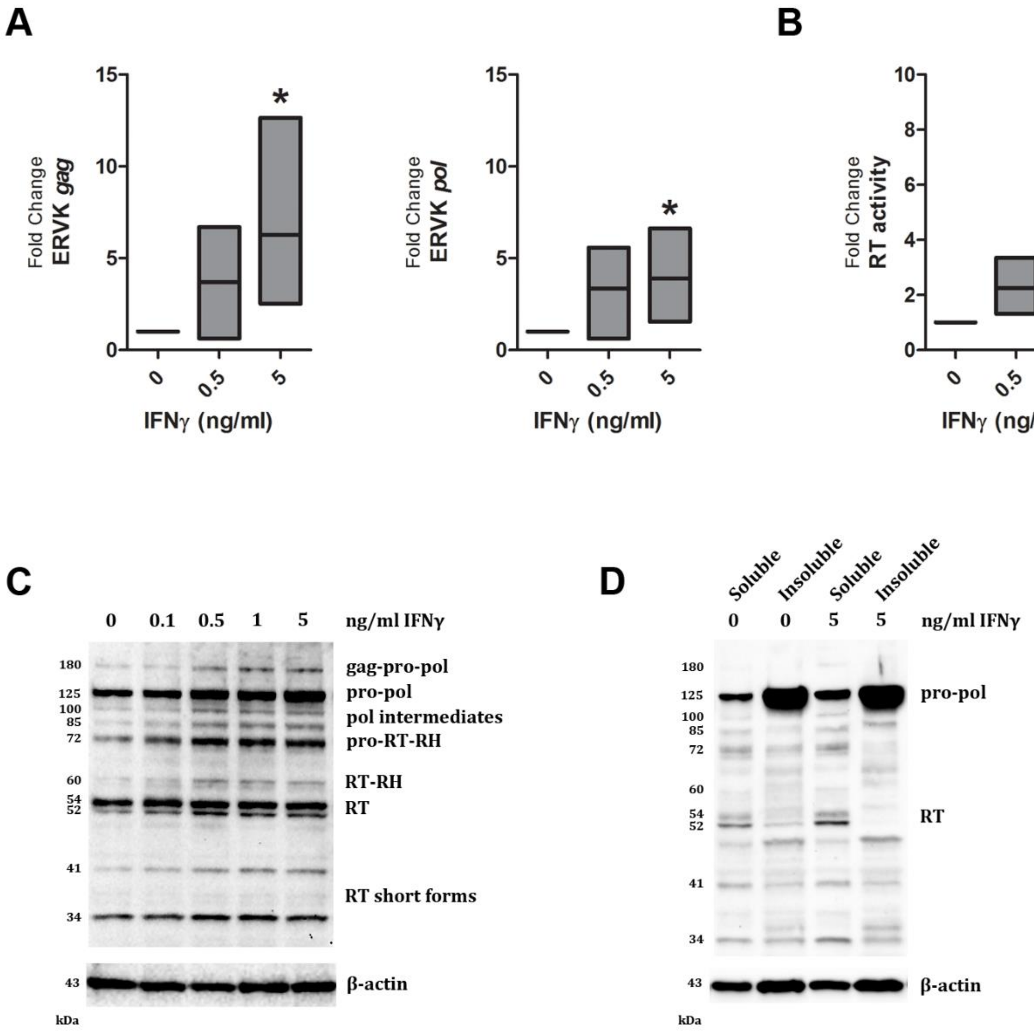


A
Fold Change ERVK gag
15
10
5
0
*
0
0.5
5
IFNγ (ng/ml)
Fold Change ERVK pol
15
10
5
0
*
0
0.5
5
IFNγ (ng/ml)
B
Fold Change RT activity
10
8
6
4
2
0
*
0
0.5
5
IFNγ (ng/ml)
C
0
0.1
0.5
1
5
ng/ml IFNγ
180
125
100
85
72
60
54
52
41
34
gag-pro-pol
pro-pol
pol intermediates
pro-RT-RH
RT-RH
RT
RT short forms
43
β-actin
kDa
D
Soluble
Insoluble
Soluble
Insoluble
0
0
5
5
ng/ml IFNγ
180
125
100
85
72
60
54
52
41
34
pro-pol
RT
43
β-actin
kDa

Figure 1. ERVK polyprotein and reverse transcriptase expression is inducible in IFNγ-treated astrocytes. The SVGA cell line was treated with increasing doses (0 to 5 ng/mL) of the cytokine IFNγ for 24 h. (**A**) IFNγ treatment enhances ERVK transcription, as measured by Q-PCR using *gag* and *pol*-specific primers ($n = 5$). ***** = $p < 0.05$; (**B**) IFNγ stimulation of astrocytes promotes elevated cellular RT activity ($n = 4$); ***** = $p < 0.05$ (**C**) Representative Western blot depicts proteins detected by a commercial anti-ERVK reverse transcriptase antibody (AbNova) or an anti-β-actin antibody control. IFNγ exposure enhances ERVK gag-pro-pol polyprotein (180 kDa), as well as several protease-cleaved forms of this viral polyprotein ($n = 4$). The two expected heterodimeric forms [44] of the ERVK RT are present in IFNγ-treated astrocytes; a 60 kDa form with an RNase H (RH) domain and a 52/54 kDa form without the RNase H domain. Several bands appear as doublets, such as the 52/54 kDa RT band, and likely represent post-translational protein modifications [38]. Short forms of the ERVK RT (41 and 34 kDa bands) may be truncated forms or represent instability and degradation of the RT protein [45]; (**D**) In both untreated and IFNγ-stimulated SVGA cells, the majority of the pro-pol polyprotein exists in an insoluble form within cells ($n = 3$). In contrast, the RT isoforms are concentrated in the soluble cytoplasmic fraction of the cell.

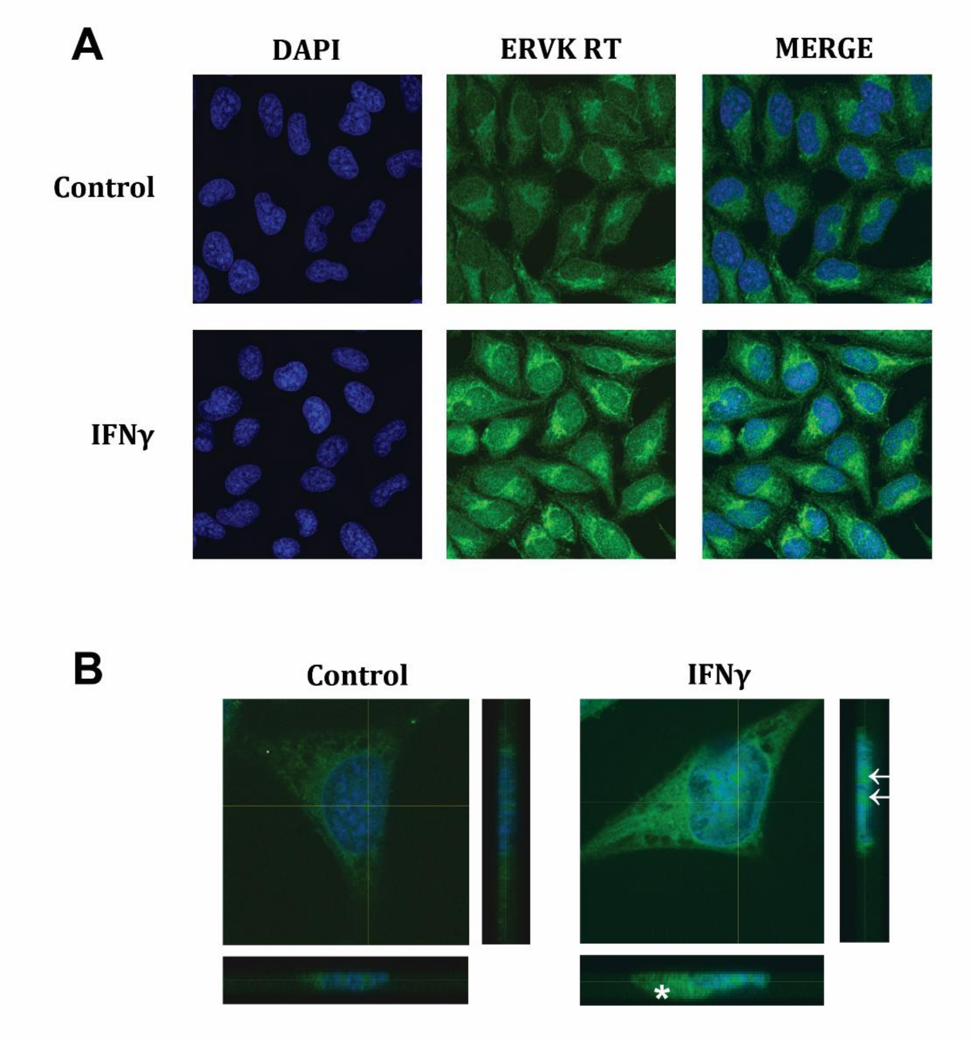
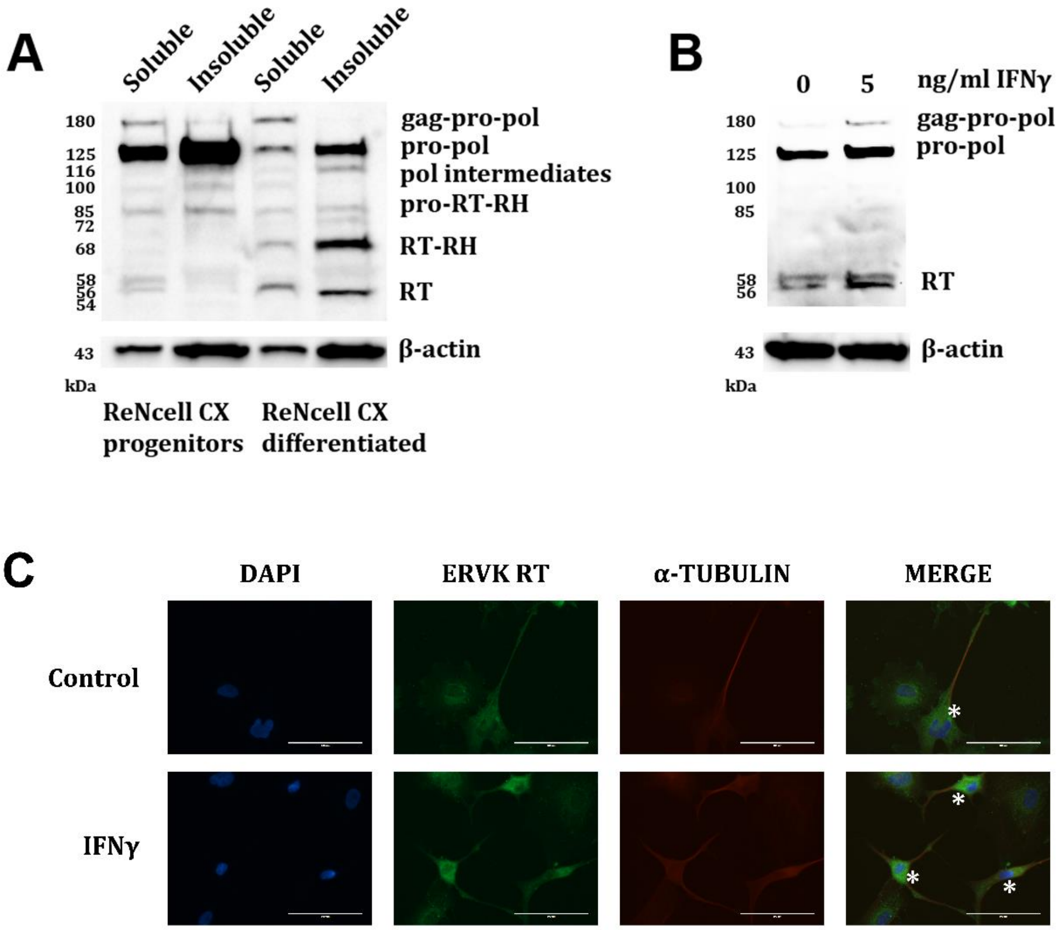

A
DAPI
ERVK RT
MERGE
Control
IFNγ
B
Control
IFNγ
*

Figure 2. IFNγ enhances ERVK RT expression in human astrocytes. The SVGA cell line was treated with 5 ng/mL of IFNγ for 24 h. Cells were immunostained using a commercial anti-ERVK reverse transcriptase primary antibody (Abnova) and fluorescently-labelled secondary antibody. Nuclei were stained with DAPI. Images were acquired using an Olympus FV1200 laser scanning confocal microscope. (**A**) Representative micrographs show basal ERVK RT staining in untreated astrocytes, while IFNγ-stimulated cells exhibit a substantial increase in ERVK RT staining as compared to the control. Magnification 200X; (**B**) Untreated and IFNγ treated astrocytes were evaluated using a 9 µm Z-stack (0.5 µm steps) which depicts ERVK RT expression throughout the entire cell. 3D projections (X, Y and Z planes of crosshair sections) confirm enhanced cytoplasmic, perinuclear and nuclear (arrows) ERVK RT staining in IFNγ treated astrocytes. Accumulation of ERVK RT in IFNγ treated cells is associated with cellular swelling (asterisk). Magnification 600X.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pro-pol
pol intermediates
pro-RT-RH
RT-RH
RT
β-actin
ReNcell CX progenitors
ReNcell CX differentiated
B
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ng/ml IFNγ
180
125
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85
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43
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gag-pro-pol
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RT
β-actin
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DAPI
ERVK RT
α-TUBULIN
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IFNγ
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Figure 3. IFNγ enhances ERVK polyprotein and RT expression in human neurons. Representative Western blots depict proteins detected by a commercial anti-ERVK reverse transcriptase antibody (Abnova) and an anti-β-actin antibody control. (**A**) Soluble and insoluble cell fractions of ReNcell CX progenitors and ReNcell CX neurons differentiated by growth factor withdrawal. Differentiated neurons express enhanced ERVK gag-pro-pol polyprotein (180 kDa), RT-RH (68 kDa) and RT (56/58 kDa) isoforms as compared to ReNcell progenitors (*n* = 2); (**B**) ReNcell CX-derived neurons were treated with 5 ng/mL IFNγ for 24 h (*n* = 1). IFNγ exposure enhances ERVK gag-pro-pol polyprotein (180 kDa), as well as RT levels in soluble whole cell lysates; (**C**) ReNcell CX-derived neurons were treated with 5 ng/mL IFNγ for 24 h (*n* = 3). Cells were immunostained using anti-ERVK reverse transcriptase primary antibody (Abnova), anti α-tubulin primary antibody (Abnova), and fluorescently-labelled secondary antibodies. Nuclei were stained with DAPI. Images were acquired using an EVOS FL microscope. Representative micrographs show basal ERVK RT staining in untreated neurons (asterisk) and glial cells, while IFNγ-stimulated neurons (but not glia) exhibit a substantial increase in ERVK RT staining in the cell body as compared to the control. Images acquired using a 40X objective.

Table 1. Fold change in ERVK polyprotein and RT band intensity normalized to β-actin loading control for Western blot in Figure 1A.

Band size (kDa)	IFNγ dose (ng/ml)				
	0	**0.1**	**0.5**	**1**	**5**
180	1.0	0.7	2.6	2.6	2.6
125	1.0	1.1	1.9	1.5	3.0
100	1.0	1.1	2.4	1.8	2.0
85	1.0	2.0	3.9	3.5	4.2
72	1.0	1.8	2.7	2.4	2.6
60	1.0	1.9	2.8	1.7	2.0
54	1.0	1.2	1.5	1.1	1.2
52	1.0	1.8	2.5	1.8	2.1
41	1.0	1.4	2.2	2.4	2.8
34	1.0	1.1	1.6	1.4	1.2

3.2 | Publication 3

NF-κB and IRF1 Induce Endogenous Retrovirus-K Expression via Interferon-Stimulated Response Elements in its 5′ Long Terminal Repeat

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The goal of this study was to elucidate the influence of TNFα and LIGHT cytokines on ERVK transcription and polyprotein/RT expression in CNS cells. This is the first report to establish that TNFα and LIGHT can markedly induce ERVK expression in neurons and astrocytes, respectively, which correlates with increased levels of IRF1 and NF-κB transcription factors in these cells. For the first time, we have determined that TNFα and LIGHT enhance IRF1 and NF-κB p65/p50 binding to the two Interferon Stimulated Response Elements (ISREs) in the ERVK 5′ LTR. We have also validated increased IRF1 and NF-κB expression in ERVK$^+$ neurons in autopsy ALS brain tissue in comparison to neuro-normal controls. This study has revealed that ERVK re-activation in ALS probably stems from augmented levels of TNFα and LIGHT in the CNS – findings with significant implications for anti-inflammatory and anti-retroviral ALS therapeutics.

Article

NF-κB and IRF1 Induce Endogenous Retrovirus-K Expression via Interferon-Stimulated Response Elements in its 5′ Long Terminal Repeat.

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Running Head: IRF1 and NF-κB promote ERVK reactivation.

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ABSTRACT

Within the human genome reside thousands of endogenous retrovirus (ERV), viral fossils of ancient germ-line infections. Evidence of ERV activity has been widely observed in health and disease. Most often cited as a bystander effect of cell culture or disease states, it is unclear as to which signals control ERV transcription and whether their expression is relevant in cellular processes. We have previously proposed that the viral promoter of endogenous retrovirus-K (ERVK) is responsive to inflammatory transcription factors. Now, we have experimental evidence to show that the likely reason ERVK is upregulated in cancer, inflammatory, infectious and neurological diseases is because of functional interferon-stimulated response elements in the viral promoter. To demonstrate that IRF1 and NF-κB isoforms drive ERVK expression, we employed over-expression assays which revealed independent and synergistic up-regulation of ERVK. Through TNFα and LIGHT cytokine treatments of human astrocytes and neurons, we show that transcriptional enhancement (Q-PCR) is mediated by IRF1 and NF-κB binding to the ERVK promoter (ChIP), and that functional ERVK viral proteins are produced (Western blot and microscopy). We further show that in ALS brain tissue, neuronal ERVK re-activation is associated with the nuclear translocation of IRF1 and NF-κB. These findings present cell-type specific signaling mechanisms behind ERVK reactivation in ALS, which extends to the pathobiology of other ERVK-associated inflammatory diseases.

INTRODUCTION

Within the human genome reside thousands of endogenous retroviruses (ERV), viral fossils of ancient germ-line infections. Evidence of ERV activity has been widely observed health and disease. Most often cited as a bystander effect of cell culture or disease states, it is unclear as to which signals control ERV transcription and whether their expression is relevant in cellular processes. We have previously proposed that the viral promoter of endogenous retrovirus-K (ERVK) is responsive to inflammatory transcription factors [1], due to the presence of two conserved interferon-stimulated response elements (ISREs).

The transcription of integrated retroviral sequences within the human genome is regulated by viral promoters called Long Terminal Repeats (LTRs) flanking either side of the core viral genome. These LTRs contain transcriptional regulatory elements that are responsive to both viral and cellular transcription factors (TFs). Interferon regulatory factors (IRFs) and nuclear factor kappa B (NF-κB) have been shown to be crucial in the transcription of Human Immunodeficiency Virus (HIV) proviruses [2,3], thus promoting HIV replication in the context of inflammation [4]. The human genome is already populated with numerous ERVK viral promoters containing IRF and NF-κB binding sites [1], however it remains unclear as to their propensity to drive ERVK transcription and expression.

In support of this paradigm for ERVK re-activation, pro-inflammatory cytokines have previously been shown to trigger viral expression in several ERVK-associated inflammatory diseases. For instance, TNFα treatment has been demonstrated to enhance ERVK (HML-2) *gag* transcription in synoviocytes obtained from patients with rheumatoid arthritis [5]. TNFα and IFNγ are able to enhance ERVW expression in peripheral blood mononuclear cells (PBMCs) obtained from patients with Multiple Sclerosis [6]. TNFα has been shown to trigger ERVW syncytin protein expression by enhancing the binding of NF-κB subunit p65 to the ERVW promoter in a human astrocytic cell line [7]. Nonetheless, how pro-inflammatory cytokines trigger ERVK expression in human cells, particularly in the central nervous system (CNS), remains to be studied.

ERVK expression is strongly enhanced in the neurodegenerative disease amyotrophic lateral sclerosis (ALS) [8]. Parallel to immune cell activation and infiltration, significantly higher levels of pro-inflammatory mediators called cytokines have been reported in the cerebrospinal fluid (CSF) and sera of ALS patients as compared to healthy controls [9-11]. These include cytokines belonging to the Tumor Necrosis Factor superfamily. The pathogenic role of Tumor Necrosis Factor alpha (TNFα) in ALS is well documented and reviewed elsewhere [12,13]. In contrast, few studies have explored the contribution of LIGHT (homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) in ALS [14-16]. Reactive microglia, astrocytes, and T cells are the major sources of these pro-inflammatory cytokines in the ALS brain [10,17,18].

There is growing recognition that TNFα and LIGHT play critical, yet divergent, roles in ALS neuropathology. Both cytokines are neurotoxic and have been associated with enhanced neuronal death. TNFα is a potent activator of the canonical nuclear factor kappa B (NF-κB) signaling pathway, culminating in the activation of p65 and p50 isoforms of this pro-inflammatory transcription factor [19]. TNFα-induced NF-κB has been shown to cause motor neuron death *in vitro*[64]. In addition, IFNγ has been demonstrated to synergize with TNFα to induce NF-κB, and enhance motor neuron death [20,21]. In line with this finding, anti-IFNγ therapy is protective and delays motor neuron damage in ALS mouse models [16]. Recently, elevated LIGHT signaling has been shown to selectively contribute to motor neuron death in ALS spinal cords [14,15]. IFNγ secreted by astrocytes is a key player in this process, as it leads to enhanced LIGHT production in spinal motor neurons [14,15]. Similar to TNFα, LIGHT is also a potent activator of the canonical, as well as the non-canonical, NF-κB pathways, leading to the activation of an alternate p52 NF-κB isoform [19]. Additionally, TNFα and IFNγ are known to synergistically activate interferon regulatory factor 1 (IRF1) expression [21]. But, the role of IRF1 activation in ALS pathology remains unexplored. Overall, the sum of these augmented cytokine signaling pathways likely results in excessive activation of NF-κB and IRF1 in the brain.

Although exacerbated TNFα, LIGHT, and IFNγ signaling pathways in the CNS converge at NF-κB and/or IRF1 dependent neuronal damage [22], the exact mechanism by which these pro-inflammatory transcription factors promote neuronal death is unclear. ERVK re-activation in neurons triggered by NF-κB/IRF1 may serve as the link between exacerbated pro-inflammatory cytokine signaling and neuronal damage in ALS.

METHODS

Patient samples

Autopsy ALS (n=5) and neuro-normal control (n=5) tissue specimens were obtained from the NIH NeuroBioBank (USA). Pathologic examination was used to confirm the clinical diagnosis of ALS. The postmortem interval of all patients was <24 hours. **Table S1** indicates the individual patient diagnosis, location of brain tissue sampling, age, gender and post-mortem interval (PMI in hours) of the samples used in this study. The brain regions analysed were prefrontal cortex (Brodmann area 9, BA9) and motor cortex (Brodmann area 6, BA6).

Immunohistochemistry of autopsy tissue

To determine the extent of ERVK RT, IRF1, and NF-κB expression patterns in the CNS of ALS patients, immunohistochemistry was performed to detect the levels and localization of these target proteins in autopsy human cortical brain tissue, as previously described [8]. Primary antibodies used were mouse anti-human ERVK RT (1:750; AbNova #H00002087-A01), rabbit anti-human IRF1 (1:100; Santa Cruz #SC497), rabbit anti-human NF-κB p65 (1:100; Abcam #ab7970), and rabbit anti-human NF-κB p50 (1:100; Abcam #ab7971). Primary antibodies were detected using 1:250 goat anti-mouse AF488 (Molecular Probes #A11017) or goat anti-rabbit AF594 (Molecular Probes #11072). Neurons were identified using a fluorescent Nissl stain (1:100; Molecular Probes # N21483). Tissues were also counterstained with 1:50,000 DAPI. Free-floating tissues were mounted onto slides and stained in a 0.1% solution of Sudan Black B. Slides were rinsed and coverslips mounted using ProLong Gold anti-fade reagent (Molecular Probes). Controls were prepared by immunostaining without the primary antibodies.

Immunostained tissues were imaged with Olympus FV1200 laser scanning confocal microscope fitted with the Olympus Fluoview version 4.0B software suite.

Cell Culture

The SVGA cell line is derived from immortalized human fetal astrocytes [23], and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum (HyClone). ReNcell CX cells (Millipore #SCC007) are immortalized human neural progenitor cells (HNPCs) [24], and were maintained in a proprietary ReNcell neural stem cell medium (Millipore) supplemented with 20 ng/ml human epidermal growth factor (EGF; PeproTech #AF10015) and 20 ng/ml human basic fibroblast growth factor (bFGF; PeproTech #AF10018B). All cell lines were maintained in a 37°C incubator containing 5% CO_2. SVGA cells were seeded into six-well plates and onto glass coverslips at a density of 300,000 cells/ml and 30,000 cells/ml, respectively, for 24 hours. To differentiate HNPCs into neurons, ReNcells were seeded in laminin (20 µg/ml; Millipore #CC095) coated six-well plates at a density of 50,000 cells/ml for 24 hours. Adhered cells were rinsed with 1X PBS and allowed to differentiate in the presence of ReNcell medium lacking growth factors for 10 days. For imaging experiments, ReNcell CX cells were cultured and differentiated into neurons in Alvetex scaffolds. Alvetex membranes (Reinnervate #AVP002) were treated with 70% Ethanol for 1 minute, rinsed with 1X PBS, and coated with 20 µg/ml laminin for 6 hours. ReNcell CX cells were seeded onto each scaffold at a density of 5×10^5 cells/well for 1 hour at 37°C and 5% CO_2, as per manufacturer's instructions. The wells were then flooded with 2 ml of ReNcell media supplemented with EGF and FGF growth factors. Twenty-four hours post-seeding, the cell culture media was replaced with that lacking growth factors. Cells were allowed to differentiate for 10 days, with partial media changes performed every 3 days.

Transient transfection of cells with constitutively active NF-κB and IRF1 constructs

IRF1-pCMVBL, NF-κB p65-pCMVBL, NF-κB p50-pCMVBL, and pCMVBL empty vector were generously provided by Dr. Rongtuan Lin (McGill University). To determine whether IRF1 and NF-κB isoforms synergize to induce ERVK transcription, SVGA cells were transfected with 1 µg of these plasmids individually or in combinations using 6 µl of Turbofect Reagent, as per manufacturer's instructions (Thermo Scientific #R0531). Cells were transfected in serum-free culture media for 4 hours, followed by addition of complete media. Cells were harvested 48 hours post-transfection. Untransfected cells and those transfected with the empty vector were used as the negative controls.

Quantitative Polymerase Chain Reaction (Q-PCR)

Total RNA was extracted and purified from cells using an Aurum Total RNA Mini Kit (Bio-Rad #732-6820). RNA concentration was measured with a NanoDrop spectrophotometer. The acceptable RNA purity was A_{260}/A_{280} 1.95 to 2.05. The iScript Reverse Transcription kit (Bio-Rad #170-8840) was used to synthesize cDNA from the extracted RNA. CFX Connect Real Time System (Bio-Rad) was utilized to perform Q-PCR in order to measure alterations in ERVK *pol* transcripts using SYBR Green detection method. The primers used were: ERVK *pol* F: 5' TGATCCCMAAAGAYTGGCCTT 3' and R: 5' TTAAGCATTCCCTGAGGYAACA 3'. 18S rRNA was used as the endogenous control (Ambion kit #1718). The data were analysed using the ΔΔCT (Livak) method, and normalized relative to the appropriate negative control. All data were graphed as mean ± standard error of measurement. GraphPad Prism was used to carry out statistical analyses including column statistics, One-way Anova test and Bonferroni post-test.

Western Blotting

SVGAs and ReNcell CX-derived neurons were treated with 0, 0.1, 0.5, 1, 5, and 10 ng/ml doses of human TNFα (PeproTech #AF-300-01A) or human LIGHT (PeproTech #AF-310-09B). Twenty four hours post-treatment, cells were harvested and lysed on ice with 50 µl of in-house lysis buffer (0.05M Tris (pH 7.4), 0.15M NaCl, 0.002M EDTA, 10% glycerol and 1% NP-40 in ultra-pure water) to extract proteins. The lysis buffer was supplemented with 1x HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). BCA assay (Thermo Scientific #PI23227) was used to determine the protein content of each sample as per manufacturer's instructions. Cell lysates were prepared for SDS-PAGE and heated at 95^{o}C for 10 minutes. Proteins (15 µg per lane) were separated by SDS-PAGE using a 10% polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk solution for one hour and probed with the desired primary antibody (1:1000 dilution) overnight at 4°C, followed by incubation at room temperature for 3 hours. Primary antibodies used were: mouse anti-human ERVK2 RT (Abnova #H00002087-A01), rabbit anti-human IRF1 (Santa Cruz #SC497), rabbit anti-human NF-κB p65 (Abcam #ab7970), rabbit anti-human NF-κB p50 (Abcam #ab32360), rabbit anti-human NF-κB p52 (Cell Signaling #4882S), and mouse anti-human β-actin (Thermo Pierce #MA5-15739; loading control). The membrane was then probed with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG secondary antibody (1:5000 dilution; Bio-Rad, #170-6516 and #170-6515) for 2 hours at room temperature. The membrane was developed with 2 ml of Luminata Crescendo Western HRP substrate (Millipore #WBLUR0500) and imaged using Bio-Rad ChemiDoc XRS+ or Protein Simple FluorChem M chemiluminescent imager. Image Lab software was used to determine the molecular weight of each band. The identity of each band was based on gag-pro-pol processing, as previously described [25].

Chromatin immunoprecipitation (ChIP)

SVGAs were seeded in 10cm dishes at an approximate density of 3 x 10^6 cells/dish for 24 hours at 37°C and 5% CO_2. Laminin-coated dishes were used to seed ReNcell CX cells at a density of 3 x 10^5 cells/dish for 24 hours at 37°C and 5% CO_2. The culture media on adhered ReNcell CX cells was then replaced with that lacking EGF and bFGF growth factors, and cells were allowed to differentiate into neurons for 10 days. SVGAs and neurons were treated with 10ng/ml human TNFα (PeproTech) or human LIGHT (PeproTech) for 8 hours, fixed with 4% paraformaldehyde, and harvested. Untreated cells were used as the negative control. Chromatin Immunoprecipitation (ChIP) was performed using Pierce Magnetic ChIP kit (Thermo Scientific #26157) as per manufacturer's instructions. IRF1 and NF-κB bound DNA segments were isolated using 3 μg of rabbit anti-human IRF1 (Santa Cruz #SC497), rabbit anti-human NF-κB p65 (Abcam #ab7970), rabbit anti-human NF-κB p50 (Abcam #ab32360), or rabbit anti-human NF-κB p52 (Cell Signaling #4882S) antibodies. Immunoprecipitation with IgG antibody was used as the negative control. QPCR was performed on the immunoprecipitated DNA using

SYBR Green detection to amplify the ISREs in the ERVK 5′ LTR. Primers for the first ISRE (nt. 380 - 392) were F: 5′-TCACCACTCCCTAATCTCAAGT-3′ and R: 5′-TCAGCACAGACCCTTTACGG-3′ and for second ISRE (nt. 563 - 575) were F: 5′- CTGAGATAGGAGAAAAACCGCCT-3′ and R: 5′-GGAGAGGGTCAGCAGACAAA-3′. Data were analyzed using the ΔΔ Ct method and normalized relative to the input and IgG controls for each condition. All data were graphed as mean ± standard error of measurement. Statistical analyses were performed in GraphPad PRISM using Two-Way Anova and Tukey's multiple comparisons test.

Fluorescent microscopy

SVGA cells and ReNcell CX-derived neurons in Alvetex scaffolds were treated with 10 ng/ml human TNFα (PeproTech) or human LIGHT (PeproTech). Untreated cells were used as the negative control. Twenty-four hours post-treatment, cells were fixed with methanol for 40 seconds, and rinsed with 1X PBS. Cells were permeabilized with 250 µl of PBS-T (PBS with 0.25% TritonX-100) and blocked with 250 µl of 3% BSA in TBS-T (TBS with 0.25% TritonX-100) for 30 minutes. Immunocytochemistry was performed using 1:750 mouse anti-human ERVK RT (Abnova #H00002087A01) primary antibody for 3 hours and 1:1000 goat anti-mouse AF488 (Molecular Probes #A11017) secondary antibody for 2 hours. Nuclei were counterstained with 1:50,000 DAPI. ReNcells were also stained with fluorescent Nissl to detect neurons (Molecular Probes #N21483). Coverslips or Alvetex membranes were mounted onto slides using ProLong Gold anti-fade reagent (Molecular Probes), and dried overnight. Controls were prepared by immunostaining without the primary antibodies. Confocal microscopy was performed using an Olympus FV1200 laser scanning confocal microscope.

RESULTS

TNFα and LIGHT enhance ERVK polyprotein and RT expression in a cell-type specific manner

Augmented levels of pro-inflammatory cytokines TNFα and LIGHT play a crucial role in ALS neuropathology [14-16,26]. Considering that ERVK re-activation coincides with pro-inflammatory signatures in ALS, we sought to determine whether these cytokines can enhance ERVK expression in human astrocytes and neurons. The treatment of human astrocytic SVGA cell line and human neurons derived from ReNcell CX cell line with TNFα or LIGHT dose-dependently enhanced ERVK polyprotein and RT levels, albeit in a cell-type specific manner. LIGHT increased ERVK protein levels most prominently in astrocytes, whereas TNFα was best able to induce ERVK expression in neurons (**Figures 1A and 2A**). We also observed enhanced ERVK polyprotein processing in these cytokine stimulated cells, which culminated in the production of the catalytic RT subunit containing an RNase H domain and the structural RT subunit without the RNase H domain (**Figures 1A and 2A**). These observations are in line with our previous finding of IFNγ-mediated ERVK polyprotein cleavage to produce a heterodimeric mature and active ERVK RT [25]. Interestingly, TNFα-treated neurons exhibited marked cleavage of the ERVK polyprotein to generate the RT-RH catalytic subunit (**Figure 2A**), suggesting that neuronal ERVK RT activity detected in ALS may optimally occur in the context of chronic TNFα exposure.

Confocal microscopy revealed that under optimal stimulating conditions, LIGHT-treated astrocytes and TNFα-treated neurons exhibited marked ERVK RT protein accumulation (**Figures 1B and 2B**). In astrocytes, ERVK polyprotein/RT formed a perinuclear ring and a large aggregate proximal to the nucleus (**Figure 1B**). Nuclear ERVK RT expression also increased and exhibited a speckled pattern (**Figure 1B**). We have previously observed similar ERVK RT staining patterns in IFNγ-treated cells [25], suggesting that ERVK polyprotein/RT aggregation is a common cellular feature occurring in the context of CNS inflammation. In addition, enhanced ERVK protein expression occurred concomitantly with the up-regulation of IRF1 and NF-κB p65, p50, and/or p52 transcription factors in astrocytes and neurons (**Figures 1A and 2A**). This finding suggests that IRF1 and NF-κB isoforms likely play a crucial role in ERVK re-activation in astrocytes and neurons, and thus, we sought to explore the mechanism behind this process.

IRF1 and NF-κB synergize to markedly enhance ERVK transcription

Previously, *in silico* analysis revealed that two Interferon Stimulated Response Elements (ISREs) are a conserved feature of ERVK promoters called 5′ Long Terminal Repeats (5′ LTR; **Figure 3A**) [1]. ISREs are known to bind Interferon Regulatory Factors, such as IRF1 [27]. The ERVK 5′ LTR also harbours numerous conserved putative NF-κB binding sites, including those that partially overlap and are adjacent to IRF1 binding sites (**Figure 3A**). The binding of IRF1 and NF-κB to their cognate sites is required for optimal transcriptional activation from the HIV-1 5′ TLR [28]. Similarly, these pro-inflammatory transcription factors may be crucial for enhancing ERVK transcription in neuroinflammatory diseases such as ALS.

To determine whether IRF1 and NF-κB cooperatively enhance ERVK transcription, we transiently transfected astrocytes with plasmids expressing constitutively active NF-κB (isoforms p65 and p50) and IRF1, individually or in combinations. The overexpression of IRF1 and NF-κB p65 alone was sufficient to significantly enhance ERVK *pol* transcription in astrocytes (**Figure 3B**). We did not observe a perceivable effect with overexpression of NF-κB p50 alone on ERVK pol RNA levels. However, co-expression of IRF1 and NF-κB p65 and p50 in astrocytes produced a marked 70 fold increase in ERVK *pol* RNA levels. These findings support the notion that IRF1 and the NF-κB p65/p50 heterodimer synergize to drive optimal transcriptional re-activation of ERVK in astrocytes. Enhanced expression of Sp1 and Sp3 transcription factors can also regulate ERVK expression (**Figure S1**), but not nearly as to the same extent as pro-inflammatory TFs.

TNFα and LIGHT enhance the binding of IRF1 and NF-κB to Interferon Stimulated Response Elements in the ERVK 5′ LTR

Pro-inflammatory cytokines, such as TNFα and LIGHT, are generally potent activators of NF-κB and also lead to IRF1 activity [19,21]. Therefore, we sought to determine whether TNFα and LIGHT-mediated induction of ERVK expression is facilitated by enhanced interactions of NF-κB and IRF1 with their cognate binding sites on the ERVK 5′ LTR. For the first time, we showcase the biological functionality of ISREs in the ERVK 5′ LTR, as they can interact with IRF1 and NF-κB isoforms. Both astrocytes and neurons exhibited basal IRF1 and NF-κB binding to both ISREs (**Figure 3 C-F**), which alludes to the basal ERVK expression observed in these cells. However, under optimal stimulating conditions, LIGHT-treated astrocytes (**Figure 3 C and D**) and TNFα-treated neurons (**Figure 3 E and F**) exhibited markedly enhanced NF-κB p65 and p50 binding to each ISRE in the ERVK promoter. We did not observe any perceivable change in the binding of NF-κB p52 isoform to the ISREs. This suggests that the canonical NF-κB p65/p50 complex, or p50 homodimers, predominantly bind the ERVK promoter and partake in proviral transcriptional re-activation. Furthermore, ChIP data did not support a role for the non-canonical p50/p52 NF-κB complex in ERVK transcription. Although the binding of IRF1 to the ISREs considerably increased with cytokine stimulation 9 and 7 fold in SVGAs and neurons, respectively, it did not reach statistical significance. Overall, these findings support the notion that TNFα or LIGHT-induced IRF1, NF-κB p65 and p50 binding to the ERVK promoter re-activates this endogenous retrovirus in the context of inflammation.

Interestingly, cytokine-mediated IRF1 and NF-κB binding to the ERVK promoter occurred in a cell-type specific manner. LIGHT, but not TNFα, significantly enhanced NF-κB p65 and p50 binding to the ISREs in astrocytes (**Figure 3 C and D**). In stark contrast, TNFα, but not LIGHT, significantly increased NF-κB p65 and p50 protein levels as well as their interaction with the ISREs on the ERVK promoter in neurons (**Figure 3 E and F**). Consistently, these results were associated with increased ERVK polyprotein/RT expression in LIGHT-treated astrocytes and TNFα-treated neurons.

The ERVK *pol* gene was used as the negative control for ChIP Q-PCR (**Figure S2**); however, transcription factor binding was detected to the ERVK *pol* region. This can be explained by extensive binding by NF-κB and IRF1 to regions other than promoters throughout the human genome [27]. Cytokine treatment did not result in notably enhanced NF-κB and IRF1 binding to the ERVK *pol* gene, which confirms that transcription factor enrichment to the ERVK promoter region is not a random event under conditions of inflammation.

IRF1 and NF-κB expression is markedly increased in ERVK$^+$ cortical neurons in ALS

We have previously demonstrated that ERVK RT expression is specifically increased in the cortical neurons of patients with ALS as compared to neuro-normal controls [8]. However, the signals that lead to neuronal ERVK RT accumulation have remained unidentified. The augmented levels of TNFα and LIGHT in the CNS is a hallmark of ALS [10,12-16]. We are the first to demonstrate that that these pro-inflammatory cytokines lead to ERVK expression in human astrocytic and neuronal cell lines. In order to validate our *in vitro* findings, we sought to determine whether cortical brain tissue from patients with ALS exhibits increased NF-κB and IRF1 nuclear localization in ERVK$^+$ neurons as compared to neuro-normal controls.

Here, we highlight that ERVK RT expression predominantly accumulated in large pyramidal neurons in the third and fifth cortical layer of prefrontal and motor cortex tissue, and associated with loss of cortical tissue organization in ALS (**Figure 4**). Weak basal ERVK expression was observed in neuro-normal cortex. Yet, a striking enhancement and expanded distribution of ERVK expression occurred in ALS cortical tissues. Inter-individual differences in ERVK expression levels were maintained when comparing prefrontal and motor cortex samples, suggesting that either genetic background (polymorphisms in proteins of key cellular pathways) or disease severity (degree of inflammation) account for differential ERVK expression. We showcase that in comparison to neuro-normal controls, motor cortex neurons in patients with ALS exhibited clear nuclear translocation of pro-inflammatory transcription factors IRF1 and NF-κB p50, and to a lesser degree p65 (**Figure 5**). Nuclear translocation of these TFs correlated with enhanced ERVK RT expression in cortical neurons. Overall, our findings strongly support the premise that ERVK re-activation in the motor cortex of patients with ALS stems from enhanced interactions of cytokine-induced IRF1 and NF-κB transcription factors with the ERVK promoter.

DISCUSSION

Several lines of evidence suggest that augmented levels of TNFα and LIGHT cytokines drive enhanced activity of pro-inflammatory transcription factors (TFs) in neurological diseases [14,15,26]. These signalling pathways may be important triggers of ERVK transcription in the CNS. Herein, we show that TNFα and LIGHT are potent inducers of ERVK polyprotein and RT expression in neurons and astrocytes, respectively. Confocal microscopy revealed a unique pattern of ERVK RT expression in cells, consisting of punctuated structures that accumulated in the perinuclear region along with the formation of a large aggregate proximal to the nucleus. This type of staining is typically seen for specialized inclusion bodies called viroplasms, which comprise the viral replication machinery [55,56]. This suggests the formation of putative ERVK viral factories in cytokine-stimulated cells. The morphology of these viroplasms also resembles that of the aggresomes, which are compartments that sequester unwanted proteins in specialized inclusions and facilitate their clearance by autophagy, thereby dissipating the cytotoxic effects of protein aggregates [57,58]. Likewise, formation of ERVK RT aggresomes may be a cellular response to protect against toxic ERVK protein accumulation. Unfortunately, the appearance of aggresomes and inclusion bodies can impair vital cellular functions, including inactivation of the proteasomal pathway responsible for clearing protein aggregates [58]. Interestingly, protein clearance pathways, such as the proteasome system and autophagy, are dysregulated in ERVK-associated neurological diseases including ALS [59]. In the absence of functional protein degradation pathways, inflammation-induced ERVK viroplasms or aggresomes may persist and propagate chronic neuronal damage.

To delineate the mechanism behind cytokine-induced ERVK re-activation, we have utilized ChIP and confirmed that the ISREs in the ERVK promoter are functional, and that enhanced binding of IRF1 and NF-κB to these elements synergistically augments ERVK gene expression in response to pro-inflammatory stimuli. The cooperative binding of IRF1 and NF-κB to their cognate sites is a conserved feature of many IRF1 and NF-κB-responsive gene promoters. For instance, synergy between IRF1 and NF-κB is required to induce the transcription from human inducible nitric oxide synthase, interleukin-15, major histocompatibility complex class I, vascular cell adhesion molecule I, and interferon β promoters [29-33]. Accordingly, overlapping or adjacent IRF1 and NF-κB binding sites have been described at these promoters, similar to that observed in the ERVK promoter [1]. IRF1 and NF-κB also synergistically activate transcription from the HIV-1 promoter, although IRF1 binding sites are not found adjacent to or overlapping with NF-κB sites [28]. In line with these findings, we have added the ERVK promoter to the growing list of IRF1 and NF-κB responsive enhancer elements.

It is interesting to note that TNFα and LIGHT enhance ERVK expression in a cell-type dependent manner. TNFα increased ERVK protein levels most prominently in neurons, whereas LIGHT was best able to induce ERVK in astrocytes. This effect can be explained by differential enrichment of NF-κB at the ERVK promoter during TNFα or LIGHT stimulation of astrocytes and neurons. TNFα, but not LIGHT, significantly increased the interaction of NF-κB p65 and p50 with the ISREs on the ERVK promoter in neurons. In contrast, LIGHT, but not TNFα, significantly enhanced NF-κB p65 and p50 binding to the ISREs in astrocytes.

Cell-type specificity of TNFα and LIGHT may also be explained by differential expression of their cognate cell surface receptors, as well as downstream signaling molecules in astrocytes and neurons. TNFα is known to be biologically active in both transmembrane as well as soluble forms [13,34]. Soluble TNFα mainly signals through TNF receptor 1 (TNFR1) [13], which is found at a lower level in astrocytes as compared to neurons (The Human Protein Atlas). Overproduction of soluble TNFα has been shown to cause neurodegeneration in the CNS [34]. Trans-membrane TNFα on the other hand mainly signals through TNFR2, which is primarily found in microglial cells [13,15]. Since, we utilized soluble TNFα in our cell line models, it is not surprising that neurons, but not astrocytes, were more responsive to this cytokine. Adaptor molecules that associate with TNF receptors, known as TRAFs, exert a second layer of control over cell-specific TNFα and LIGHT signaling. TRAF3 is basally expressed in neurons, but not in glial cells (The Human Protein Atlas), and has been shown to be much more inducible in neurons as compared to astrocytes [35]. TRAF3 is a negative regulator of LIGHT signaling as it inhibits the function of LTβ receptor, which results in NF-κB inactivity [35]. In contrast, TRAF3 has no effect on TNFα-induced NF-κB signaling [35]. Neuronal expression of TRAF3 may have inhibited LIGHT-induced NF-κB signaling, leading to a lack of any perceivable effect on ERVK expression in our neuronal models.

The NF-κB class of transcription factors function as heterodimers or homodimers comprised of various combinations of subunits p65, Rel B, c-Rel, p50, and p52 [36]. The most common NF-κB species found in human neurons are the canonical p65/p50 and the non-canonical p50/p50 complexes [37]. Different NF-κB dimers recognize slightly different binding sequences with high affinity [38]. For instance, p50 homodimers bind the consensus decamer GGGGATYCCC, where Y is a pyrimidine base, while p65/p50 heterodimers have high affinity for NF-κB sites with AT rich centres [38]. Since, majority of the NF-κB binding sites on the ERVK 5′ LTR are GC rich (60 to 80% GC content) [1], the ERVK promoter is likely most responsive to p50 homodimers. Indeed, ChIP experiments revealed the most dramatic enrichment in the binding of NF-κB p50 at the ISREs in the ERVK promoter in both astrocytes and neurons. Nevertheless, p50 may be present in homodimeric or heterodimeric complexes with p65 at the ERVK promoter.

Our findings suggests that the canonical p65/p50 complex most likely activates ERVK in astrocytes. This is because we observed a marked increase in ERVK pol RNA levels only with the co-overexpression of IRF1 and NF-κB p65 and p50 in astrocytes, and not with IRF1 and p65 or IRF1 and p50 combinations. In addition, enhanced ERVK RT expression associated with increased levels of all of these TFs in astrocytes. In contrast, our findings support the role of non-canonical p50 homodimers, rather than p65/p50 heterodimers, in ERVK re-activation in neurons. In support of this claim, enhanced levels of mature ERVK RT were observed despite the lack of any significant increase in NF-κB p65 protein levels upon TNFα stimulation of neurons. Further support emanates from the finding that the cortical neurons in ALS tissues exhibited enhanced nuclear translocation of p50 rather than p65, concomitantly with ERVK RT accumulation.

Previous studies have demonstrated that p50 homodimers are global transcriptional repressors, as p50 lacks a transcriptional activation domain (TAD) [36]. Accordingly, p50 homodimers repress the expression of a variety of human genes including *TNFα* and *IL-6* [39]. NF-κB p50 homodimers are also known to repress HIV-1 transcription, leading to retroviral latency [39]. In stark contrast, our findings argue for an activating role of p50 homodimers on ERVK transcription. Interestingly, this may account for enhanced ERVK expression which precedes rebounds of HIV-1 re-activation in cells latently infected with this exogenous retrovirus [40]. Since p50 lacks a TAD, it can only stimulate transcription when complexed with other NF-κB subunits containing a TAD, or alternatively with other co-activators such as Bcl-3, C/EBP, Sp1, or TFII-I that bridge the p50 homodimers to the transcriptome by recruiting transcription initiation machinery [41-43]. Thus, NF-κB p50 must be present in a complex with other transcriptional co-activators at the ERVK 5′ LTR. Indeed, ERVK promoter harbors binding sites for co-activators such as Sp1 and TFII-I in the vicinity of NF-κB sites [1], suggesting that transcription factors other than NF-κB p65 and p50 and IRF1 likely partake in the complexity of LTR-driven ERVK re-activation.

Moreover, in a study by Zhou et al., NF-κB p50 was demonstrated to play a much more prominent role in neuronal survival as compared to p65 [37]. In the presence of glutamate toxicity, marked increase in p50 nuclear levels was required to enhance neuronal survival [37]. Spinal cord neurons were also determined to be more vulnerable to apoptosis upon the inhibition of nuclear p50 translocation *in vitro* [44]. Neuronal survival in these studies can be accounted for by homodimeric p50-mediated induction of *bcl-2* transcription, as well as blockade of caspase 3 cleavage – both of which decrease the propensity of cells to apoptotic cell death [44,45]. Thus, under conditions of cellular stress, p50 homodimers elicit a protective survival response in neurons; unfortunately, this may inadvertently cause neuronal ERVK transcriptional re-activation mediated by p50, as well as accumulation of ERVK proteins which are normally cleaved and degraded by caspase activation [46].

As compared to neuro-normal controls, ERVK RT expression was markedly enhanced in large pyramidal neurons in BA9 prefrontal and BA6 motor cortex of ALS tissues. Pyramidal neurons in the motor cortex normally exhibit constitutive basal NF-κB activity, which is required to maintain neuronal plasticity [47]. In the presence of already active NF-κB, pro-inflammatory stimuli including TNFα and LIGHT, which culminate in IRF1 activation, may be sufficient to drive ERVK re-activation in pyramidal neurons. IRF1 activation in cortical neurons has previously been demonstrated in the context of neurotropic viral infections, and serves as a protective mechanism limiting viral replication and spread within the CNS [48]. Acute and chronic viral infections have been associated with the etiology of several ERVK-associated neurological diseases, including schizophrenia, multiple sclerosis and ALS [49-52]. However, a causative link between these viral infections and the pathology of neurodegenerative diseases is a highly debated topic. Viruses may cause transient infections, leading to anti-viral immune activation which eventually culminates in the clearance of the infectious agent. However, based on our findings, IRF1 and NF-κB may trigger ERVK re-activation. Anti-viral response against ERVK may create a feed forward loop, generating repetitive cycles of NF-κB/IRF1-induced ERVK expression and inflammatory response against ERVK-expressing neurons, leading to neuronal injury. Previously, the envelope protein of ERVW has been demonstrated to trigger innate immune signaling and the secretion of pro-inflammatory cytokines, thereby driving NF-κB activation [53]. This TF further activated LTR-driven transcription of ERVW, generating a vicious cycle of latent ERV re-activation and uncontrolled inflammation [6,7]. Likewise, ERVK re-activation in cortical neurons may perpetuate chronic tissue damage.

In addition to immune-mediated neuronal damage, multiple retroviral proteins have been shown to exert direct neurotoxic effects. For instance, the overexpression of ERVW envelope protein induces endoplasmic reticulum stress, leading to neuroinflammation and production of free radicals with ensuing demyelination and axonal injury in multiple sclerosis [54]. Similarly, the expression of ERVK proteins may also prove to be toxic for neurons. However, whether ERVK re-activation in neurological diseases is responsible for neuroinflammation and cell death is yet to be elucidated.

It is now well established that exacerbated TNFα and LIGHT signaling pathways in ALS converge at NF-κB and possibly IRF1 dependent neuronal damage [22]; however, the exact mechanism by which these pro-inflammatory transcription factors promote neuronal death has remained obscure. Our findings suggest that neuronal ERVK protein expression and aggregation triggered by the synergistic action of NF-κB and IRF1 may serve as the link between exacerbated pro-inflammatory cytokine signaling and tissue damage. Consequently, squelching ERVK activity through antiretroviral or immunomodulatory regimens may hinder virus-mediated neuropathology and improve the symptoms of ALS.

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Author Contributions

All authors contributed to the study design and wrote the manuscript. R.N.D., M.M. and J.F.P performed the experiments and analysed the data. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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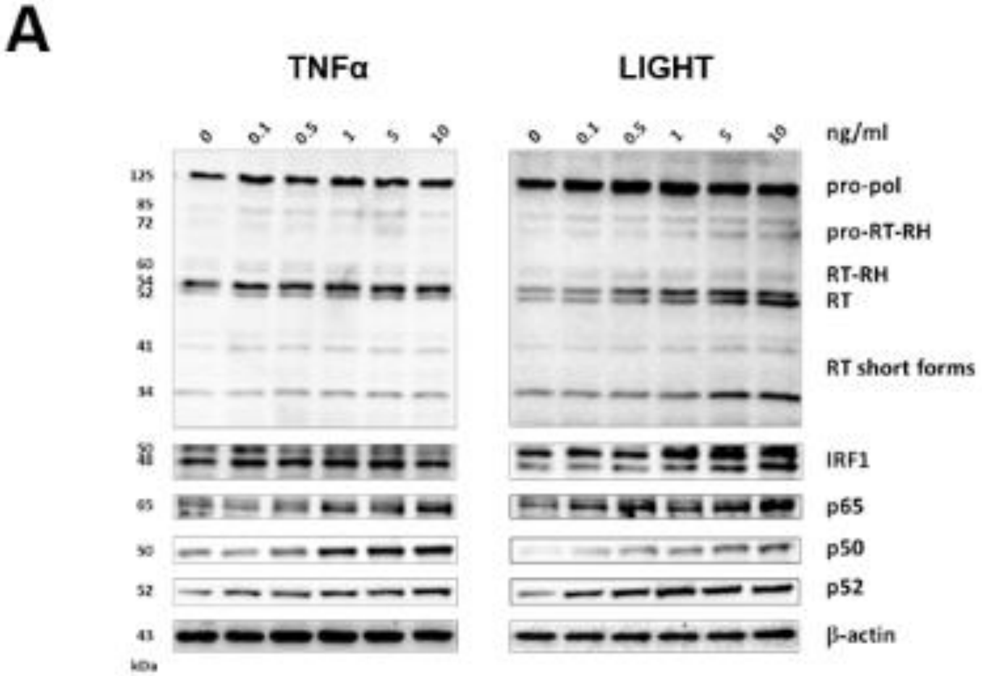
A
TNFα
LIGHT
0
0.1
0.5
1
5
10
ng/ml
125
85
72
60
54
52
41
34
50
48
65
50
52
43
kDa
pro-pol
pro-RT-RH
RT-RH
RT
RT short forms
IRF1
p65
p50
p52
β-actin

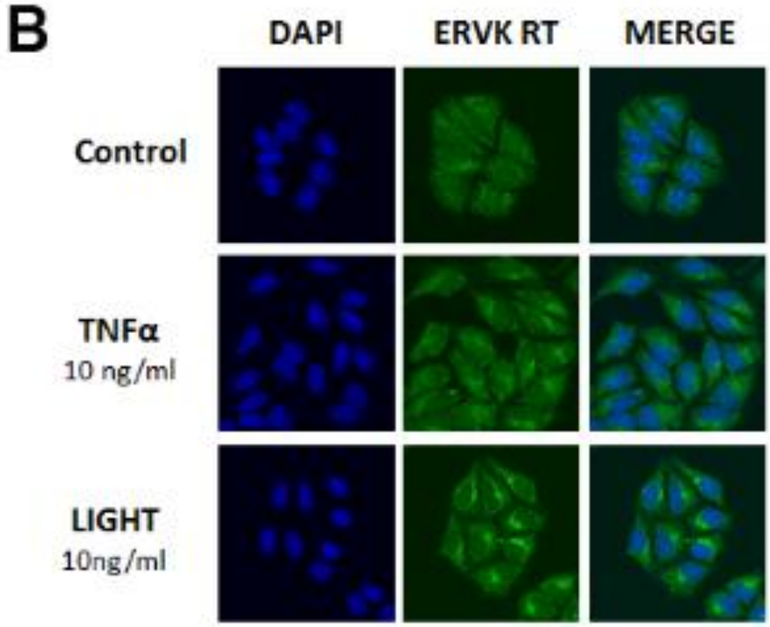
B
DAPI
ERVK RT
MERGE
Control
TNFα
10 ng/ml
LIGHT
10ng/ml

Figure 1. LIGHT, but not TNFα, markedly enhances ERVK polyprotein and RT expression in astrocytes. SVGA cells were treated with various doses of TNFα or LIGHT for 24 hours. Western blot and confocal microscopy were used to detect alterations in ERVK RT, IRF1, and NF-κB p65, p50, or p52 protein levels. (A) Western blot depicts that LIGHT strongly induced ERVK polyprotein expression and cleavage to produce the small 52/54 kDa RT without RNase H and the larger 60 kDa RT with RNase H, concomitantly with up-regulation of IRF1 and NF-κB protein levels. In comparison, TNFα slightly enhanced ERVK polyprotein and RT subunit expression. β-actin was used as the loading control (n=3). (B) Representative confocal micrographs depicting marked LIGHT-mediated induction of ERVK RT expression. ERVK RT aggregates deposited proximal to the nucleus and formed a perinuclear ring (n=3).

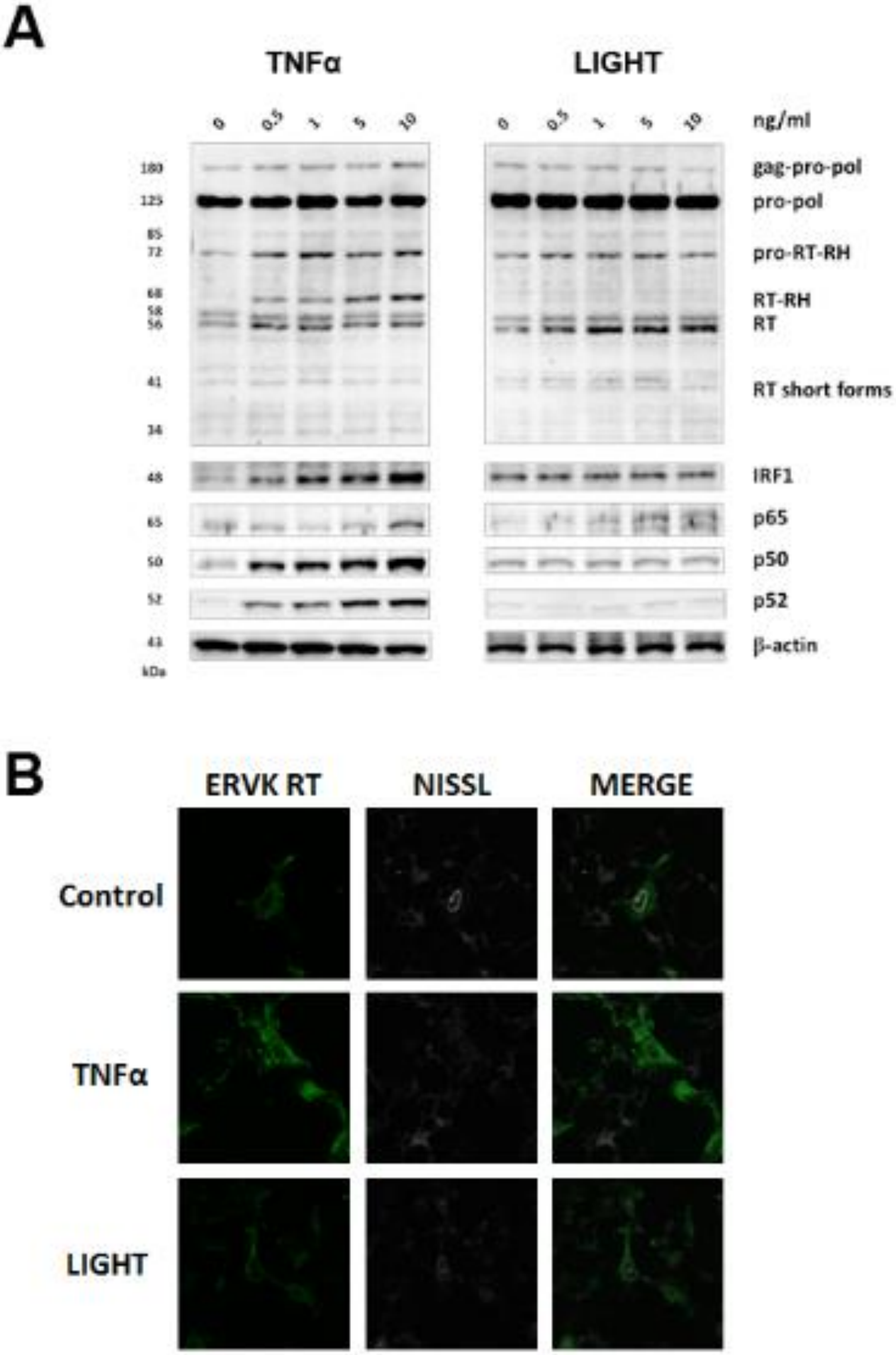
A
TNFα
LIGHT
0
0.5
1
5
10
0
0.5
1
5
10
ng/ml
180
125
85
72
68
58
56
41
34
48
65
50
52
43
kDa
gag-pro-pol
pro-pol
pro-RT-RH
RT-RH
RT
RT short forms
IRF1
p65
p50
p52
β-actin
B
ERVK RT
NISSL
MERGE
Control
TNFα
LIGHT

Figure 2. TNFα, but not LIGHT, markedly enhances ERVK polyprotein and RT expression in neurons. ReNcell CX-derived neurons were treated with various doses of TNFα or LIGHT for 24 hours. Western blot and confocal microscopy were used to detect alterations in ERVK RT, IRF1, and NF-κB p65, p50, or p52 protein levels. (A) Western blot depicts that TNFα strongly induced ERVK polyprotein expression and cleavage to produce the small 56/58 kDa RT without RNase H and the larger 68 kDa RT with RNase H, concomitantly with up-regulation of IRF1 and NF-κB protein levels. In comparison, LIGHT enhanced the expression of RT subunit without RNase H, but not RT with RNaseH domain, suggesting that optimal RT activity may occur during exposure of neurons to TNFα. β-actin was used as the loading control (n=3). (B) Representative confocal micrographs depicting TNFα-mediated induction of ERVK RT expression in neurons. Fluorescent Nissl stain was used to identify neurons (n=3).

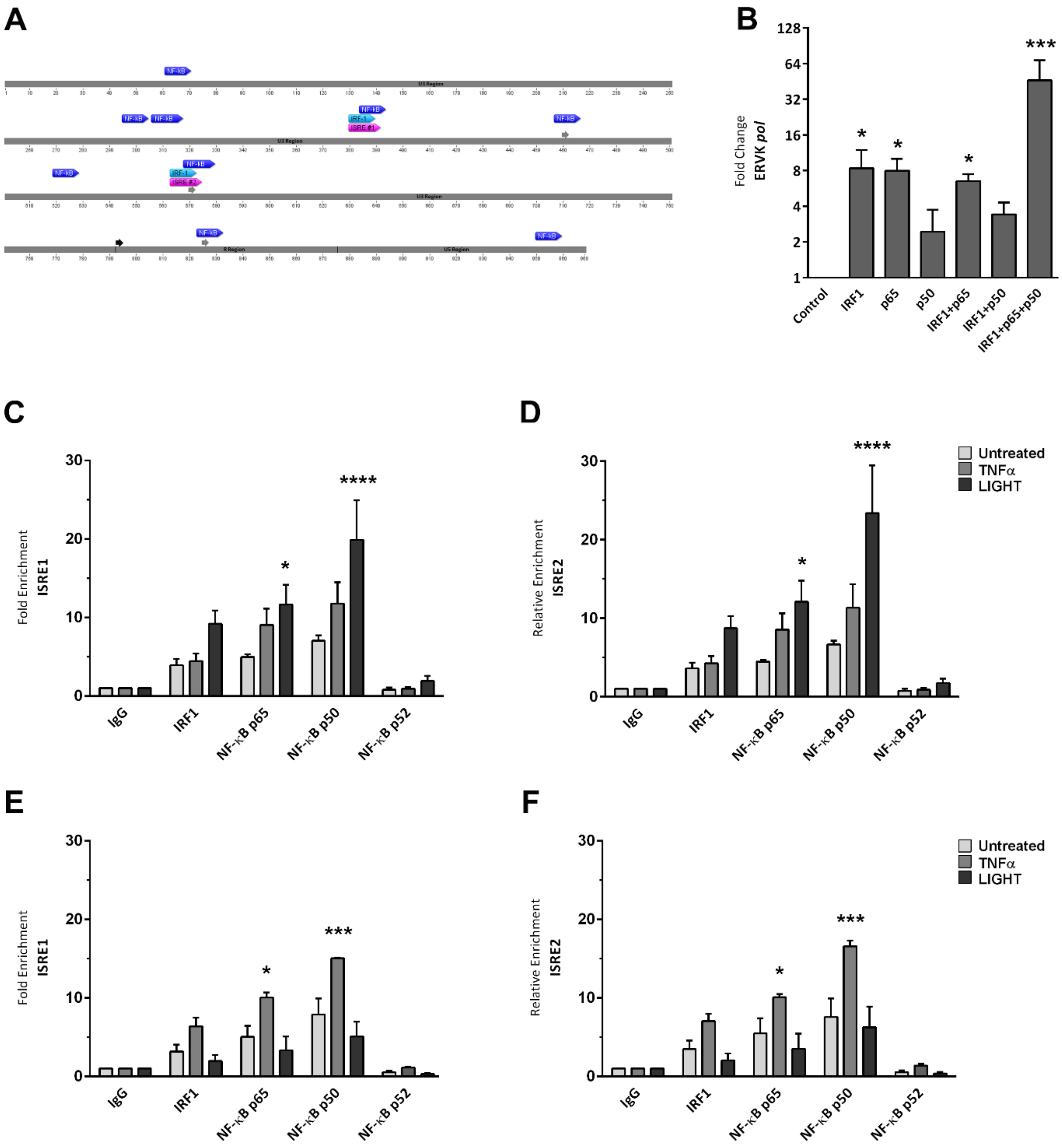
A
NF-κB
IRF-1
ISRE #1
ISRE #2
U3 Region
R Region
U5 Region
B
Fold Change ERVK pol
128
64
32
16
8
4
2
1
Control
IRF1
p65
p50
IRF1+p65
IRF1+p50
IRF1+p65+p50
*
*
*
***
C
Fold Enrichment ISRE1
D
Relative Enrichment ISRE2
E
Fold Enrichment ISRE1
F
Relative Enrichment ISRE2
30
20
10
0
IgG
IRF1
NF-κB p65
NF-κB p50
NF-κB p52
*
****
***
Untreated
TNFα
LIGHT

Figure 3. NF-κB and IRF1 interact with the ISREs in the ERVK 5′ LTR, and synergize to enhance ERVK gene transcription. (A) In silico-predicted IRF1 and NF-κB binding sites, including two Interferon Stimulated Response elements (ISREs) in the ERVK 5′ LTR. Adapted from [1]. **(B)** IRF1 and NF-κB p65 and p50 synergize to significantly enhance ERVK pol transcription in astrocytes. SVGA cells were transfected with 10 µg of empty vector (negative control), as well as plasmids encoding IRF1 and NF-κB isoforms individually and in combinations, for 48 hours. The modulation in ERVK pol RNA levels was measured by using SYBR Green detection through Q-PCR, and data were normalized relative to the negative control (*p<0.05 ***p<0.001; n=3). 18sRNA was used as the endogenous control. Although IRF1 and NF-κB p65 alone were sufficient to significantly induce ERVK pol transcription, IRF1 and NF-κB p65 and p50 synergized to further enhance ERVK pol RNA levels up to 70 fold. **(C-F)** TNFα and LIGHT markedly enhance the binding of IRF1 and NF-κB p65 and p50 to both ISREs in the ERVK 5′ LTR in a cell-type specific manner. Chromatin was extracted from SVGAs and ReNcell CX-derived neurons treated with TNFα (10 ng/ml) or LIGHT (10 ng/ml) for 8 hours. Chromatin Immunoprecipitation (ChIP) was performed with anti-human IRF1, or NF-κB p65, p50, or p52 antibodies. Q-PCR was used to amplify immunoprecipitated ISRE sequences within the ERVK 5′ LTR using SYBR Green detection. Fold enrichment of transcription factors at each ISRE was first normalized to the input control and then to the IgG negative control. All transcription factors were bound to the ISREs at basal levels. However, the binding of NF-κB p65 and p50 was significantly enhanced with LIGHT treatment, but not with TNFα treatment in astrocytes **(C-D)** (n=3; *p<0.05, ****p<0.0001). In contrast, the binding of NF-κB p65 and p50 was significantly enhanced with TNFα treatment, but not with LIGHT treatment in neurons **(E-F)** (n=2; *p<0.05, ***p<0.001).

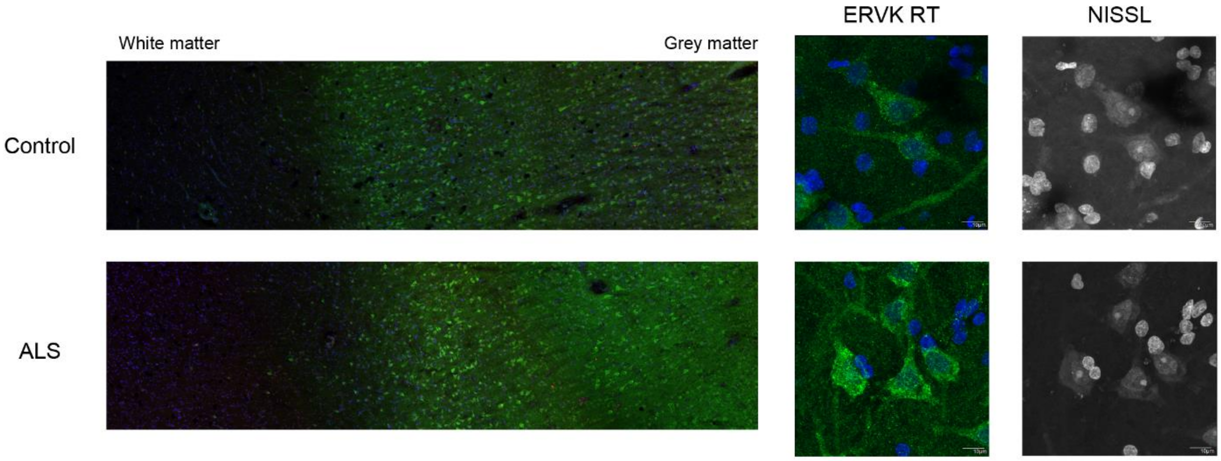
ERVK RT
NISSL
White matter
Grey matter
Control
ALS

Figure 4. Representative images of ERVK reverse transcriptase (RT) in cortical brain tissue. Prefrontal cortex autopsy tissues (Brodmann area 9, NIH NeuroBioBank) of an individual with cancer (neuro-normal) and a patient with ALS were immunostained for ERVK RT expression (green). Mosaic tiling (left panel, 10X magnification) reveals enhanced ERVK RT expression in deep cortical tissue (V) and upper cortical tissue (III) layers in ALS tissue. Nissl stained cells reveal ERVK RT staining in large pyramidal neurons. Nuclear DAPI staining (blue). Scale bar on 40X magnified images (right) is 10µm.

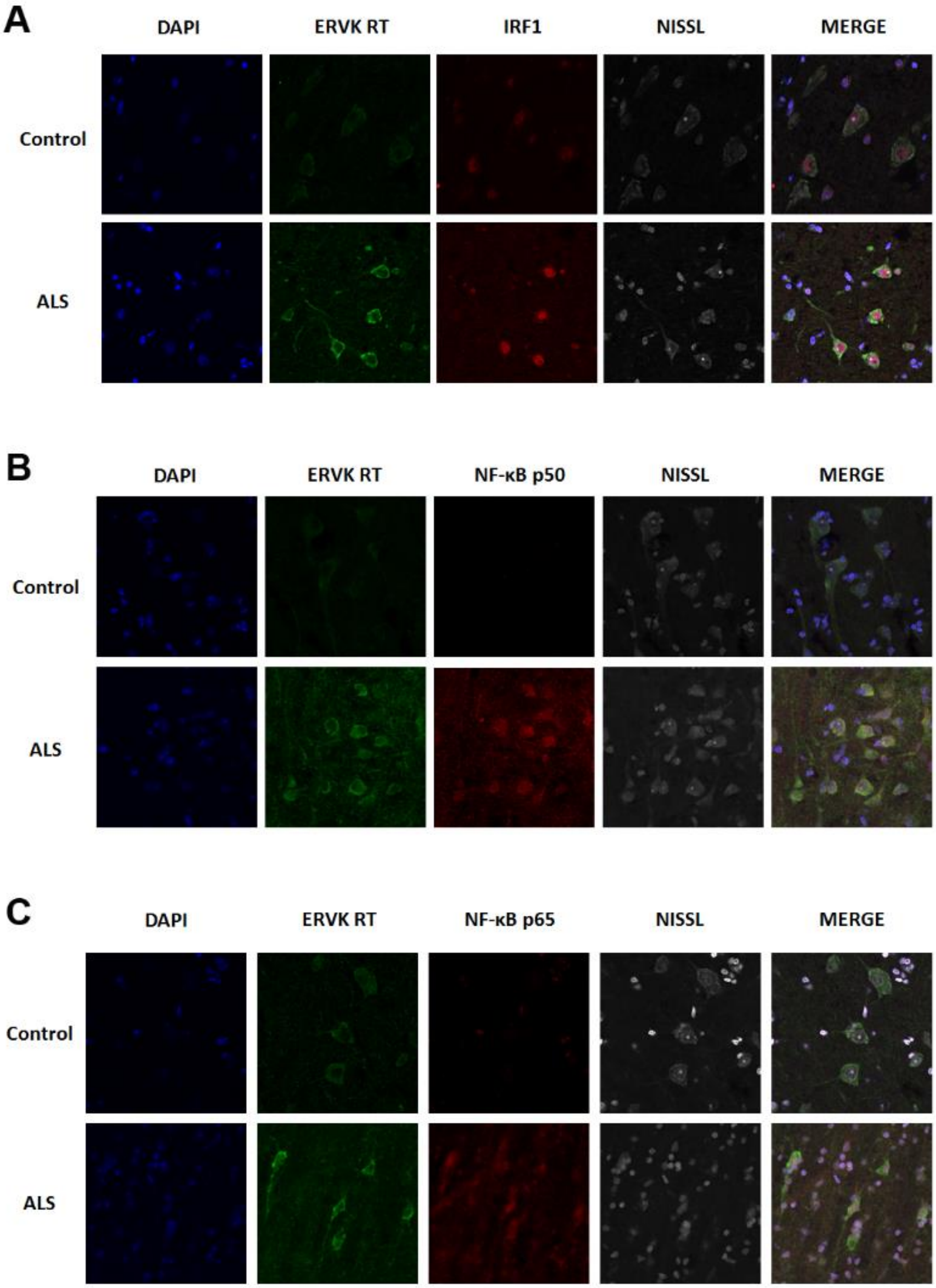
A
DAPI
ERVK RT
IRF1
NISSL
MERGE
Control
ALS
B
DAPI
ERVK RT
NF-κB p50
NISSL
MERGE
Control
ALS
C
DAPI
ERVK RT
NF-κB p65
NISSL
MERGE
Control
ALS

Figure 5. The expression of ERVK RT is markedly enhanced in cortical neurons of patients with ALS and associates with increased levels of and enhanced nuclear translocation of IRF1 and NF-κB. Representative confocal micrographs of ERVK RT, IRF1 and NF-κB p50 protein detection in Brodmann area 6 motor cortex tissue from an ALS patient and from a neuro-normal control (n=5). ERVK RT expression increased in the perinuclear region and in the axons of large pyramidal neurons in ALS motor cortex. This occurred concomitantly with increased expression of **(A)** IRF1, **(B)** NF-κB p50 and **(C)** NF-κB p65 nuclear translocation in cortical neurons.

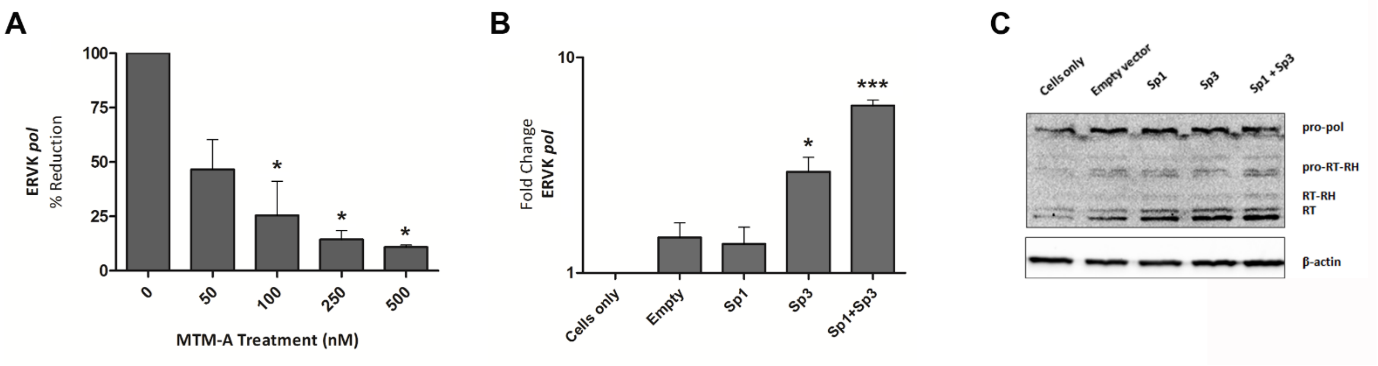
A
100
75
50
25
0
ERVK pol
% Reduction
*
*
*
0
50
100
250
500
MTM-A Treatment (nM)
B
10
1
Fold Change
ERVK pol
*
***
Cells only
Empty
Sp1
Sp3
Sp1+Sp3
C
Cells only
Empty vector
Sp1
Sp3
Sp1 + Sp3
pro-pol
pro-RT-RH
RT-RH
RT
β-actin

Figure S1. Sp1 and Sp3 enhance ERVK transcription and protein expression. (A) Reduced ERVK *pol* expression 24 hours after Mithramycin-A (MTM-A) treatment of chronically ERVK-expressing NCCIT cells. MTM-A is known to abrogate Sp1 activity. ERVK expression was measured by Q-PCR and data were normalized relative to the untreated cells. 500nM MTM-A inhibited ERVK expression in NCCIT cells by 90%. Statistical analysis was performed using One-way Anova and Bonferroni post-test (n=3; *p<0.05). **(B)** SVGAs were transfected with 1 ug of Sp1 and/or Sp3 plasmids for 48 hours using Turbofect reagent. ERVK *pol* expression was measured by Q-PCR using SYBR Green detection, and data were normalized relative to the untreated cells. 18s rRNA was used as the endogenous control. Sp1 alone did not significantly up-regulate ERVK *pol* transcription. Sp3 alone induced ERVK *pol* RNA levels by 3 fold. In the presence of both Sp1 and Sp3, ERVK expression was increased 6 fold. Statistical analysis was performed using One-Way Anova and Bonferroni post-test (n=3; *p<0.05 ***p<0.005). **(C)** Western blot depicting the sequential cleavage of the gag-pro-pol polyprotein, culminating in the production of prototypical ERVK RT isoforms. ERVK RT protein levels increased in the presence of Sp1 and Sp3 individually and in combination. β-actin was used as the loading control (n=3).

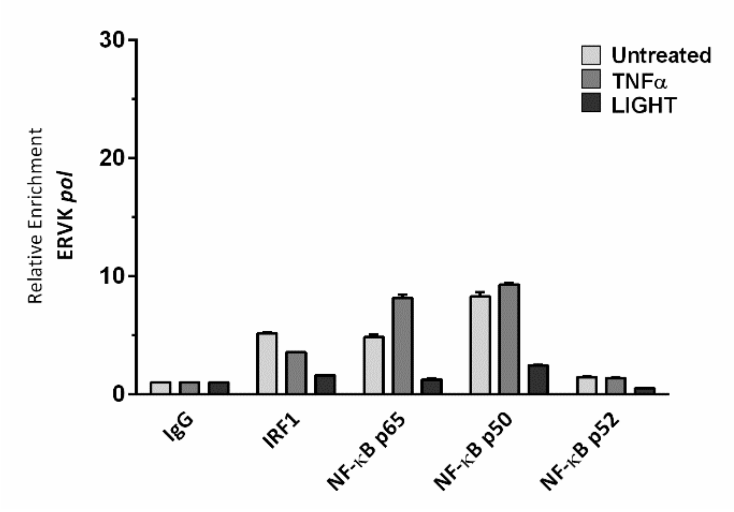
30
20
10
0
Relative Enrichment
ERVK *pol*
Untreated
TNFα
LIGHT
IgG
IRF1
NF-κB p65
NF-κB p50
NF-κB p52

Figure S2. Chromatin was extracted from SVGAs treated with TNFα (10 ng/ml) or LIGHT (10 ng/ml) for 8 hours. Chromatin Immunoprecipitation (ChIP) was performed with anti-human IRF1, or NF-κB p65, p50, or p52 antibodies. Q-PCR was used to amplify immunoprecipitated ERVK *pol* (negative control) using SYBR Green detection. Fold enrichment of transcription factors at ERVK *pol* was first normalized to the input control and then to the IgG negative control. IRF1 as well as NF-κB p65 and p50 were bound to the ERVK *pol* region at basal levels. NF-κB p65 binding increased with TNFα treatment. All transcription factor binding decreased with LIGHT treatment (n=2).

Table S1. Cortical Brain Tissue Specimens

Case	Diagnosis	Cause of Death	Tissue	Age (yrs)	Gender	PMI
2776	ALS	ALS (progressive bulbar palsy)	BA6, BA9	76	F	8.6
5215	ALS	ALS	BA6, BA9	59	M	12.5
5187	ALS	ALS, Hypertension, Chronic urinary tract infection	BA6, BA9	69	M	13.2
5212	ALS	ALS	BA6, BA9	50	M	21
5216	ALS	ALS	BA6, BA9	53	M	16.8
4660	Normal	Pancreatic cancer, Diabetes, Hypertension	BA6, BA9	73	F	18.5
3371	Normal	Lung Cancer	BA6, BA9	52	M	16
4514	Normal	Lung Cancer, Chronic obstructive pulmonary disease	BA6, BA9	66	M	17.3
3565	Normal	Cardiomyopathy	BA6, BA9	76	M	11
3221	Normal	Chronic obstructive pulmonary disease	BA6, BA9	90	M	17.8

BA6: Brodmann area 6 motor cortex

BA9: Brodmann area 9 prefrontal cortex

PMI: post-mortem interval

TDP-43 Regulates Human Endogenous Retrovirus-K Transcription and Viral Protein Accumulation: Implications for HIV-Associated Neurocognitive Disorders and ALS

Mamneet Manghera, Jennifer Ferguson-Parry, Avindra Nath, and Renée Douville

To be submitted to *Nature Neuroscience* or *Annals of Neurology,* September 2015

The goal of this study was to evaluate the influence of altered TDP-43 activity on ERVK transcription and proteinopathy in astrocytes and neurons. We have shown that neurons exhibiting TDP-43 accumulation have a marked increase in ERVK protein levels in autopsy brain tissue from HIV-infected individuals and those with ALS. We also determined that overexpression of wild-type, but not truncated forms of TDP-43 induced ERVK transcription. Aggregating forms of TDP-43 however promoted the cytoplasmic accumulation and aggregation of ERVK proteins. TDP-43 was found to bind the ERVK promoter, and increased binding in the context of inflammation or proteasome inhibition enhanced ERVK transcription. Thus, enhanced ERVK transcription and protein accumulation likely stems from TDP-43 dysregulation in ALS and HIV associated neurocognitive disorder.

Article

TDP-43 Regulates Human Endogenous Retrovirus-K Transcription and Viral Protein Accumulation: Implications for HIV-Associated Neurocognitive Disorders and ALS.

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Running Head: TDP-43 regulates endogenous retrovirus-K expression

Word count: 5,807

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Source of support: ALS Association, Manitoba Medical Services Foundation, Research Manitoba, Canada Foundation for Innovation, University of Winnipeg, National Institutes of Health (NIH)

ABSTRACT

The involvement of TDP-43 in retrovirus replication, and specifically HIV infection, remains controversial. We evaluated whether TDP-43 exerts an effect on endogenous retrovirus-K (ERVK) expression. Using autopsy tissue from HIV$^+$ individuals and patients with amyotrophic lateral sclerosis (ALS), we show marked enhancement of ERVK in TDP-43$^+$ neurons. Human astrocytes and neurons further demonstrated cell-type specific differences in their ability to express and clear ERVK proteins during inflammation and proteasome inhibition. Astrocytes, but not neurons, were able to clear excess ERVK proteins through an autophagic response. Overexpression of wild-type, but not ALS-associated aggregating forms of TDP-43 induced ERVK transcription. TDP-43 bound the ERVK promoter in the context of inflammation or proteasome inhibition. However, only aggregating forms of TDP-43 promoted the cytoplasmic accumulation and aggregation of ERVK proteins. Our findings support the paradigm that TDP-43 heterozygosity enhances neuropathology, and that ERVK proteinopathy is a novel aspect of TDP-43 misregulation in neurodegenerative diseases.

Keywords: Human Immunodeficiency virus (HIV); Endogenous retrovirus (ERVK); TAR DNA-binding protein 43 (TDP-43); HIV-associated neurocognitive disorders (HAND); Amyotrophic Lateral Sclerosis (ALS)

INTRODUCTION

Human retroviruses are neurotrophic, causing clinically silent central nervous system (CNS) lesions long before diagnosis [1]. Despite antiretroviral therapy, neurocognitive disorders continue to complicate the clinical treatment of Human immunodeficiency virus (HIV) infection[2]. More severe forms of HIV-associated neurocognitive disorders (HAND) are now less frequent, but the prevalence of mild neurocognitive deficits – those affecting daily living activities – have increased among HIV$^+$ populations [1]. Both HIV and human T-cell leukemia virus (HTLV) have also been associated with an increased incidence of ALS-like syndromes [3, 4]. Anti-retroviral therapy in HIV-infected patients has been reported to reverse the symptoms of ALS-syndrome [5, 6]. A pathological link between HAND and ALS is the re-activation of neuron-expressed human endogenous retrovirus-K (ERVK) [7, 8].

The human genome is actually a composite of human and viral genes, with over 8% of our DNA occupied by endogenous retroviruses (ERVs) [9, 10]. ERVK is one of the most recent entrants into the human genome [11], and its expression has been linked to inflammatory, infectious and neurological diseases [12]. Inflammatory signals and cell-specific transcription factors are crucial for driving differential ERVK expression in tissues [13, 14]. Infectious agents, such as HIV, can also trigger ERVK expression [8, 15, 16]. Once re-activated, ERVK virions can transfer viral genomes to neighbouring cells [17], but unlike HIV, there is no evidence of viral replication cycles that could lead to human-to-human transmission of ERVK.

HIV enters the CNS shortly after initial infection, yet it remains unknown whether slow progressive pathological changes or abrupt alterations following systemic immunosuppression trigger neurodegeneration. Microglia, macrophages and astrocytes can support productive HIV infection and promote chronic pro-inflammatory responses (reviewed in [18]). HIV infection is known to re-activate ERVK expression in proliferating peripheral blood mononuclear cells [19]. A longitudinal study by Contreras-Galindo *et al.* indicates that increased ERVK expression in HIV-infected patients precedes spikes of HIV replication [20]. Clinically, patients who fail to respond to HAART therapy or receive sub-optimal therapeutic doses also exhibit higher ERVK expression [20, 21]. However, ERVK re-activation is not limited to the periphery, as it is also a hallmark of HIV neuroinvasion[8]. Cortical neurons of HIV$^+$ individuals exhibit enhanced ERVK (HML-2) envelope protein expression as compared to HIV$^-$ controls, which is postulated to be neuroprotective [8].

We are now only beginning to recognize the pathological contributions of ERVs in neurological disease. ERV expression was commonly cited as a bystander effect of disease processes, but it is now becoming evident that transfer of ERVK RNA and proteins within the host may trigger innate immune responses [17, 22]. Another unexplored facet of ERVK re-activation is what cells do with excess viral protein accumulation. Protein deposition is characteristic of several neurological diseases, where a failure to clear excess and aggregating cellular proteins is associated with neuronal dysfunction and a lack of structural connectivity correlating with clinical symptom severity [23, 24].

Several gene mutations within different pathways converge into the common feature of TDP-43 positive neuropathology in ALS [25, 26]. Only 5-10% of familial and 1% of sporadic ALS cases carry TDP-43 mutations [27, 28], eluding to the fact that wild-type TDP-43 is incorrectly processed in the majority of patients with ALS[26]. An important aspect contributing to altered proteostasis and TDP-43 proteinopathy is impairment of both the ubiquitin proteasome system (UPS), autophagy and stress granule pathways, which are disrupted in ALS [26, 29]. TDP-43 is known to tightly auto-regulate its expression, in order to maintain the homeostatic RNA-protein complex formation [25]. Yet, studies have documented enhanced TDP-43 expression in *ex vivo* patient tissues and fluids [7, 30-33]. Our previous findings demonstrate that enhanced nuclear TDP-43 protein expression co-localizes and correlates with the extent of ERVK$^+$ neurons in ALS patients [7]. Several studies have shown that cell stress and pathogenic ALS mutations can contribute to elevated TDP-43 levels, thus promoting neurodegeneration (reviewed in [34, 35]). Conversely, in the context of glutamate accumulation, elevation of nuclear TDP-43 lends protection against cortical neuronal death [36].

One challenge of understanding how TDP-43 fits into the pathways involved in neurodegeneration is its multiple cellular functions. TDP-43 is a global regulator of RNA metabolism with defined roles in transcription, splicing, stability, transport, translation, microRNA maturation, as well as protein homeostasis. In addition to the role of TDP-43 in RNA regulation [37], its DNA binding capacity has been shown to repress HIV transcription by binding to specific sequences within proviral DNA [38]. Recently, this finding was put into question, as TDP-43 over-expression had no effect on HIV levels in human immune cells and could even weakly enhance Tat-dependent HIV replication in HeLa cells [39]. Both articles examining the role of TDP-43 on HIV replication did not address cell-specific TDP-43 proteinopathy, nor validate their findings in *ex vivo* tissues. Moreover, the influence of TDP-43 on ERVK expression remains unexplored.

METHODS

Patient samples

Tissue specimens were obtained from the California NeuroAIDS Tissue Consortium (CNTC), the Texas Repository for AIDS Neuropathogenesis Research (TRANR), the National NeuroAIDS Tissue Consortium (NNTC), the Human Brain and Spinal Fluid Resource Center (HBSFRC), the Rocky Mountain MS Center (RMMSC) and the Johns Hopkins School of Medicine Brain Bank (JHSMBB). **Table S1** indicates the individual patient diagnosis, location of brain tissue sampling, age, gender and post-mortem interval (PMI in hours) of the samples used in this study. Among confirmed cases with HIV infection (as indicated by CD4 count, plasma and cerebral spinal fluid viral loads), brain tissue samples were further classified as having HIV-encephalitis based of neuropathological examination.

Immunohistochemistry and immunoblotting of autopsy tissue

To determine the extent of ERVK expression patterns in the CNS of HIV-infected patients, we used a previously described immunohistochemistry technique to detect RT protein levels and localization in autopsy tissue from human cortical brain tissue [41]. Density of immunostaining was quantified using ImageJ software. Antibodies against the ERVK reverse transcriptase protein (AbNova #H00002087-A01) and human TDP-43 (Protein Tech #10782-2-AP) were used for immunohistochemistry, and the same TDP-43 antibody for western blotting. For immunoblot sample preparation, small pieces of brain tissue were disrupted with a pestel and whole cell extracts were prepared using RIPA buffer (50mM Tris, 100mM NaCl, 100 mM EDTA, 1% SDS, 0.5% sodium deoxycholate, 1% NP40 and protease inhibitor cocktail). Whole cell extracts were cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C before use in western blot. Primary antibodies were detected using IRDye secondary antibodies and immunoblot visualised with an Odyssey scanner (LI-COR Biosciences). Odyssey software was used to measuring the optical density of the TDP-43-specific bands versus the β-actin control of each sample. Comparisons between patient groups were performed using the nonparametric Mann–Whitney U-test. Evaluation of correlation was performed by calculating the Spearman's rank correlation coefficient. P-values less than 0.05 were considered statistically significant. Analyses were performed with GraphPad Prism version 5 (GraphPad Software, La Jolla, California, USA).

Cell Culture

The SVGA cell line is derived from immortalized human fetal astrocytes[73], and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum (HyClone). ReNcell CX cells (Millipore, #SCC007) are immortalized human neural progenitor cells (HNPCs) [74], and were maintained in a proprietary ReNcell neural stem cell medium (Millipore) supplemented with 20 ng/ml human epidermal growth factor (EGF; Peprotech #AF10015), 20 ng/ml human basic fibroblast growth factor (bFGF; Peprotech #AF10018B). All cell lines were maintained in a 37°C incubator containing 5% CO_2. SVGA cells were seeded into six-well plates and onto glass coverslips in twelve-well plates at a density of 300,000 cells/ml and 30,000 cells/ml, respectively, and grown for 24 hours. To differentiate HNPCs into neurons, ReNcells were seeded in laminin (20 µg/ml; Millipore #CC095) coated six-well plates at a density of 50,000 cells/ml for 24 hours. Adhered cells were rinsed with 1X PBS and allowed to differentiate in the presence of ReNcell medium lacking EGF and bFGF for 10 days. For imaging experiments, ReNcell CX cells were cultured and differentiated into neurons in Alvetex scaffolds. Alvetex membranes (Reinnervate #AVP002) were treated with 70% Ethanol for 1 minute, rinsed with 1X PBS, and coated with 20 µg/ml laminin for 6 hours. ReNcell CX cells were seeded onto each scaffold at a density of 5×10^5 cells/well for 1 hour at 37°C and 5% CO_2, as per manufacturer's instructions. The wells were then flooded with 2 ml of ReNcell media supplemented with EGF and FGF growth factors. Twenty-four hours post-seeding, the cell culture media was replaced with that lacking growth factors. Cells were allowed to differentiate for 10 days, with partial media changes performed every 3 days. SVGAs and neurons were treated with 10 ng/ml dose of human TNFα (PeproTech, #AF300-02) and/or 3µM MG132 (Sigma) for 24 hours. Plated untreated cells were used as negative controls.

Transient transfection of wild-type and mutant TDP-43 constructs

For transcript and total protein quantification, 4 µl of Lipofectamine LTX with 4 µl of Plus Reagent was used to transfect cells with 0.5, 1, 2, and 4 µg doses of TDP-43 Notag 1 or Notag 6 constructs (Addgene), as per manufacturer's instructions (Life Technologies). For imaging experiments, cells were transfected with 0.5 µg of TDP-43 tomato alone or in combination with 1.0 µg of TDP-43 Notag 1, 2, 3, or 6 constructs (Addgene), using 2 µl of Lipofectamine LTX with 1 µl of Plus Reagent. Cells were transfected in serum-free culture media for 4 hours, followed by addition of complete media for a total incubation of 24 hours. Untransfected cells and those transfected with LTX and Plus Reagent only were used as negative controls.

Chromatin immunoprecipitation (ChIP)

SVGAs were seeded in dishes at an approximate density of 3 x 10^6 cells/dish for 24 hours at 37°C and 5% CO_2. Dishes were coated with 130 µg of laminin (Millipore) overnight. ReNcell CX cells were seeded at a density of 3 x 10^5 cells/dish for 24 hours at 37°C and 5% CO_2, and then allowed to differentiate into neurons in media lacking FGF and EGF growth factors (Peprotech) for 10 days. Cells were treated with 10ng/ml TNFα (Peprotech) and/or 3µM MG132 (Sigma Aldrich) for 18 hours, fixed with 4% paraformaldehyde, and harvested. Chromatin Immunoprecipitation (ChIP) was performed using Pierce Magnetic ChIP kit (Thermo Scientific) as per manufacturer's instructions. TDP-43 bound DNA segments were isolated using 3 µg of ChIP-validated rabbit anti-human TDP-43 (Proteintech #I2892-I-AP) antibody. IgG antibody was used as the negative control. QPCR was performed on the immunoprecipitated DNA using SYBR Green detection to amplify the ERVK 5' LTR (nt. 800 - 968). Primers used were F: 5'-

TACTAAGGGAACTCAGAGGCCG-3′ and R: 5′-TAGACACCAGTGAAGGGGTGG-3′. Data were analyzed using the ΔΔ Ct method and normalized relative to the input and IgG controls for each condition. All data were graphed as mean ± standard error of measurement. Statistical analyses were performed in GraphPad PRISM using One-Way Anova and Tukey’s multiple comparisons test.

Quantitative Polymerase Chain Reaction (Q-PCR)

Total RNA was extracted and purified from cells using an Aurum Total RNA Mini Kit (Bio-Rad, #732-6820). RNA concentration was measured with a NanoDrop spectrophotometer. The acceptable RNA purity was $A_{260}/A_{280} > 2.0$. The iScript Reverse Transcription kit (Bio-Rad, #170-8840) was used to synthesize cDNA from extracted RNA. CFX Connect Real Time System (Bio-Rad) was employed to perform Q-PCR in order to measure alterations in *TARDBP*, ERVK *gag* and *pol* transcripts using SYBR Green detection method. The primers used were: TDP-43 F: 5′-GTACGGGGATGTGATGGATG-3′ and R: 5′-CTGCGCAATCTGATCATCTG-3′, ERVK *gag* F: 5′ TCGGGAAACGAGCAAAGG 3′ and R: 5′ GAATTGGGAATGCCCCAGTT 3′, and ERVK *pol* F: 5′ TGATCCCMAAAGAYTGGCCTT 3′ and R: 5′ TTAAGCATTCCCTGAGGYAACA 3′. 18S rRNA was used as the endogenous control (Ambion kit #1718). The data were analysed using the ΔΔCT (Livak) method. All data were graphed as mean ± standard error of measurement. GraphPad Prism was used to carry out statistical analyses including column statistics, One-way Anova Friedman test, and Dunn’s post-test.

Western Blotting

Cells were lysed on ice with 50 µl of in-house lysis buffer (0.05M Tris (pH 7.4), 0.15M NaCl, 0.002M EDTA, 10% glycerol and 1% NP-40 in ultra-pure water) to extract the soluble proteins, followed by extraction of insoluble proteins in 50 µl of RIPA buffer (10% 1X TBS, 1% SDS, 1% NP-40 and 0.5% DOC in ultra-pure water). Both buffers were supplemented with 1x HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). BCA assay (Thermo Scientific, #PI23227) was used to determine the protein content of each sample as per manufacturer’s instructions. Cell lysates were prepared for SDS-PAGE and heated at 95^{o}C for 10 minutes. Proteins (15 µg per lane) were separated by SDS-PAGE using a 10% polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk solution for one hour and probed with primary antibody overnight at 4°C, followed by incubation at room temperature for 3 hours. Primary antibodies were: mouse anti-human ERVK2 RT primary antibody (1:1000 dilution; Abnova, #H00002087-A01), rabbit anti-human TDP-43 (Thermo Scientific #PA5-17011), G3BP1, mouse-anti-human LC3B (MBL, #M1523), mouse anti-human β-actin (Thermo Pierce, #MA5-15739; loading control). The membrane was then probed with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG secondary antibody (1:5000 dilution; Bio-Rad, #170-6516 and #170-6515) for 2 hours at room temperature. The membrane was developed with 2 ml of Luminata Crescendo Western HRP substrate (Millipore; #WBLUR0500) and imaged using Bio-Rad ChemiDoc XRS+ or Protein Simple FluorChem M chemiluminescent imager. Image Lab software was used to determine the molecular weight of each band, as well as their density relative to that of the negative control. Band densities were normalized relative to the β-actin loading control. The identity of each band was based on gag-pro-pol processing, as previously described [13].

Fluorescent microscopy

Twenty-four hours post-transfection or treatment, cells were fixed with methanol for 40 seconds, and rinsed with PBS. Cells were permeabilized with 250 µl of PBS-T (PBS with 0.25% TritonX-100) and blocked with 250 µl of 3% BSA in TBS-T (TBS with 0.25% TritonX-100) for 30 minutes. Immunocytochemistry was performed using 1:1000 mouse anti-human ERVK RT (Abnova #H00002087A01), rabbit anti-human TDP-43 (Thermo Scientific #PA5-17011) and/or G3BP1 primary antibodies for 3 hours and 1:1500 goat anti-mouse AF488 and goat anti-rabbit AF594 (Molecular Probes #A11017 and #A11072) secondary antibodies for 2 hours. Nuclei were counterstained with 1:25000 DAPI. ReNcells were also stained with fluorescent Nissl (Molecular Probes #N21483). Coverslips were mounted onto slides using Prolong gold anti-fade reagent (Molecular Probes), and dried overnight. Controls were prepared by immunostaining without the primary antibodies. Confocal microscopy was performed using Olympus FV1200 confocal microscope. For each image acquired, number of cells with aggregates and total number of cells were counted in order to calculate percent aggregation under each condition using ASW4.0 software suite. In each image, the boundaries of cells with and without aggregates were also outlined. The mean intensity of ERVK RT and DAPI staining was then recorded for these cells. The ratio of these intensities was calculated to give an indication of overall change in ERVK RT levels with the overexpression of wild-type and truncated TDP-43. Statistical analyses were performed in GraphPad PRISM using One-Way Anova and Dunn's multiple comparisons test.

RESULTS

ERVK reverse transcriptase and TDP-43 are strongly upregulated in HIV infection

We have previously demonstrated that ERVK reverse transcriptase (RT) expression is elevated in neurons of patients with Amyotrophic Lateral Sclerosis (ALS) [7]. Here, we sought to determine the extent of ERVK expression in the CNS of HIV-infected patients by detecting RT protein levels and localization pattern in autopsy tissue from parietal cortical tissue of HIV patients with encephalitis (HIV-E/HAND, n=6,), HIV patients without encephalitis (HIV, n=9) and chronic systemic illness (Controls, n=7) (**Table 1**).

As HIV-E is a focal disease, different regions may have varying levels of HIV replication. Yet, in both HIV-infected groups, increased ERVK RT protein was detectable, but surprisingly expressed at similar levels (**Figure 1A**). A caveat of using excised brain specimens is sampling bias, as not every tissue sample may be representative of the brain pathology as a whole; therefore, we sought to determine the extent of HIV replication in each tissue specimen examined and stratified the tissues from HIV infected individuals based on the presence or absence of HIV Gag p24 positive cells. ERVK RT expression was significantly up-regulated in brain tissue with p24 reactivity (**Figures 1B and 1C**). This result is consistent with previous findings in peripheral blood mononuclear cells demonstrating that ERVK expression is triggered by productive HIV infection [19, 40]. Moreover, ERVK titers are known to be elevated in HIV$^+$ patients who failed to respond to HAART therapy [20], suggesting that ERVK expression in the CNS may also reflect inadequate response to antiretroviral treatment. Interestingly, plasma viral loads were related to ERVK RT measurements in cortical tissue of HIV-infected patients (Spearman's correlation p=0.052), supporting the idea that systemic HIV replication favours ERVK expression in both brain and PBMC. Neocortical productive HIV infection is characteristic of HIV encephalitis / HAND[1], and may also be a significant factor in milder neurocognitive disorders. As robust ERVK expression was found in several HIV^+ patients not clinically diagnosed with HIV-E/HAND, pathological but modest HIV replication could drive ERVK activity in the CNS, which may precede overt clinical symptoms of neurological impairment.

Our study of ERVK RT expression in ALS patients revealed that the degree of ERVK re-activation was strongly correlated with TDP-43 *in vivo*[41]. Aberrant cytoplasmic aggregates of ubiquitin-associated and hyper-phosphorylated TDP-43 are a common event in ALS cortical tissues[42, 43]. Measurement of TDP-43 protein expression by immunohistochemistry (**Figure 1D**) or western blot (**Figure S1**) analysis revealed increased levels in HIV^+ specimens compared to controls. Furthermore, TDP-43 and ERVK RT proteins were co-expressed in the majority of neuronal cells (**Figure 1D**). To quantitatively examine the expression pattern, co-labeled counting and density measurements of ERVK versus TDP-43 staining in neurons was evaluated. **Figures 1E and 1F** demonstrate that there is a significant positive correlation between TDP-43 expression and ERVK expression in tissue (Spearman's correlation p<0.0001, n=22) and within individual neurons (Spearman's correlation p<0.0001, n=40). Enhanced nuclear TDP-43 expression in cortical neurons (6-fold) was also associated with increased TDP-43 phosphorylation (4-fold) (**Figure S1**). This data suggests that specific posttranslational modifications may alter TDP-43 protein turnover in the nucleus, as well as the formation of TDP-43 aggregates [42-44].

Astrocytes and neurons differentially clear ERVK protein accumulation

We observed neuronal ERVK expression in both HIV infection and ALS. Intriguingly, no other brain cells appeared to accumulate ERVK proteins, as opposed to cortical neurons. To address how astrocytes and neurons cope with ERVK re-activation, we designed an induction protocol based on inflammation and proteasome inhibition, two components of ALS [26, 45, 46] and HAND [47-49] neuropathology. In both diseases, chronic inflammation and alterations in protein ubiquitination culminates in a defect of proteasome function, decreased protein turnover and synaptic dysfunction in neurons. TNFα treatment of human astrocytic cells (SVGAs) had no perceivable effect on the induction or cleavage of ERVK polyprotein into prototypic active subunits [13] (**Figure 2A**). Proteasome inhibition by MG132 dramatically enhanced ERVK polyprotein levels in astrocytes. In conjunction, TNFα and MG132 caused the degradation of ERVK proteins, as well as cleavage of TDP-43 into insoluble 35 and 25 kDa forms. This degradative process in astrocytes appeared to be mediated through the autophagic pathway, as evidenced by LC3B cleavage. Conversely, the same treatments in human ReNcell CX differentiated neurons yielded disparate results, with no evidence of effective autophagic clearance of ERVK proteins with dual TNFα and MG132 treatment (**Figure 2B**). This was despite the fact that MG132 treatments resulted in TDP-43 and LC3B cleavage, indicating the initiation of autophagy. Moreover, co-treatment with TNFα and MG132 enhanced the prototypic cleavage of ERVK polyprotein into functional RT subunits.

To validate that these observations were associated with protein aggregation or protein clearance, cells were visualized for ERVK RT and TDP-43 by confocal microscopy. SVGA cells treated with TNFα exhibited slightly enhanced ERVK expression, which was not evident using western blot analysis (**Figure 2C**). Deposition of ERVK polyprotein/RT occurred proximal to the nucleus in an asymmetric fashion. MG132 treatment caused both the nuclear and cytoplasmic aggregation of TDP-43, in conjunction with substantially greater ERVK RT expression. Cytoplasmic ERVK RT and TDP-43 co-localized in aggregates adjacent to the cell nucleus. SVGA cells given dual TNFα and MG132 treatment had less ERVK RT protein than MG132 treatment alone, suggesting inflammatory signals helped drive an effective protein degradation pathway in the context of proteasome inhibition. Differentiated neurons exhibited more robust expression of ERVK RT at basal levels and when stimulated by either TNFα and/or MG132 (**Figure 2D**). However, MG132 treatment of ReNcell CX neurons resulted in ERVK protein aggregation and colocalization with TDP-43 aggregates, most markedly with TNFα and MG132 co-treatment.

TDP-43 is a transcriptional activator of ERVK

The ERVK LTR contains numerous putative TDP-43 binding sites (**Figures 3A and S3**), similar to those identified on the HIV-1 LTR [38]. Considering the enhanced expression of nuclear TDP-43 in cortical neurons of individuals with ALS and HIV infection, we sought to determine whether TDP-43 played a transcriptional role in the re-activation of ERVK. To address this question, SVGAs and differentiated ReNcells were transfected with wild-type and C-terminal mutant TDP-43 constructs[50]. Over-expression of a full-length TDP-43 construct (Notag 1[50]) dose-dependently enhanced ERVK transcription in both astrocytes and neurons, independently of additional stimuli (**Figure 3B**). Neuronal cultures were more susceptible to wild-type TDP-43-mediated transcriptional activation, with a stronger ERVK *gag* and *pol* enhancement at 10-100-fold lower levels of TDP-43 over-expression as compared to astrocytic cultures. In stark contrast, N-terminal truncated TDP-43 (Notag 6[50]) failed to enhance ERVK transcription (**Figure 3C**). This indicates that wild-type TDP-43 is a transcriptional activator of ERVK expression and may complex with other transcription factors to drive ERVK expression under inflammatory conditions. TPD-43 is known to trigger pathogenic NF-κB mediated pathways in neurons [51, 52], and our data support this paradigm, extending it to include ERVK-driven pathology.

TDP-43 is known to be both an RNA and DNA binding protein. The ERVK promoter contains numerous putative TDP-43 binding sites, primarily in the U5 799-968 bp region (**Figure 4A and S2**). To confirm that TDP-43 bound the ERVK LTR, chromatin immunoprecipitation (ChIP) was performed (**Figure 4 and S3**). **Figure 4E** reveals that TDP-43 binds the U5 region of the ERVK promoter, with enhanced binding during TNFα stimulation or proteasome inhibition ($p<0.05$) in astrocytes. With simultaneous TNFα and MG132 treatment of SVGA cells, TDP-43 undergoes cleavage into the 35 and 25 kDa forms (**Figure 2A**), and fails to bind the ERVK LTR. TDP-43 cleavage coincided with a reduction in inflammation-driven ERVK transcription (**Figure 4A and 4C**), suggesting that TDP-43 co-activates the ERVK LTR along with NF-κB. In contrast, enhanced TDP-43 binding to the ERVK LTR was not observed in TNFα stimulated neurons (**Figure 4F**), with no evidence of enhanced ERVK transcription in an inflammatory context (**Figure 4B and 4D**). With MG132 treatment, TDP-43 was enriched on the ERVK LTR (**Figure 4F**), without evidence of an effect on transcription (**Figure 4B and 4D**). With simultaneous TNFα and MG132 treatment of neurons, partial TDP-43 cleavage occurs (**Figure 2C**), and there is less TDP-43 bound to the ERVK promoter despite exposure to MG132 (**Figure 4F**). Under all treatment conditions in neurons, there was no effect on TDP-43 mRNA expression (data not shown). This suggests that TDP-43 can only induce ERVK expression in neurons when it is expressed significantly above physiological levels.

Mutant forms of TDP-43 promote cytoplasmic ERVK RT protein aggregation

In ALS, the majority of TDP-43 mutations occur in the C-terminal glycine-rich domain. However, both N-terminal and C-terminal TDP-43 mutants can precipitate TDP-43 aggregation [50, 53]. Transient transfection of SVGA cells with a fluorescently-tagged TDP-43 construct resulted in predominantly nuclear and to a lesser degree diffuse cytoplasmic TDP-43 localization (**Figure 5**). When this indicator protein was expressed in conjunction with wild-type (Notag 1) or domain-deletion aggregating forms of TDP-43 (Notag 2,3,6), we observed a marked enhancement of ERVK expression and aggregation with mutant TDP-43, as compared to wild-type TDP-43. Cytoplasmic TDP-43 aggregates co-localized with ERVK RT aggregation, although the extent of ERVK protein aggregates exceeded beyond the boundaries of TDP-43 foci. Quantification of ERVK protein expression was normalized against DAPI staining, revealing that only mutant forms of TDP-43 induced ERVK RT expression in cells. Wild-type TDP-43 could promote protein aggregate formation in 14.6% of transfected cells, but was ineffectual at increasing ERVK RT protein levels. In contrast, N-terminal and C-terminal truncated TDP-43 constructs resulted in 24-31% of cells displaying protein aggregation, with a notable enhancement of ERVK RT expression in the majority of cells containing aggregates. Deletion of the N-terminus of TDP-43 conferred the greatest effect on ERVK accumulation (Notags 2 & 3). This suggests that although both C and N-terminal truncated forms of TDP-43 can precipitate proteinopathy, the 35 kDa form of TDP-43 may be the most effective in promoting viral protein accumulation. These findings further support the notion that ERVK proteinopathy may occur in neurological disease characterized by TDP-43 deregulation, such as ALS, HAND and frontotemporal dementia (FTLD).

ERVK RT localizes to stress granules

ERVK RT co-localized with TDP-43 aggregates in our TDP-43 overexpression models in astrocytes (**Figure 5**). The ERVK RT and TDP-43 staining pattern in cells overexpressing these proteins resembled that of stress granules (SGs) [54]. Therefore, we immunostained astrocytes for the stress granule marker G3BP1 [55]. In astrocytes, large G3BP1$^+$ stress granules are formed in cells treated with TNFα and MG132 (**Figure 6**A). In this case, the ERVK RT/polyprotein colocalizes with G3BP1, suggesting that this viral RNA binding protein is present in association with stress granules. When ERVK RT expression is compared with the autophagosome marker LC3B (**Figure 6B**), there is some evidence of colocalization, but not to the same extent as with G3BP1. Interestingly, in human autopsy tissue from ALS patients (**Figure 6C**), we observed a substantial increase in G3BP1. G3BP1 levels may be supported by the enhanced TDP-43 expression [54], as seen in ERVK+ cortical neurons in ALS [7]. Yet, in these cortical neurons, there was no clear colocalization of G3BP1 and ERVK RT; we propose that this segregation of SGs and viroplasm may underlie enhanced ERVK viral protein expression in ALS. Together, our data point to the fact that stress granule formation may regulate ERVK expression, in conjunction with autophagic and proteasomal degradation of ERVK proteins. Deregulation of stress granule formation is now considered a potential mechanism for disruption of neuronal homeostasis and motor neuron death in ALS. Our data suggest that ERVK proteinopathy may be a crucial player in this degenerative process.

DISCUSSION

The expression of ERVs in the CNS has been associated with neurodegenerative diseases, such as ALS, Multiple Sclerosis and Schizophrenia, and is proposed to mediate specific pathological contributions to these diseases [56-58]. Our findings clearly suggest that the neuronal ERVK expression in HIV-infected individuals may also promote a unique pathological component of HIV-associated neurological disorders. Enhanced ERVK expression in neurons occurred in the majority of HIV$^+$ individuals, and more strongly within brain tissue exhibiting HIV replication. Our results support previous observations in PBMC from patients with HIV infection [20, 21], that a failure to respond to HAART therapy or receiving sub-optimal therapy may be associated with increased ERVK expression – both in the periphery and as our data suggest, in the CNS. Thus, the development of antivirals suppressing HIV replication in the brain may also be indirectly neuroprotective by limiting ERV-mediated pathology.

Inhibition of the ubiquitin-proteasome system, as seen in ALS, encourages the formation of stress granules [59]. Many viruses manipulate the cellular stress response by disrupting the formation of RNA granules [55]. Usurping key RNA binding proteins or degrading stress granules can facilitate viral replication. TDP-43 is known to interact with the HIV Gag-pol polyprotein, as part of Staufen1 ribonucleoprotein (RNP) complexes [60]. The expression of TDP-43 in RNPs overlaps with both HIV viral RNA and Gag expression. Both RNP and protein aggregates are subject to autophagic clearance. Overlapping aggregation of TDP-43 and ERVK polyprotein in both astrocytes and neurons suggests that inflammatory signals and/or proteasome impairment promote ERVK protein accumulation. Both translated viral RNA and ERVK RT-RNA interaction could promote the formation of protein and RNP aggregates in the cytoplasm. Recruitment of TDP-43 to ERVK RNPs is consistent with a viral evasion strategy to promote ERVK replication. Whereas astrocytes were able to clear the build-up of viral protein accumulation by unconventional ERVK polyprotein cleavage, neuronal cultures showed an inability to clear ERVK proteins. In fact, dual inflammation and proteasome inhibition promoted the prototypic cleavage of functional ERVK RT isoforms. The abundance of ERVK proteins within neurons may reflect cell-type specific differences in the selectivity and speed of autophagic mechanisms. This may increase the vulnerability of human neurons to viral protein inclusion formation during ERVK reactivation. Due to neuronal longevity and cytoarchitecture in the cerebral cortex, any genetic or functional impairment in protein or RNP clearance pathways may promote the maintenance of ERVK activity during and after the resolution of neuroinflammation.

Stress granules (SG) and processing bodies (PB) are two forms of cytoplasmic RNA-protein complexes, which regulate gene expression by translational arrest and RNA degradation, respectively [55]. Large SGs dock with PBs, facilitating RNA degradation [61]. Virus tend to disrupt RNA granules as a mechanism to promote replication [55]. Formation of stress granules has also increasingly been implicated in ALS pathology [29]. The stress granule protein G3BP1 is regulated by TDP-43 [54, 62] and is essential for SG-PB docking [61]. TDP-43 has previously been shown to colocalize with stress granule markers, and overexpression of wild type as well as mutant TDP-43 can enhance the formation of stress granules, although TDP-43 mutants result in more stress granules formed per cell [63]. But, the localization of ERVK proteins to stress granules has not been examined. Herein, we have demonstrated that ERVK RT also co-localizes with the stress granule marker G3BP1 in response to TNFα and MG132 stimulation of astrocytes, suggesting that ERVK proteins can be recruited to stress granules. G3BP1 expression was also significantly enhanced in ERVK$^+$ cortical neurons of patients with ALS as compared to neuro-normal controls, but no co-localization between ERVK RT and G3BP1 was observed. Exclusion of ERVK RT from stress granules in neurons may reflect a viral evasion strategy to escape degradative processes. As TDP-43 is an RNA binding protein, it may bind ERVK nucleic acids and shuttle them to SGs as an antiviral mechanism, to impede viral RNA translation. Despite the fact that TDP-43 mutants have an increased SG association, they may not be able to interact with ERVK RNA. In ALS, TDP-43 mutations or cleavage may result in a failure to traffic viral RNA to SGs, allowing the translation of ERVK polyprotein in neurons.

We also postulate that TDP-43 cleavage may be enhanced by ERVK protease activity in neurons, as prototypic polyprotein cleavage events occurred concomitantly with the generation of a 35 kDa TDP-43 fragment. Coxsakievirus B3 protease has been shown to mediate TDP-43 cleavage at position Q327 resulting in an N-terminal 35 kDa product and an 8 kDa C-terminal fragment [64]. Protease cleavage of RNP-associated proteins, such as TDP-43, is a characteristic immune evasion strategy of many positive-stranded RNA viruses, as early induction and subsequent disassembly of stress granules limits innate antiviral response [55]. In contrast, here we have shown that human astrocytes displayed evidence of both prototypic ERVK polyprotein cleavage mediated by the viral protease, but under conditions of inflammation and proteasome inhibition, non-prototypic cleavage of the polyprotein occurred, suggesting that cellular proteases can also digest ERVK viral proteins. A robust cleavage of all soluble and insoluble TDP-43 into the 35 kDa TDP-43 fragment in astrocytes during TNFα and MG132 treatment is likely indicative of a caspase, calpain or cathepsin-mediated TDP-43 cleavage event [65-67], which may have also generated non-prototypic ERVK polyprotein cleavage. This may represent an intrinsic cellular mechanism to protect astrocytes from the accumulation of endogenous retrovirus proteins. It appears that neurons fail to initiate this digestive pathway, both in *in vitro* culture and as evidenced by the accumulation of ERVK RT in neurons of HIV$^+$ individuals and patients with ALS. Viral protease activity may further explain the predictable spread of misfolded TDP-43 down the axon and across synapses through interconnected neural networks, as the transfer may involve the activity of virion-associated protease [68].

TDP-43 heterozygous missense mutations have been identified in familial and sporadic ALS [69-71]. Our data suggest that intact TDP-43 can transcriptionally activate the ERVK LTR, and that mutant TDP-43 can promote the aggregation of ERVK RT proteins. This TDP-43-mediated enhancement of viral protein production in conjunction with the aggregating effects of truncated forms of TDP-43 may promote viral proteinopathy in individuals carrying select heterozygous TDP-43 alleles. This effect may be further precipitated by the enhancement of ERVK expression by inflammatory signalling in ALS and cerebral HIV infection. In support of these observations, it has been recently shown that human wild-type TDP-43 potentiates Q331K-mutant TDP-43 driven motor neuron loss in a humanized mouse model [72]. The aggressive phenotype of dual wild-type and mutant TDP-43 expression in this model resulted in the loss of both spinal and layer V neurons of the cortex. In addition, TDP-43(WTxQ331K) mice exhibited enhanced nuclear clearance of TDP-43.

In contrast with previous evidence of TDP-43-mediated transcriptional repression of HIV-1 promoter, here we showcase that TDP-43 is a transcriptional activator of ERVK. TDP-43 over-expression, specifically aggregating forms, promotes ERVK proteinopathy in human astrocytes and neurons. These findings significantly challenge the perspective on protein deposition in the neuropathogenesis of HAND and ALS, by including ERVK proteinopathy as a novel mechanism of neuronal damage in these diseases. Consequently, quenching ERVK activity in these conditions may improve disease symptoms, and reverse viral-mediated pathology. This may be achieved through the use of anti-retroviral drugs, immunomodulatory regimens, and/or drugs that enhance autophagy.

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Author Contributions

All authors contributed to the study design and wrote the manuscript. R.N.D., M.M. and J.F.P performed the experiments and analysed the data. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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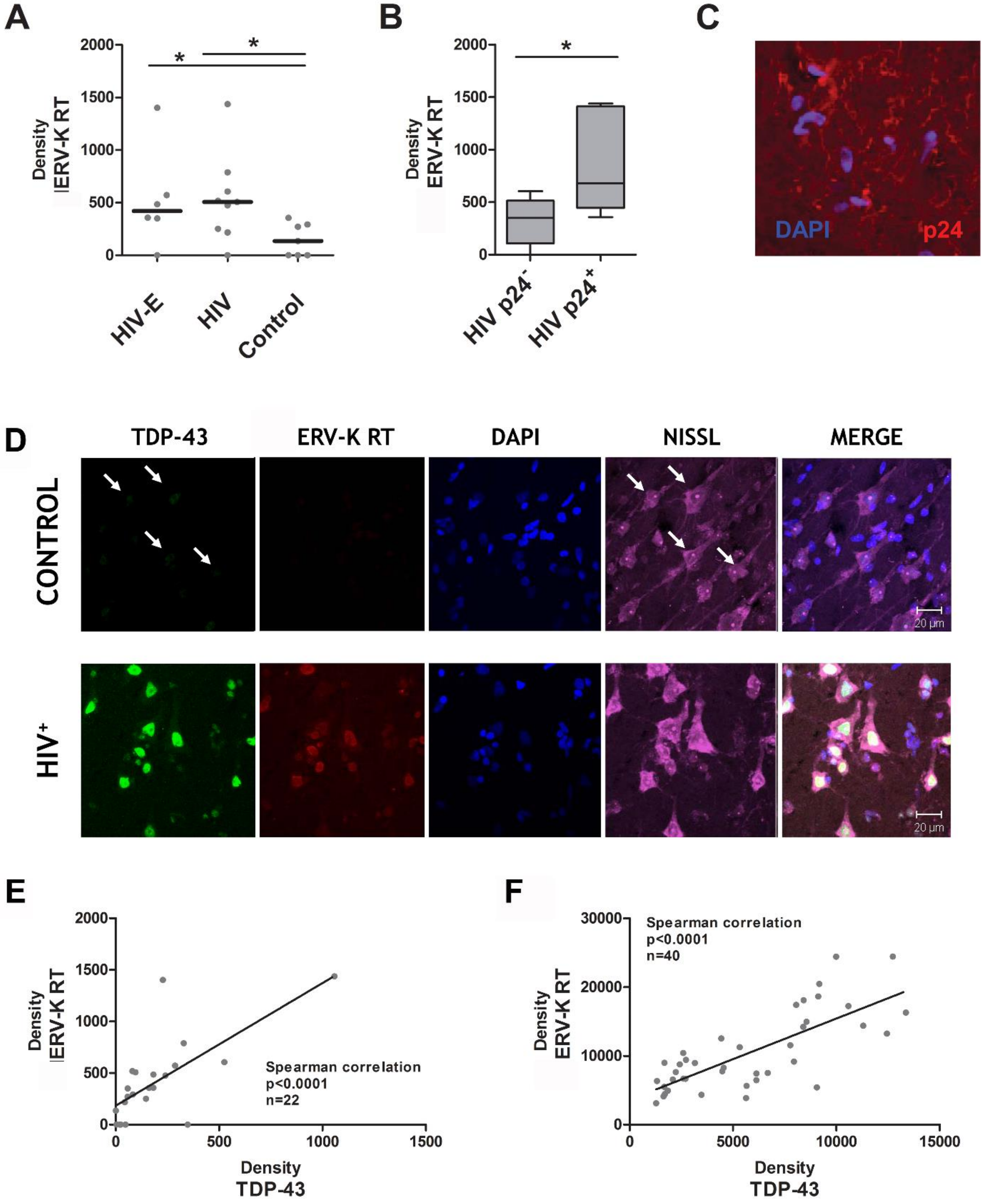

A
Density
ERV-K RT
2000
1500
1000
500
0
*
*
HIV-E
HIV
Control
B
Density
ERV-K RT
2000
1500
1000
500
0
*
HIV p24⁻
HIV p24⁺
C
DAPI
p24
D
TDP-43
ERV-K RT
DAPI
NISSL
MERGE
CONTROL
HIV⁺
20 μm
20 μm
E
Density
ERV-K RT
2000
1500
1000
500
0
0
500
1000
1500
Density
TDP-43
Spearman correlation
p<0.0001
n=22
F
Density
ERV-K RT
30000
20000
10000
0
0
5000
10000
15000
Density
TDP-43
Spearman correlation
p<0.0001
n=40

Figure 1: Human endogenous retrovirus-K reverse transcriptase is induced in cortical tissue during HIV infection. HIV-infected individuals, with HAND/HIV-encephalitis (HIV-E) or without HIV-E (HIV) expressed greater levels of human endogenous retrovirus-K (HERV-K) reverse transcriptase protein in their cortical tissue, as compared to patients deceased with chronic systemic disease (Control) (**A**). HIV replication in cortical tissue, as measured by HIV p24 protein immunostaining, is associated with significantly higher HERV-K reverse transcriptase expression (**B and C**). Mann-Whitney derived t-test, * $p<0.05$. Significant correlation of neuronal HERV-K reverse transcriptase and TDP-43 protein levels in HIV^+ patients. Representative immunohistochemistry images of TDP-43 protein, human endogenous retrovirus-K reverse transcriptase (HERV-K RT), nucleic as measured by DAPI staining and neurons as measured by Nissl staining in the cortical brain tissue of HIV infected patients (HIV^+) versus patients with systemic disease (Control) (**D**). ImageJ analysis was used to quantify the density of HERV-K RT and TDP-43 staining within individual tissue samples (**E**) and within individual neurons (**F**) of HIV infected and controls cortical brain specimens.

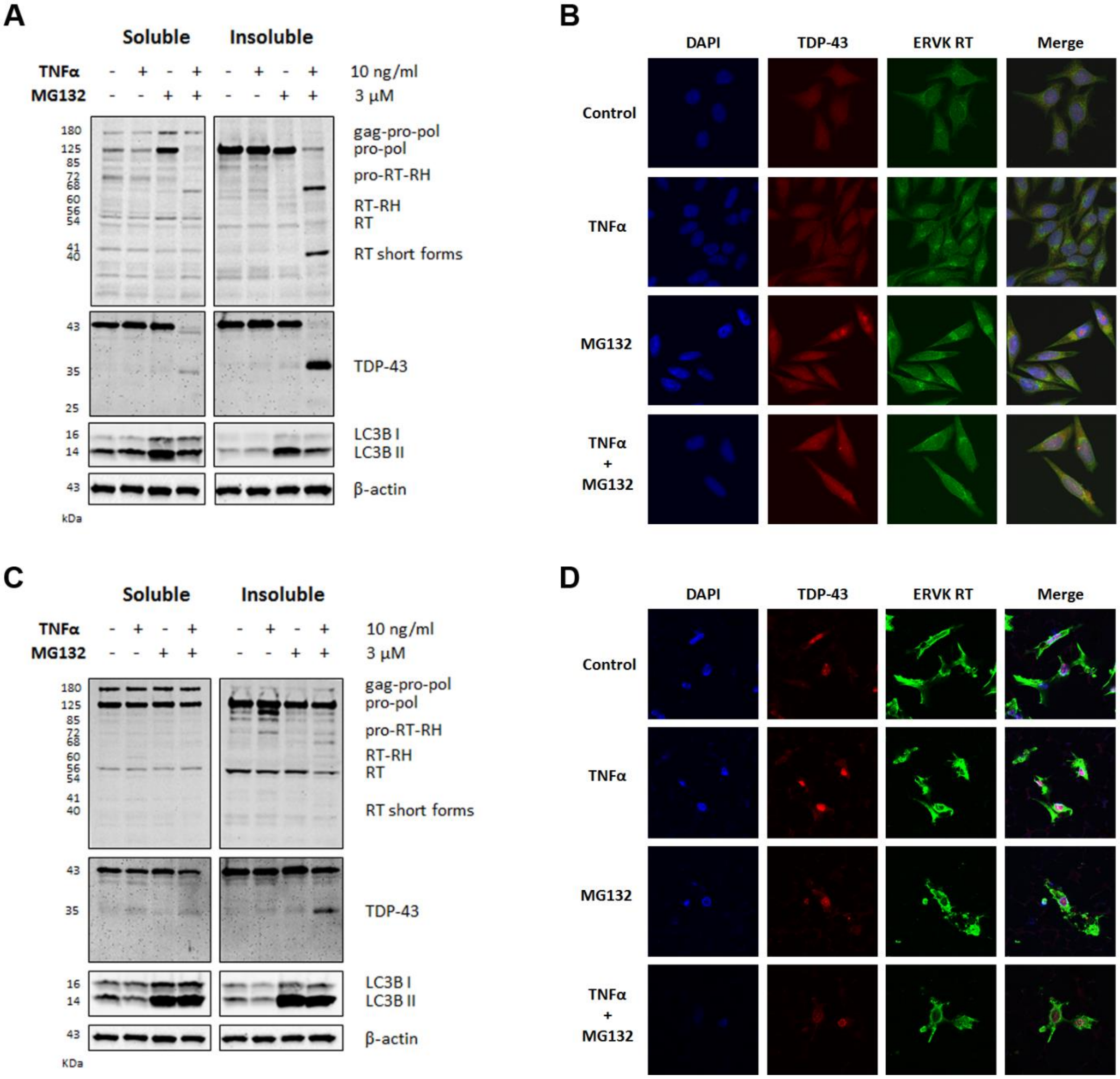
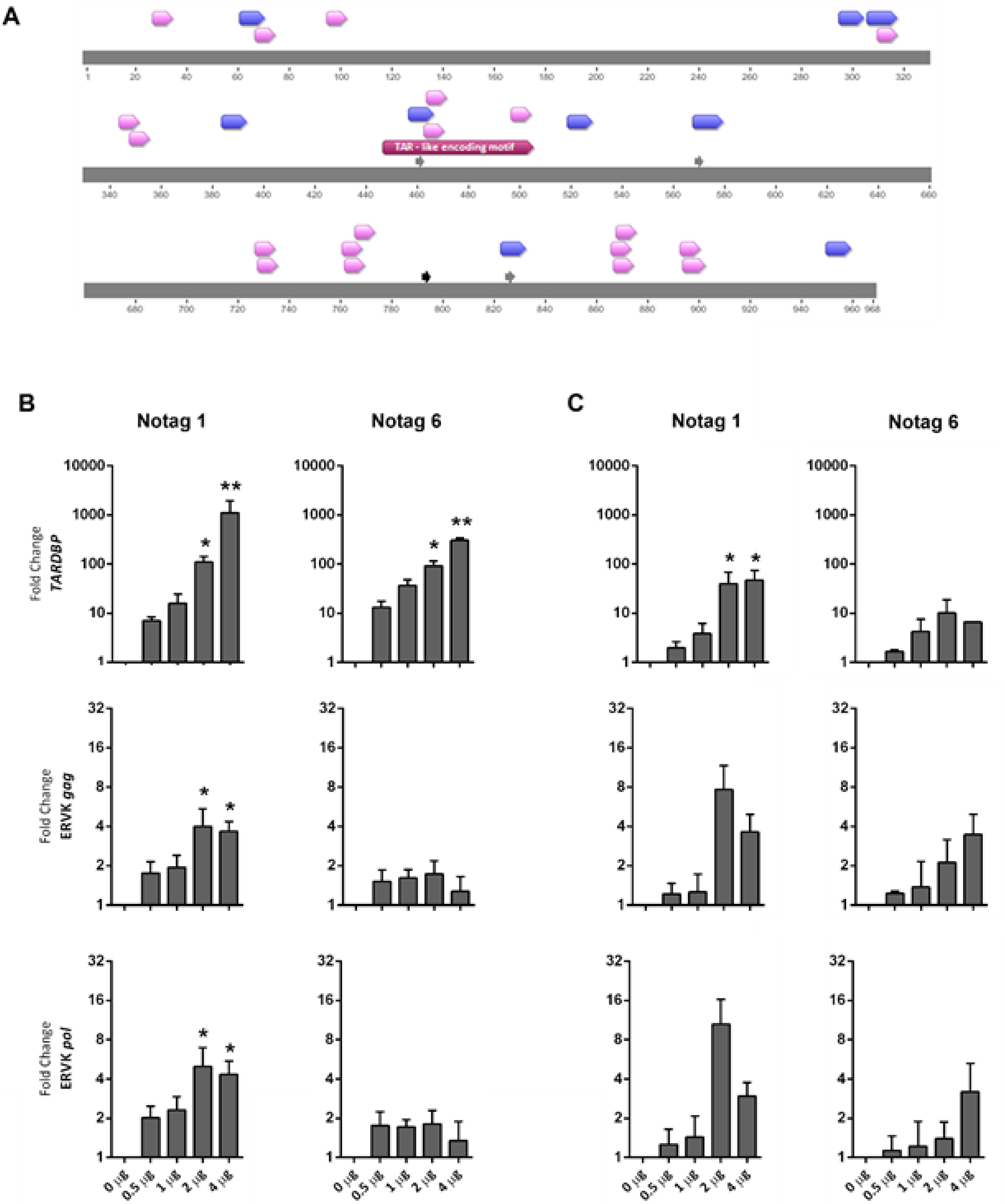

A
Soluble
Insoluble
TNFα - + - + - + - + 10 ng/ml
MG132 - - + + - - + + 3 μM
180
125
85
72
68
60
56
54
41
40
43
35
25
16
14
43
kDa
gag-pro-pol
pro-pol
pro-RT-RH
RT-RH
RT
RT short forms
TDP-43
LC3B I
LC3B II
β-actin
B
DAPI
TDP-43
ERVK RT
Merge
Control
TNFα
MG132
TNFα + MG132
C
Soluble
Insoluble
TNFα - + - + - + - + 10 ng/ml
MG132 - - + + - - + + 3 μM
180
125
85
72
68
60
56
54
41
40
43
35
16
14
43
KDa
gag-pro-pol
pro-pol
pro-RT-RH
RT-RH
RT
RT short forms
TDP-43
LC3B I
LC3B II
β-actin
D
DAPI
TDP-43
ERVK RT
Merge
Control
TNFα
MG132
TNFα + MG132

Figure 2: Astrocytes and neurons differentially clear ERVK protein accumulation. SVGA and ReNcell CX-derived neurons were treated with TNFα and MG132 individually or in combination for 24 hours (n=3). Western blot was performed on the soluble and insoluble protein fractions to detect alterations in ERVK RT, TDP-43, and LC3B protein levels relative to untreated cells. β-actin was used as the loading control. **(A)** In SVGA cells, TNFα had no discernible effect on ERVK RT expression. MG132-mediated proteasome inhibition dramatically enhanced ERVK polyprotein/RT levels. SVGA cells were able to clear ERVK and TDP-43 protein accumulation through autophagy, as indicated by enhanced LC3B-I cleavage into LC3B-II with TNFα and MG132 combination treatment**. (B)** Representative confocal micrographs of SVGAs treated with TNFα and/or MG132 (n=3), recapitulating MG132-mediated ERVK polyprotein/RT and TDP-43 aggregation. ERVK protein aggregates deposited proximal to the nucleus. TNFα slightly enhanced ERVK deposition, which was not evident in western blot. SVGA cells treated with a combination of TNFα and MG132 exhibited less ERVK polyprotein/RT expression and aggregation. **(C)** Unlike SVGAs, TNFα, but not MG132 treatment of neurons, enhanced ERVK RT expression. Neurons were also unable to degrade ERVK polyprotein/RT through autophagy, although LC3B cleavage was observed (n=3). **(D)** These findings were also confirmed using confocal microscopy, as ERVK polyprotein/RT aggregation persisted in neurons regardless of TNFα and MG132 dual treatment (n=3).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100
120
140
160
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200
220
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260
280
300
320
TAR - like encoding motif
340
360
380
400
420
440
460
480
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520
540
560
580
600
620
640
660
680
700
720
740
760
780
800
820
840
860
880
900
920
940
960
968
B
C
Notag 1
Notag 6
Notag 1
Notag 6
Fold Change TARDBP
Fold Change ERVK gag
Fold Change ERVK pol
10000
1000
100
10
1
32
16
8
4
2
1
*
**
0 µg
0.5 µg
1 µg
2 µg
4 µg

Figure 3. TDP-43 is a transcriptional activator of ERVK. Depiction of TDP-43 (pink) and κB (purple) binding sites on a consensus ERVK LTR, as predicted by Promo software (**A**). The sequence of the HIV-1 TAR-DNA element was used to identify a potential TAR-like encoding motif spanning the nucleotides 448 to 505 within the consensus ERVK 5′ LTR. Conventional and three alternative transcriptional start sites are depicted by black and grey arrows, respectively. Wild-type TDP-43 drives ERVK expression to a greater extent in neurons than in astrocytes (**B and C**). SVGA and ReNcell CX-derived neurons were transfected with various doses of plasmids encoding wild-type (Notag 1 construct) or truncated TDP-43 (Notag 6 construct) for 24 hours (n=3). Q-PCR was performed on RNA extracts using SYBR green detection. ΔΔCt method was used to calculate fold change in TARDPBP (TDP-43 gene), ERVK *pol* and ERVK *gag* transcription relative to untransfected negative control. 18s rRNA was used as the endogenous control. Western blot was also performed on the soluble and insoluble protein fractions to detect alterations in ERVK RT and TDP-43 levels relative to untreated cells. Over-expression of wild-type TDP-43 dose-dependently enhanced ERVK *gag* and *pol* transcription in both astrocytes (*p<0.05 **p<0.01; n=3) (**B**) and neurons (n=2) (**C**). In contrast, mutated TDP-43 failed to significantly enhance ERVK transcription in astrocytes (*p<0.05 **p<0.01; n=3) (**B**) and in neurons (n=2) (**C**).

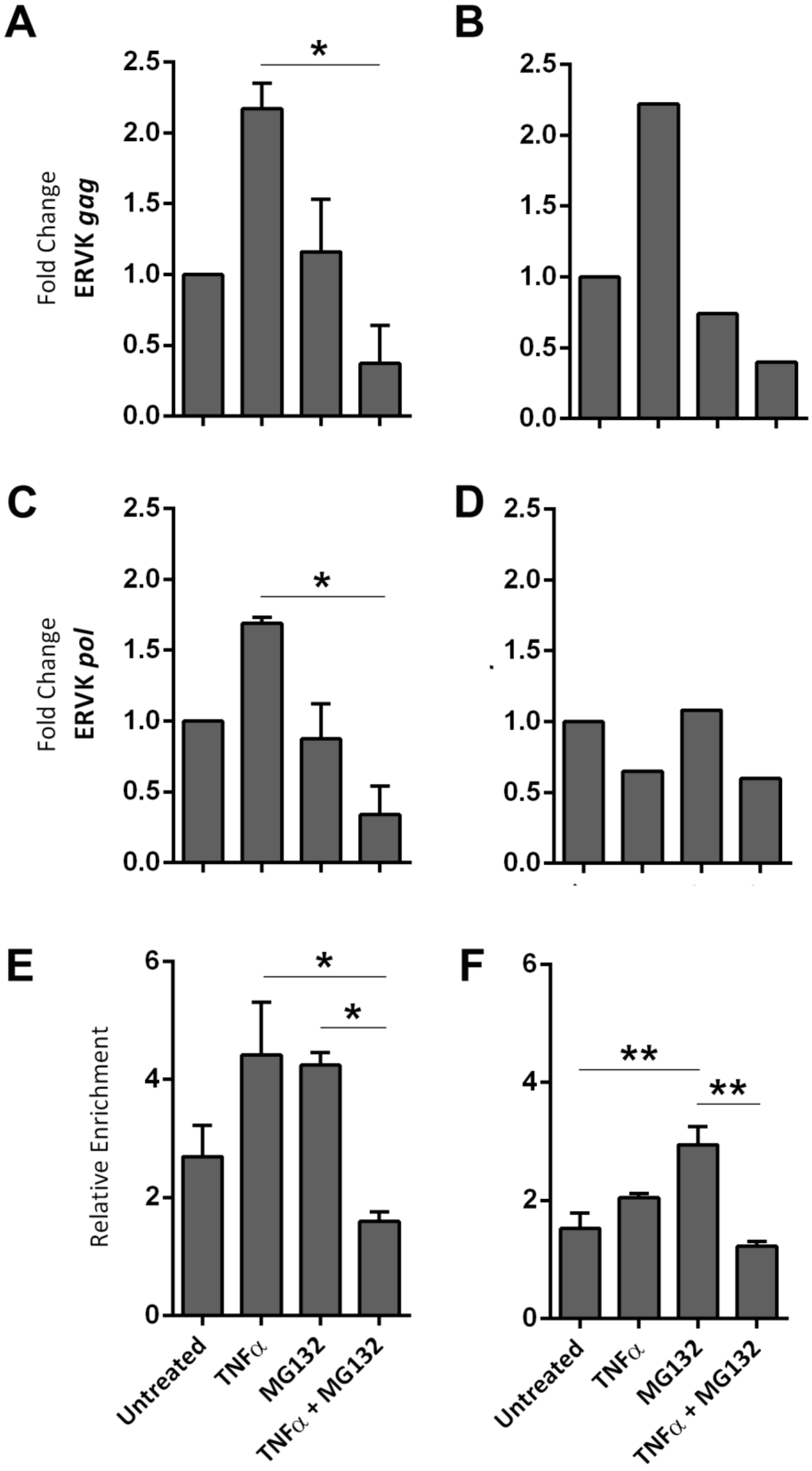
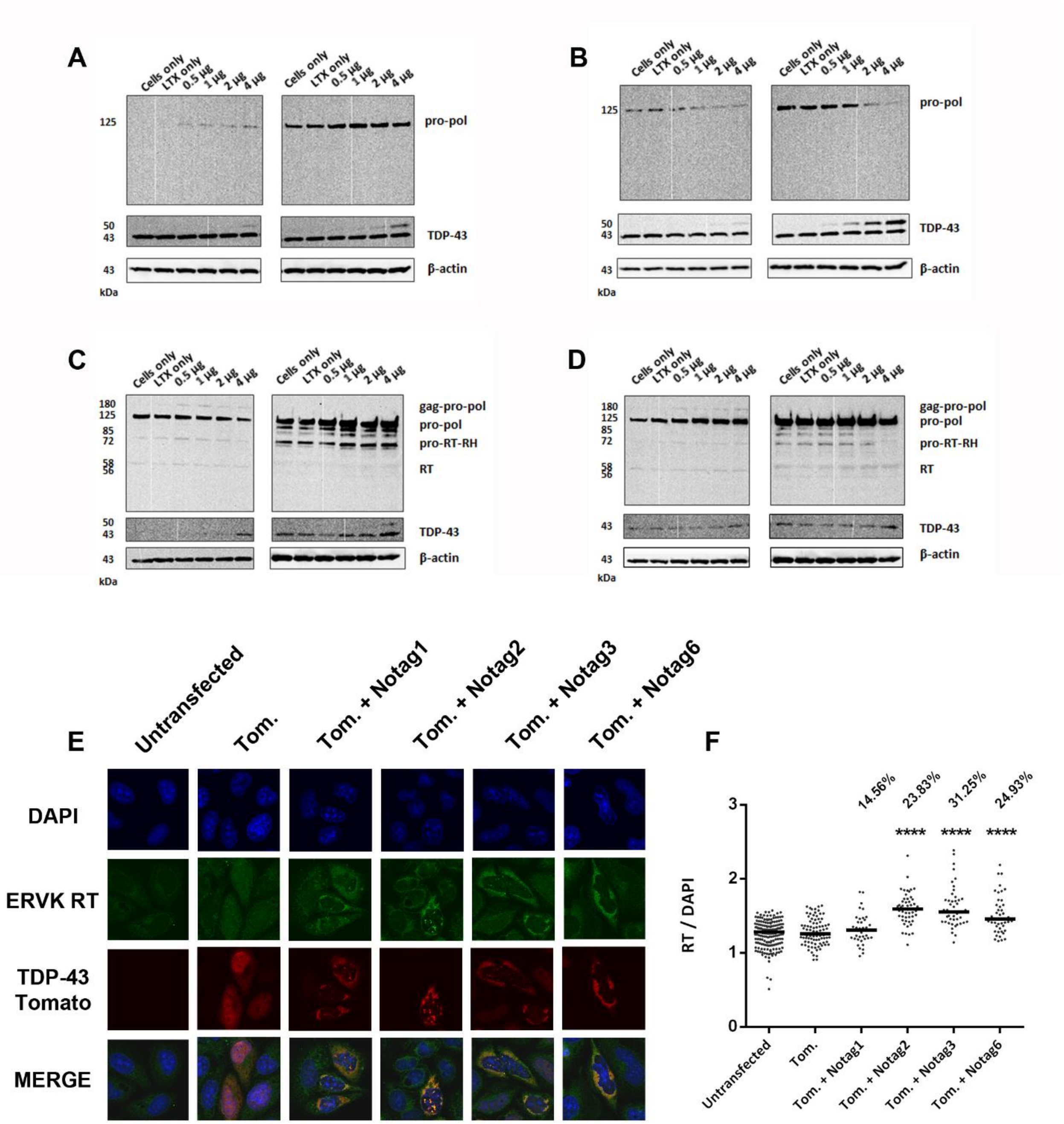

A
B
C
D
E
F
Fold Change ERVK *gag*
Fold Change ERVK *pol*
Relative Enrichment
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
*
**
Untreated
TNFα
MG132
TNFα + MG132

Figure 4. TDP-43 binds the ERVK LTR. Q-PCR was also performed on whole cell RNA extracts to determine alterations in ERVK *gag* and *pol* transcription relative to untreated cells using ΔΔCt method. 18s rRNA was used as the endogenous control. Decreased binding of TDP-43 to the ERVK promoter coincided with a reduction in ERVK transcription in astrocytes (*p<0.05; n=3) **(A, C, E)** and neurons (p values; n=1) **(B, D, F)**. Chromatin isolated from TNFα and/or MG132 treated SVGAs and ReNcell CX-derived neurons was subjected to chromatin immunoprecipitation with anti-human TDP-43 antibody or IgG antibody control **(E and F)**. QPCR was performed on immunoprecipitated DNA to amplify ERVK 5′LTR using SYBR Green detection. For each condition, fold Enrichment in TDP-43 binding was calculated relative to the input first and then IgG control. TNFα as well as MG132 treatment of SVGAs **(E)** and MG132 treatment of neurons **(F)** led to enhanced binding of TDP-43 to the ERVK promoter, which was abolished with TDP-43 cleavage during dual treatment of cells with TNFα and MG132 (*p<0.05 **p<0.01; n=3).


A
Cells only
LTX only
0.5 µg
1 µg
2 µg
4 µg
125
pro-pol
50
43
TDP-43
43
β-actin
kDa
B
Cells only
LTX only
0.5 µg
1 µg
2 µg
4 µg
125
pro-pol
50
43
TDP-43
43
β-actin
kDa
C
Cells only
LTX only
0.5 µg
1 µg
2 µg
4 µg
180
125
85
72
58
56
gag-pro-pol
pro-pol
pro-RT-RH
RT
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TDP-43
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0.5 µg
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gag-pro-pol
pro-pol
pro-RT-RH
RT
43
TDP-43
43
β-actin
kDa
E
Untransfected
Tom.
Tom. + Notag1
Tom. + Notag2
Tom. + Notag3
Tom. + Notag6
DAPI
ERVK RT
TDP-43 Tomato
MERGE
F
14.56%
23.83%
31.25%
24.93%
****
****
****
RT / DAPI
3
2
1
0
Untransfected
Tom.
Tom. + Notag1
Tom. + Notag2
Tom. + Notag3
Tom. + Notag6

Figure 5. Wild-type and mutant forms of TDP-43 significantly differ in their capacity to enhance ERVK RT protein aggregation. SVGAs (**A and B**) and ReNcells (**C and D**) were transfected with either wild-type (Notag 1) (**A and C**) or mutant TDP-43 (Notag 6) constructs (**B and D**). Western blot was performed on the soluble (left) and insoluble (right) protein fractions to detect alterations in ERVK RT and TDP-43 protein levels relative to untreated and untransfected cells. β-actin was used as the loading control. **(A, B)** In SVGA cells, overexpression of wild-type TDP-43 enhanced ERVK polyprotein expression, whereas overexpression the C-terminal truncated TDP-43 mutant reduced ERVK expression. **(C, D)** In stark contrast, wild-type TDP-43 promoted insoluble ERVK polyprotein expression, and the Notag6 construct enhanced the cytoplasmic availability of the ERVK polyprotein. **(E,F)** SVGA cells were transfected with a combination of fluorescently-tagged wild-type TDP-43 indicator plasmid (TDP-43 tomato) and constructs encoding wild-type (Notag 1) or mutant forms of TDP-43 (Notag 2, Notag 3, or Notag 6). 24 hours post-transfection, cells were fixed with methanol and immunostained using anti-human ERVK RT antibody. Cells were counterstained with DAPI. Confocal micrographs were acquired using Olympus Fluoview confocal microscopy suite. Untransfected cells or those transfected with TDP-43 tomato only were used as negative controls. **(E)** Representative micrographs depict that over-expression of mutant TDP-43 markedly enhanced ERVK RT expression and aggregation, as compared to wild-type TDP-43. Cytosolic TDP-43 aggregates co-localized with ERVK RT aggregates (n=3). **(F)** ERVK RT expression was also quantified in these cells and normalized to DAPI staining. Only mutant forms of TDP-43 were found to significantly enhance ERVK RT expression and aggregation, with N-terminal truncated TDP-43 fragments (Notag 2 and 3) conferring the greatest effect on ERVK accumulation (****$p<0.0001$; n=3).

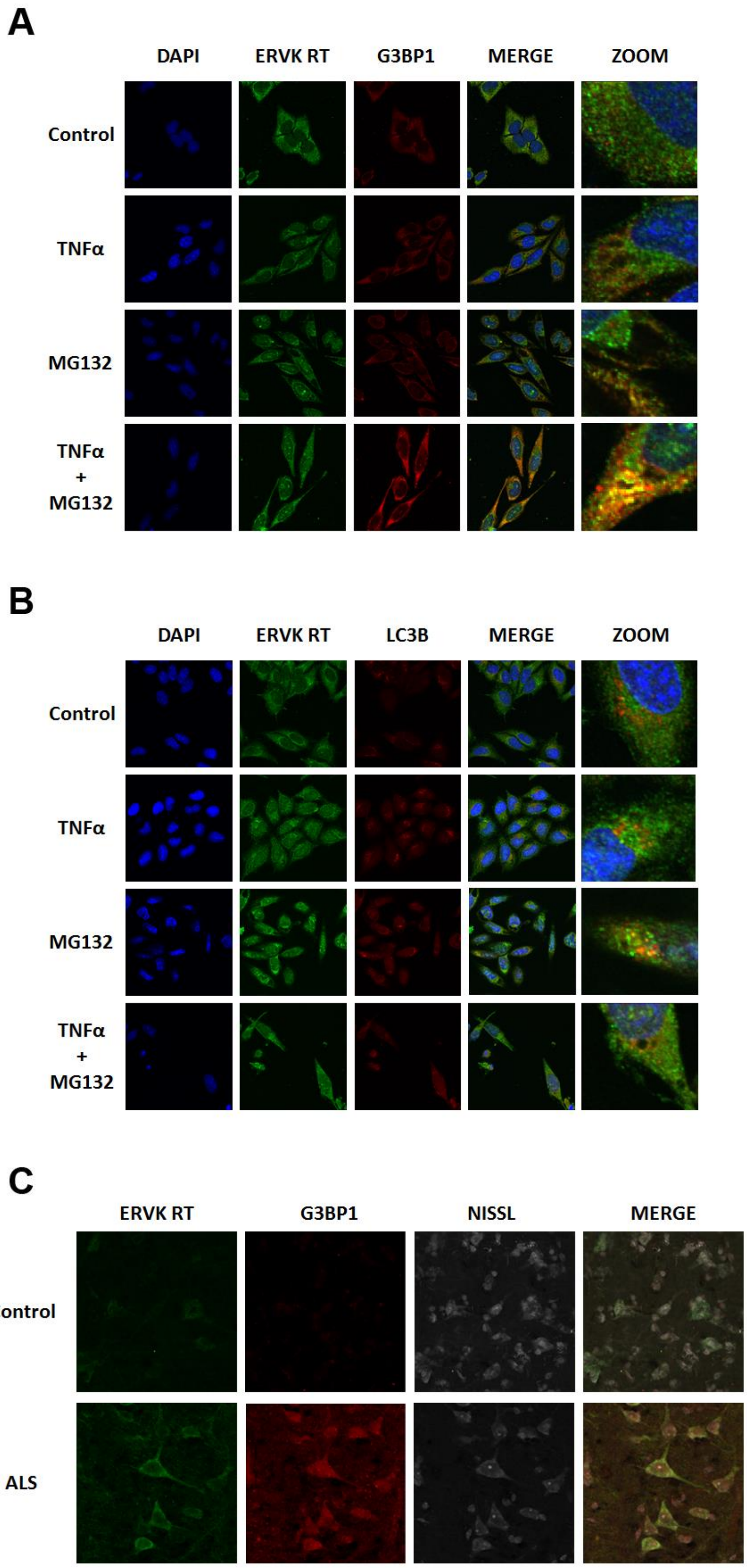
A
DAPI
ERVK RT
G3BP1
MERGE
ZOOM
Control
TNFα
MG132
TNFα
+
MG132
B
DAPI
ERVK RT
LC3B
MERGE
ZOOM
Control
TNFα
MG132
TNFα
+
MG132
C
ERVK RT
G3BP1
NISSL
MERGE
Control
ALS

Figure 6. ERVK is localized to G3BP1$^+$ stress granules. SVGA cells were treated with TNFα (10 ng/ml) and/or MG132 (3uM) for 24 hours, methanol-fixed, and immunostained for ERVK RT, G3BP1, or LC3B (n=1). **(A)** Representative confocal micrographs depict markedly enhanced co-localization of ERVK RT and G3BP1 in astrocytes treated with a combination of TNFα and MG132. **(B)** In comparison, ERVK RT did not co-localize as strongly with the autophagy marker LC3B, suggesting specific shuttling of ERVK RT to stress granules**. (C)** Representative confocal micrographs of Brodmann's area 6 motor cortex from a patient with ALS and a neuro-normal control. Cortical neurons in ALS tissues exhibited marked increase in G3BP1 levels concomitantly with enhanced ERVK RT levels. However, a lack of co-localization between ERVK RT and G3BP1 was evident, and may represent viral evasion strategy to escape degradative processes.

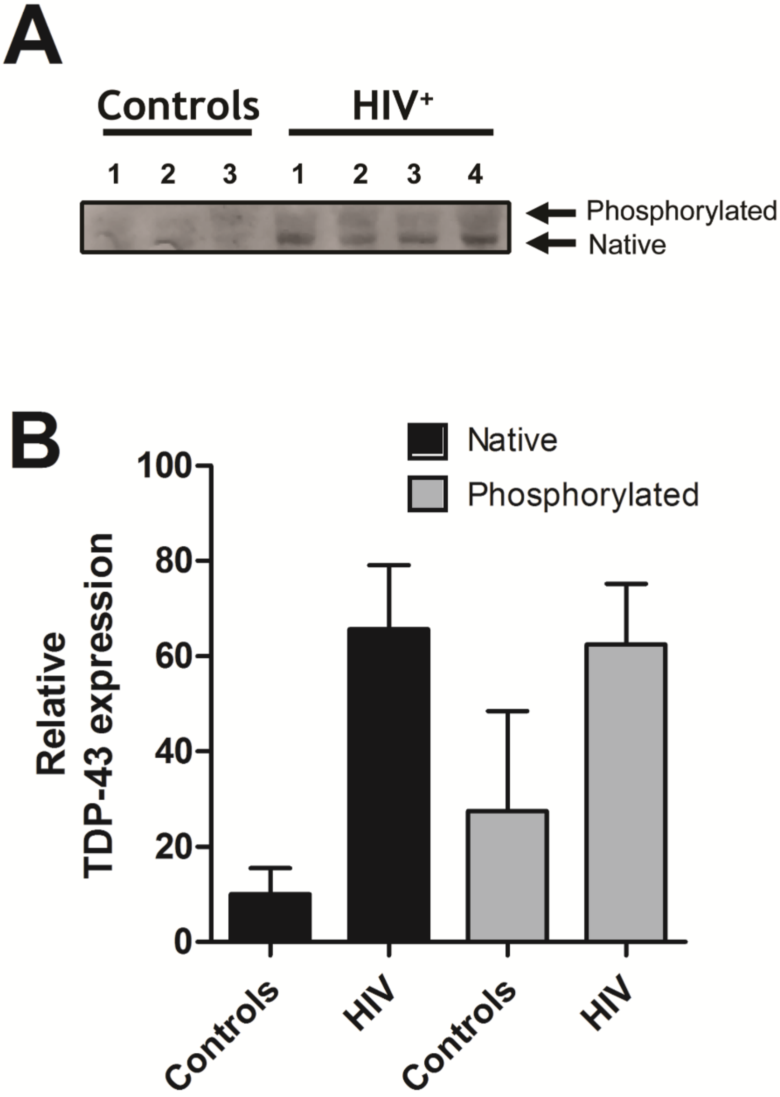
A
Controls
HIV+
1
2
3
1
2
3
4
Phosphorylated
Native
B
Native
Phosphorylated
Relative TDP-43 expression
100
80
60
40
20
0
Controls
HIV
Controls
HIV

Figure S1: Neuronal TDP-43 is over-expressed and phosphorylated during HIV infection. Western blot analysis of whole cell lysates from cortical brain tissue reveals increased native and phosphorylated TDP-43 expression in HIV infected patient samples (1-4) as compared to controls (1-3) (**A**). Densitometry measurements of the immunoblot bands confirm that both native and phosphorylated TDP-43 levels are enhanced in cortical tissue specimens from HIV positive individuals as compared to chronic systemic disease controls (**B**).

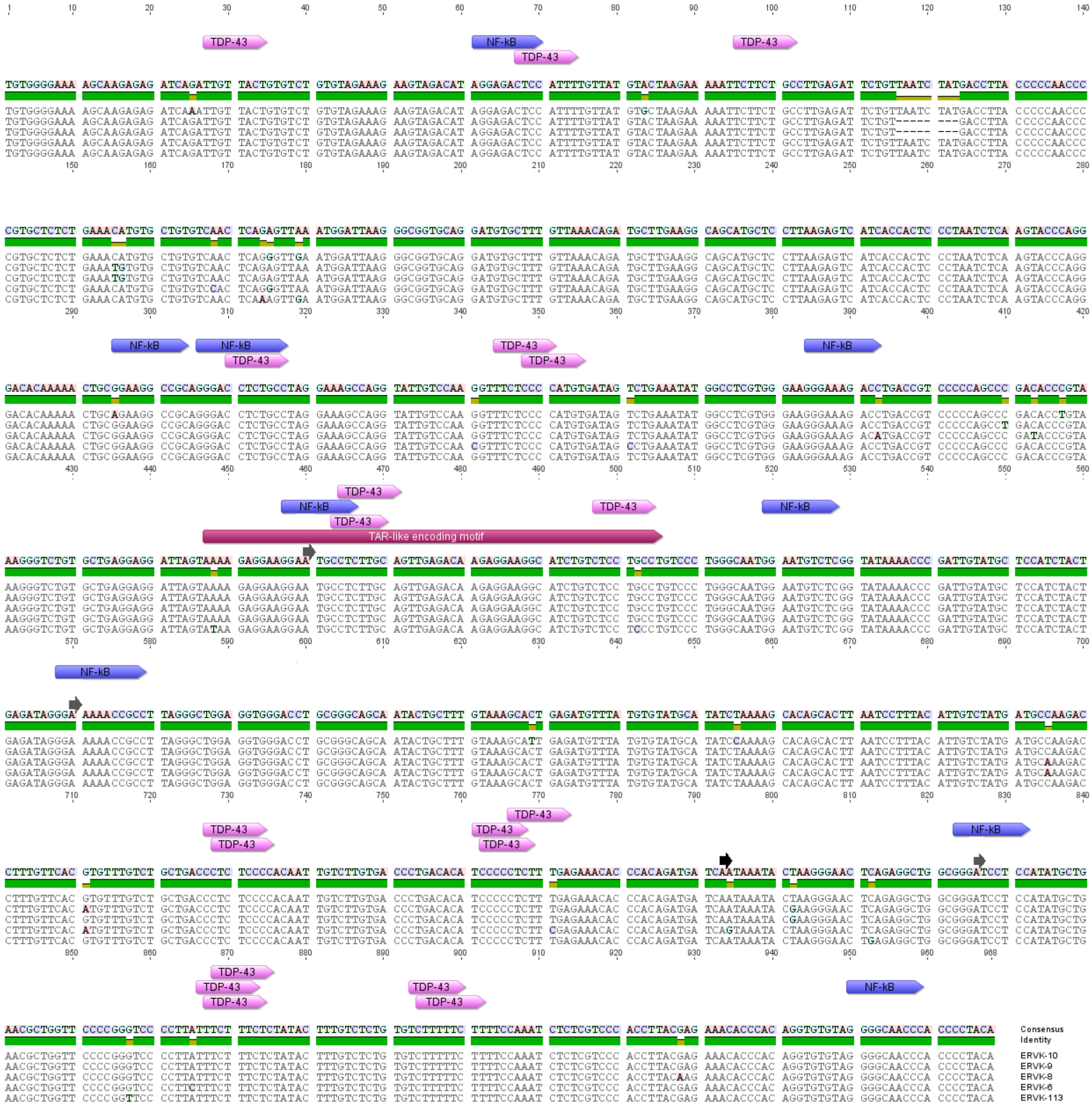
TDP-43
NF-kB
TAR-like encoding motif
Consensus
Identity
ERVK-10
ERVK-9
ERVK-8
ERVK-6
ERVK-113

Figure S2: TDP-43 and κB sites within the ERVK LTR, as predicted by Promo software. *In silico* examination of the conserved TDP-43 binding sites within five prototypic ERVK 5′ LTRs. The ERVK 5′ LTR consensus sequence was constructed using individual ERVK LTRs in the following order (GenBank accession numbers in brackets): ERVK-10 (M12854.1), ERVK-9 (former HERV-K109) (AF164615.1), ERVK-8 (former HERV-K115) (AY037929.1), ERVK-6 (former HERV-K108) (AF074086.2) and ERVK-113 (JF742069.1). The sequences of the TDP-43 DNA binding sites were obtained from [38]. The sequence of the HIV-1 TAR-DNA element was obtained from GenBank (accession number AM076891.1) and used to identify a potential TAR-like encoding motif spanning the nucleotides 448 to 505 within the consensus ERVK 5′ LTR. Conventional (793bp) and three alternative (460, 570, and 826 bp) transcriptional start sites are depicted by black and grey arrows, respectively [14]. Sequence alignment and annotation were performed using Geneious software [75].

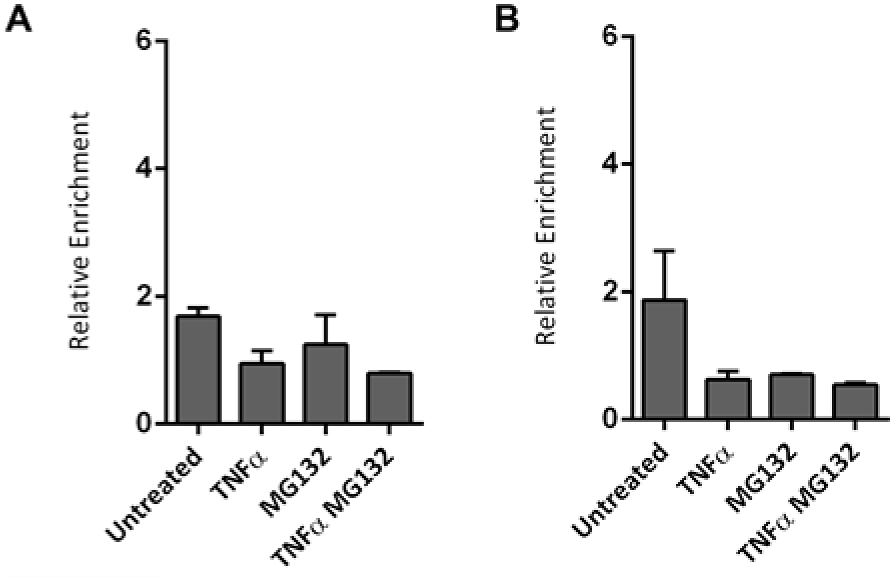
A
Relative Enrichment
0
2
4
6
Untreated
TNFα
MG132
TNFα MG132
B
Relative Enrichment
0
2
4
6
Untreated
TNFα
MG132
TNFα MG132

Figure S3. **No perceivable change was observed in TDP-43 binding to the RIG-I promoter.** Chromatin isolated from TNFα and/or MG132 treated SVGAs **(A)** and ReNcell CX-derived neurons **(B)** was subjected to chromatin immunoprecipitation with anti-human TDP-43 antibody or IgG antibody control. QPCR was performed on immunoprecipitated DNA to amplify the RIG-I promoter (negative control) using SYBR Green detection. For each condition, fold enrichment in TDP-43 binding was calculated relative to the input first and then IgG control.

Table 1: Cortical Brain Tissue Specimens

Case	Brain Bank	Diagnosis	Tissue	Age (yrs)	Gender	PMI	CD4	Plasma viral load	CSF viral load
CB164	CNTC	HIV+	Parietal lobe	35	M	5	211	2827	78
CC128	CNTC	HIV+	Parietal lobe	47	M	12	306	4953	ND
CE132	CNTC	HIV+/HIV-E	Parietal lobe	39	M	7	26	ND	0
7101868276	TRANR	HIV+/HIV-E	Parietal cortex	41	M	7.6	37	>500000	>55695
7100616568	TRANR	HIV+/HIV-E	Parietal cortex	32	M	16.2	77	489796	3041500
7101847783	TRANR	HIV+	Parietal cortex	59	M	11.3	8	54439	735
7101997784	TRANR	HIV+	Parietal cortex	41	M	15.3	76	711	644
7100928374	TRANR	HIV+	Parietal cortex	38	F	10.8	3	104358	93
7100598287	TRANR	HIV+	Parietal cortex	50	M	13	121	<50	<10
1093	NNTC	HIV+/possible HAD	Parietal cortex	56	M	6	365	25669	ND
2005	NNTC	HIV+	Parietal cortex	44	F	3	66	>75000	249
2012	NNTC	HIV+/HIV-E	Parietal cortex	49	M	16	273	ND	<50
5007	NNTC	HIV+/possible HAD/HIV-E	Parietal cortex	37	F	3	91	>75000	ND
5008	NNTC	HIV+/possible HAD/HIV-E	Parietal cortex	53	M	7	128	8641	54061
6081	NNTC	HIV+/probable HAD	Parietal cortex	35	M	2	ND	ND	ND
3565	HBSFRC	Cardiomyopathy	Cortex	76	M	11			
27	RMMSC	Coronary artery disease	Cortex	69	M	6.5			
712	JHSMBB	Unknown	Prefrontal cortex	44	F	20			
23895	JHSMBB	Unknown	Occipital cortex	ND	ND	ND			
000	JHSMBB	Unknown	Motor cortex	ND	ND	ND			
33	RMMSC	Pulmonary disease	Cortex	69	M	4.5			
795	JHSMBB	Huntington's disease	Sensory Cortex	48	F	9			
2776	NBB	ALS	BA6 Motor Cortex	76	F	8.6			
5215	NBB	ALS	BA6 Motor Cortex	59	M	12.5			
5187	NBB	ALS	BA6 Motor Cortex	69	M	13.2			
4660	NBB	Cancer	BA6 Motor Cortex	73	F	18.5			
3371	NBB	Cancer	BA6 Motor Cortex	52	M	16			
4514	NBB	Cancer	BA6 Motor Cortex	66	M	17.3			

Tissue specimens were obtained from the California NeuroAIDS Tissue Consortium (CNTC), the Texas Repository for AIDS Neuropathogenesis Research (TRANR), the National NeuroAIDS Tissue Consortium (NNTC), the Human Brain and Spinal Fluid Resource Center (HBSFRC), the Rocky Mountain MS Center (RMMSC), the Johns Hopkins School of Medicine Brain Bank (JHSMBB) and the NIH Neurobiobank (NBB). Post-mortem interval (PMI) is indicated in hours. Brodmann’s area 6 (BA6). No data (ND).

4. DISCUSSION

Aberrant ERVK transcription and protein expression in the CNS has been implicated in the pathogenesis of several neuroinflammatory diseases, including ALS and HAND. However, the mechanisms that promote ERVK transcription and viral protein accumulation in the context of neuroinflammation have not been comprehensively analyzed. Thus, the overarching goal of this work was to elucidate the cellular pathways responsible for ERVK re-activation in these associated neurological diseases, as well as to validate findings from our *in vitro* models by examining biomarkers in *ex vivo* human autopsy tissues.

4.1 | Transcriptional regulation of ERVK

4.1.1 | ERVK promoter contains functional ISREs, which bind NF-κB and IRF1 to enhance ERVK gene transcription during neuroinflammation

Several lines of evidence suggest that augmented levels of TNFα, LIGHT, and IFNγ cytokines drive enhanced activity of pro-inflammatory transcription factors (TFs) in neurological diseases, which may be important triggers of ERVK transcription in the CNS[19,59,68]. We have previously examined ERVK (HML-2) 5′ LTRs *in silico* and identified conserved putative binding sites for IRF1 and NF-κB, including two Interferon Stimulated Response Elements (ISREs)[68]. However, the biological functionality of these predicted ISREs has not been elucidated. We therefore set out to determine whether the aforementioned cytokines can enhance ERVK gene transcription and protein expression in astrocytes and neurons, and whether this effect is mediated by increased interactions of NF-κB and/or IRF1 with the ERVK 5′ LTR.

For the first time, we have utilized chromatin immunoprecipitation (ChIP) to showcase that the ERVK 5′ LTR harbors two conserved functional ISREs which can interact with pro-inflammatory TFs NF-κB and IRF1. Both astrocytes and ReNcell CX-derived neurons exhibited basal NF-κB and IRF1 binding to the ISREs in ERVK 5′ LTRs, which alludes to the basal ERVK expression in these cells (**Publication 3; Figure 3**). Under optimal stimulating conditions, LIGHT-treated astrocytes and TNFα-treated neurons exhibited markedly enhanced NF-κB p50 and p65 binding to each ISRE in the ERVK promoter (**Publication 3; Figure 3**). Transcription factor binding was associated with increased ERVK polyprotein and RT expression in these cells (**Publication 3; Figures 1 and 2**). Although the binding of IRF1 to the ISREs considerably increased with cytokine stimulation (9 and 7 fold in SVGAs and neurons, respectively), it did not reach statistical significance with three experimental replicates. Together, these findings suggest that NF-κB and IRF1 binding to the ERVK 5′ LTR has an important role in transcriptional re-activation of this provirus in the context of neuroinflammation.

A caveat of our ChIP experiments was the lack of a promoter deficient in IRF1 and/or NF-κB binding sequences to serve as the negative control. This is because the human genome is extensively laden with binding sites for these TFs[113,114]. For instance, there are 14,000 estimated NF-κB binding sites in the human genome[114]. There are also numerous NF-κB responsive genes in a single cell type; for example, human pancreatic cells alone contain over 500 genes targeted by this TF[115]. Additionally, NF-κB binding is not restricted to promoter regions, as a significant level of binding has been detected in intronic regions with ChIP experiments[116]. Likewise, we also observed enrichment of NF-κB and IRF1 proteins in DNA regions other than promoters, such as the ERVK *pol* gene (**Publication 3, Figure S2**). Thus, it is inherently difficult to identify IRF1 and/or NF-κB deficient regions of the human genome that may be used as negative controls in ChIP Q-PCR.

Moreover, the interaction of transcription factors with their cognate sites on DNA is a very complex and a dynamic process, and not all binding events lead to functional outcomes[117–119]. For instance, cooperativity between TFs is one of the crucial determinants of a successful transcriptional output at a given promoter[117–119]. IRF1 and NF-κB-responsive human and viral gene promoters are no exception, as synergy between IRF-1 and NF-κB is required to induce the transcription from human inducible nitric oxide synthase, interleukin-15, and interferon β promoters, as well as from the HIV-1 5′ LTR[65]. Accordingly, partially overlapping or adjacent IRF1 and NF-κB binding sites have been described at these promoters[65,120]. The ERVK 5′ LTR also harbours similar binding sequence arrangement for these TFs (**Publication 3, Figure 3A**). Cooperativity between TFs can activate a given promoter because co-bound TFs can recruit common cofactors, such as p300/CBP and TFIID, leading to the formation of transcription initiation complexes[117]. The protein-protein interactions between transcription factors can further stabilize transcription initiation complexes, leading to an optimal effect on target gene expression[117–119]. IRF1 and NF-κB binding to their respective sites in the interferon β promoter is known to form a stable nucleoprotein complex, called an enhanceosome[121]. IRF1 and NF-κB cooperatively recruit p300/CBP to the enhanceosome, which leads to synergistic transcriptional activation of the interferon β gene. In line with these findings, herein we show that the binding of NF-κB p65/p50 and IRF1 to the ERVK promoter is simultaneously required to synergistically enhance proviral transcription. This is because transient transfections of astrocytes with plasmids encoding active NF-κB isoforms and IRF1 produced a significant 70 fold increase in ERVK pol RNA levels only when these transcription factors were co-expressed, and not when overexpressed individually (**Publication 3, Figure 3B**). This data supports the notion that a combination of NF-κB p65/p50 and IRF1 transcription factors can work in concert on the ERVK 5′ LTR to optimally drive the transcriptional re-activation of ERVK.

Pro-inflammatory cytokine-mediated induction of ERVK also translates to the protein level in astrocytes and neurons, as TNFα, LIGHT, and IFNγ dose-dependently enhance ERVK polyprotein/RT levels, albeit in a cell-type specific manner. IFNγ was able to induce ERVK polyprotein/RT expression equally well in both astrocytes and neurons (**Publication 2, Figures 1, 2, and 3**). TNFα increased ERVK protein levels most prominently in neurons (**Publication 3, Figure 2**), whereas LIGHT was best able to induce ERVK in astrocytes (**Publication 3, Figure 1**). This effect can be explained by differential enrichment of NF-κB at the ISREs in the ERVK promoter during TNFα or LIGHT stimulation of astrocytes and neurons. TNFα, but not LIGHT, significantly increased NF-κB p65 and p50 protein levels as well as their interaction with the ISREs on the ERVK promoter in neurons (**Publication 3, Figures 2 and 3 E-F**). In stark contrast, LIGHT, but not TNFα, significantly enhanced NF-κB p65 and p50 binding to the ISREs in astrocytes (**Publication 3, Figure 3 C-D**).

Such cell-type specificity of TNF superfamily cytokines, TNFα and LIGHT, may also be explained by differential expression of their cognate cell surface receptors, as well as downstream signaling molecules in astrocytes and neurons. TNFα is known to be biologically active in both transmembrane as well as soluble forms[122,123]. Soluble TNFα mainly signals through TNF receptor 1 (TNFR1)[122], which is found at a lower level in astrocytes as compared to neurons (The Human Protein Atlas). Overproduction of soluble TNFα has been shown to cause neurodegeneration in the CNS[123]. Trans-membrane TNFα on the other hand mainly signals through TNFR2, which is primarily found in microglial cells[122,101]. Since, we utilized soluble TNFα in our cell line models, it is not surprising that neurons, but not astrocytes, were more responsive to this cytokine. Adaptor molecules that associate with TNF receptors, known as TRAFs, exert a second layer of control over cell-specific TNFα and LIGHT signaling. TRAF3 is basally expressed in neurons, but not in glial cells (The Human Protein Atlas), and has been shown to be much more inducible in neurons as compared to astrocytes[124]. TRAF3 is a negative regulator of LIGHT signaling as it inhibits the function of LTβ receptor, which results in NF-κB inactivity[124]. In contrast, TRAF3 has no effect on TNFα-induced NF-κB signaling[124]. Neuronal expression of TRAF3 may have inhibited LIGHT-induced NF-κB signaling, leading to a lack of any perceivable effect on ERVK expression in our neuronal models.

In order to further validate our *in vitro* findings, we have utilized fluorescent confocal microscopy to determine whether the cortical brain tissue from patients with ALS exhibits increased IRF1/NF-κB nuclear localization in ERVK$^+$ neurons as compared to neuro-normal controls. The cortical neurons in ALS brain tissues indeed show enhanced nuclear translocation of IRF1 and NF-κB p50, and to a lesser extent p65, as compared to the controls (**Publication 3, Figure 5**). Nuclear translocation of these TFs correlated with enhanced ERVK RT expression in cortical neurons. Overall, ALS-associated pro-inflammatory cytokines are likely responsible for ERVK re-activation in the cortical neurons of patients with this neurodegenerative disorder.

It is now well established that exacerbated TNFα, LIGHT, and IFNγ signaling pathways in the CNS converge at NF-κB and/or IRF1 dependent neuronal damage[51]; however, the exact mechanism by which these pro-inflammatory transcription factors promote neuronal death remains unclear. Our findings suggest that ERVK re-activation in neurons triggered by the synergistic action of NF-κB and IRF1 may serve as the link between exacerbated pro-inflammatory cytokine signaling and neuronal damage. The detection of ERVK nucleic acids and proteins by innate immune sensors may drive anti-retroviral responses and further NF-κB/IRF1 activation, culminating in the production of pro-inflammatory cytokines by astrocytes, glial cells, and infiltrating T cells. This may create a feed forward loop, generating repetitive cycles of NF-κB/IRF1-induced ERVK expression and inflammatory response against ERVK-expressing neurons, leading to neuronal injury. Previously, the envelope protein of ERVW has been demonstrated to trigger innate immune signaling and the secretion of pro-inflammatory cytokines, driving NF-κB activation[125]. This TF further activated LTR-driven transcription of ERVW, generating a vicious cycle of latent ERV re-activation and uncontrolled inflammation[104,105]. In addition, multiple retroviral proteins have been shown to exert neurotoxic effects. For instance, the overexpression of ERVW envelope protein induces endoplasmic reticulum stress, leading to neuroinflammation and production of free radicals with ensuing demyelination and axonal injury[126]. HIV-1 proteins gp120 and Tat can lead to the activation of neuronal proteases, which cleave post-synaptic density proteins and cause synaptic dysfunction[39]. Similarly, the expression of ERVK proteins may also prove to be toxic for neurons. However, whether ERVK re-activation in neurological diseases is responsible for neuroinflammation and cell death is yet to be elucidated.

4.1.2 | TDP-43 interacts with the ERVK promoter and acts as a transcriptional activator of ERVK

In addition to NF-κB and IRF1, other transcription factors may also play an important role in regulating ERVK transcription. TDP-43 is a global transcriptional regulator, and has previously been shown to modulate HIV gene expression[83,84]. The overexpression of endogenous TDP-43 has been strongly co-related with higher levels of ERVK *pol* transcripts in the neurons of ALS patients[31], suggesting that TDP-43 may influence ERVK transcription. Indeed, *in silico* analysis of ERVK 5′ LTRs has revealed conserved putative TDP-43 bindings sites throughout this proviral promoter, although the functionality of these sites remains to be verified empirically. Therefore, we sought to determine whether accumulation of TDP-43 enhances its interactions with the ERVK 5′ LTR, and leads to up-regulation of ERVK gene transcription and protein levels in astrocytes and neurons.

For the first time, we have shown that TDP-43 binds the ERVK promoter and acts as a transcriptional activator of ERVK. In support of this claim, overexpression of the wild-type TDP-43, but not a mutated form, significantly enhanced ERVK *gag* and *pol* transcription in a dose dependent manner in astrocytes and neurons (**Publication 4, Figure 3**). In addition, TDP-43 accumulation, achieved through proteasome blockade via MG132 treatment of cells, associated with increased nuclear localization of TDP-43 and its interaction with the ERVK 5′ LTR in astrocytes and neurons (**Publication 4, Figures 2B, 2D, and 4**). This coincided with enhanced levels of ERVK polyprotein/RT in astrocytes but not in neurons (**Publication 4, Figure 2**). It is important to note that transcription factor binding on a promoter does not necessarily imply a gene expression output, as many other factors govern the outcome of transcription factor binding to DNA. This includes, but is not limited to, the presence or absence of other transcriptional co-modulators, ability of transcription factors to interact and dimerize, appropriate spatiotemporal organization of their binding sites, as well as the length of time they are bound to their cognate sites on a promoter[117,118,119]. A lack of effect on ERVK transcription in neurons despite enhanced TDP-43 binding to the ERVK promoter may be explained by these factors. Moreover, TDP-43 cleavage stimulated by TNFα and MG132 treatment diminished the binding of TDP-43 to the ERVK promoter, leading to a dramatic decrease in ERVK transcripts and polyprotein/RT levels in astrocytes (**Publication 4, Figure 4**). Although this effect was also observed in neurons, it was much less prominent as compared to that observed in astrocytes (**Publication 4, Figure 4**).

Cell-type dependent regulation of host as well as viral gene expression is a common feature of many cellular transcription factors[80]. Thus, it is not surprising that TDP-43 was able to enhance ERVK expression in the astrocytic cell line much more strongly as compared to ReNcell CX-derived neurons. In addition, a given transcription factor can also exert a differential influence on the expression of its target gene depending on the promoter context, including where it binds on the promoter when multiple binding sites are present and its interactions with other transcription factors[80,127,128]. Likewise, TDP-43 may differentially bind to its respective sites in the ERVK promoter in astrocytes *versus* neurons. It may also differentially interact with other transcription factors, such as NF-κB p65[111], and influence its binding to the ERVK promoter in these cells. Astrocytic cells used in these experiments expressed higher basal levels of IRF1 and NF-κB p65 proteins in comparison to neurons. A comparatively greater number of TDP-43 – NF-κB p65 interactions in astrocytes may enhance the levels of active NF-κB p65 above the required threshold, leading to ERVK transcriptional activation in the presence of IRF1. Consequently, these factors may account for the ability of TDP-43 to serve as a strong ERVK transcriptional activator in astrocytes but only a weak one in neurons.

Furthermore, the genetic background of cell lines has previously been shown to cause transcriptional heterogeneity[129]. Likewise, the genetic background of the ReNcell CX cell line used to derive neurons may also influence the ability of endogenous TDP-43 to enhance ERVK transcription in these cells. For instance, there may be variations in ERVK LTRs or alternatively functional single nucleotide polymorphisms in transcription factors (for example NF-κB) between SVGAs and ReNcell CX cells, which may influence the level of TDP-43-mediated transcription of ERVK. A potential solution to this issue would be the use of additional astrocytic and neuronal cell lines to evaluate the influence of TDP-43 accumulation on ERVK transcription. Unfortunately, there is a lack of human astrocytic and neuronal cell lines with normal human karyotype; thus, alternative cell lines cannot be employed to study ERVK, as abnormalities in chromosome structure and number are known to significantly influence ERVK biology.

Overall, these studies have allowed us to expand our understanding of the mechanisms behind transcriptional re-activation of ERVK in ALS. We have demonstrated that augmented activity of ALS-associated transcription factors – NF-κB, IRF1, and TDP-43 – can enhance ERVK expression in astrocytes and neurons through their increased interactions with the ERVK promoter. Although enhanced NF-κB and IRF1 activity is a common feature of many inflammatory diseases, ERVK re-activation may proceed through different transcriptional mechanisms in other ERVK-associated diseases. Yet unexplored transcription factors and cellular signaling pathways may play a crucial role in this process, as the ERVK promoter is laden with binding sites for numerous other transcriptional regulators, such as activating protein-1 (AP-1), Signal Transducer and activator of transcription (STAT proteins), and other interferon regualtory factors (IRF3, IRF7)[68]. The influence of these host factors and disease-specific proteins on ERVK transcription was not in the scope of the current studies and will have to be tested empirically in the future.

4.2 | Processing of the ERVK polyprotein culminates in the production of active heterodimeric RT under neuroinflammatory conditions

Enhanced expression and activity of the ERVK reverse transcriptase (RT) protein has been implicated in several inflammatory and neurodegenerative diseases. Most importantly, elevated levels of ERVK RT have been observed in the cortical neurons of patients with ALS[31]. This observation is consistent with the detection of increased RT activity in the CSF and serum of individuals with ALS[35,53]. Thus, augmented ERVK RT expression is a promising biomarker for diseases associated with ERVK re-activation, including ALS. However, little work has been done to identify ERVK RT isoforms, their production resulting from ERVK polyprotein cleavage, and their functionality in health and disease.

Using gag-pro-pol polyprotein processing in exogenous retroviruses (HIV) as a model (**Figure 3**), we are the first to describe enhanced ERVK polyprotein cleavage culminating in the generation of conventional RT subunits under inflammatory conditions in neurons and astrocytes. Similar to HIV polyprotein processing, multiple protease cleavage steps produced intermediate protein products before ERVK RT subunits were finally released. As with exogenous retroviral RT enzymes[72,76,77], the formation of two different sized ERVK RT isoforms was detected in astrocytes and neurons. Astrocyte-derived ERVK RT consisted of a 52 or 54 kDa structural isoform without RNase H and a 60 kDa catalytic isoform containing an RNase H domain (**Publication 2, Figure 1C; Publication 3, Figure 1A**). In comparison, neuron-derived ERVK RT exhibited an increase in mass, comprising 56 or 58 kDa structural isoform and a 68 kDa catalytic isoform (**Publication 2, Figure 3A and B; Publication 3, Figure 2A**). Additionally, the size of ERVK RT with RNase H domain was previously determined to be approximately 65 kDa[130]. This variation in sizes may be attributed to the cell types used as sources of ERVK RT, as like other proteins, ERVK RT may also acquire differential post-translational modifications depending on the cell type[131]. Nonetheless, our findings depict that ERVK RT is likely to be a heterodimer consisting of a smaller structural subunit without RNase H and a larger catalytically active subunit with RNase H, similar to RT encoded by exogenous retroviruses. However, a caveat of this study is that the sizes and the identities of the cleavage products as well as the ERVK RT subunits were inferred from HIV polyprotein cleavage and RT structure, and still remain to be verified empirically. This may be achieved by separating and collecting the ERVK polyprotein cleavage products by high pressure liquid chromatography, performing amino acid analysis, and confirming their identity by mass spectrometry[132].

Interestingly, the structural RT subunit was found to be expressed at basal levels in both astrocytes and neurons, and dose dependently increased with cytokine treatment of cells. However, the catalytically active RT subunit was only expressed in the presence of cytokines, suggesting that optimal ERVK RT activity likely occurs under neuroinflammatory conditions (**Publication 2, Figure 1C; Publication 3, Figure 2A**). In support of this claim, RT enzymatic activity in whole cell extracts was significantly enhanced with cytokine treatment as compared to untreated cells (**Publication 2, Figure 1B**); however, it does not specifically reflect ERVK RT activity, but rather global cellular RT activity. Due to a lack of enzymatic assays specifically for ERVK RT, it is currently not possible to precisely measure the activity of this ERVK protein. Alternatively, currently available enzymatic assays designed to measure exogenous retroviral RT activity[132] can be modified to specifically measure the activity of ERVK RT in our cell line models, which has previously been achieved for a reconstructed infectious clone of ERVK (HML-2)[133].

Nonetheless, the ability of ERVK to produce an active viral RT enzyme has important implications for ERVK biology, as well as for the role of this endogneous retrovirus in the associated inflammatory diseases. Since RT catalyzes conversion of retroviral RNA into DNA – a step necessary for the integration of retroviral genome into the host DNA – the presence of enzymatically active ERVK RT suggests that ERVK reintegration events may be possible as long as the activity of viral integrase is retained. Accordingly, insertional polymorphisms in ERVK (HML-2) loci have been reported in humans[134], raising the possibility that ERVK (HML-2) family may be active and undergoing reintegration in present-day humans. In addition, enzymatically active RT may facilitate retrotransposition of ERVK, which may perturb the function of critical host genes and contribute towards disease[130]. There is evidence that endogenous RT activity and retrotransposition can alter gene expression patterns in human cells, including neurons[135,136]. Likewise, enhanced ERVK RT activity in cortical neurons of patients with ALS may contribute to neurodegeneration by disrupting the expression of critical genes through retrotransposition events.

Moreover, RT activity will generate RNA-DNA hybrids and eventually cytoplasmic dsDNA during reverse transcription of ERVK RNA genome in astrocytes and neurons. These RNA and DNA intermediates may be transmitted in a cell to cell fashion[137], as ERVK assembles virions with RNA or DNA genomes. It has been shown that ERVK virions can package a synthetic ERVK HML-2 genetic probe and transmit it to other cells[29]. These newly transmitted ERVK nucleotide sequences may be detected by PRRs, such as Retinoic acid inducible gene I (RIG-I) and IFN inducible protein 16 (IFI16)[138]. ERVK RNA:DNA hybrids generated in a cell may also serve as viral PAMPs for innate immune detection by Toll like receptor 9 (TLR9)[139] or orphan DNA sensors[140]. The interactions of these PRRs with their cognate ligands culminates in the activation of NF-κB and IRFs, and the production of pro-inflammatoy cytokines, which aid in restricting viral replication. However, ERVK may exploit this process for its further activation, generating a feed forward loop consisting of NF-κB/IRF1-induced ERVK expression and innate immune response against ERVK-expressing neurons, causing chronic neuronal damage observed in ALS and other ERVK-associated neurological diseases.

Furthermore, sequential ERVK polyprotein processing lends credibility to the fact that ERVK encodes and produces a functional viral protease enzyme[141]. However, whether ERVK protease was responsible for cleaving the ERVK polyprotein to generate mature RT in our studies remains to be elucidated experimentally. Alternatively, an unidentified cellular protease may have been responsible for cleaving ERVK polyprotein. Nonetheless, the production of active viral protease has significant implications for ERVK biology and persistence of ERVK expression in associated diseases. For instance, ERVK virions are generally thought to be non-infectious because of their inability to produce an active protease and thus form mature virus particles[30]. However, mature ERVK virions have been detected in cancer cell lines[28], but their infectivity is yet to be clearly determined. Recently, ERVK virions derived from these cell lines were demonstrated to transmit ERVK sequences to uninfected cells[29], challenging the prevailing notion that ERVK virions are non-infectious. In line with this, biologically functional ERVK protease may lead to the production of mature and infectious ERVK virions in human tissues; re-infection of host cells may lead to virus-mediated or immune-mediated cellular damage and contribute to disease pathology. In addition, similar to HIV protease, ERVK protease may also allow viral PAMPs to escape immune detection through protease-mediated degradation of key innate immune sensors, such as RIG-I[61]. This may foster persistence of inducible ERVK expression in the context of inflammation, which is likely to be detrimental for the CNS.

4.3 | Non-prototypic ERVK polyprotein cleavage may indicate cellular response to degrade ERVK polyprotein/RT and mitigate ERVK activity

In addition to prototypic polyprotein cleavage pattern leading to the production of active ERVK RT in our studies, we also observed that ERVK polyprotein can be completely cleaved to generate non-prototypic protein fragments. This was achieved through treatment of astrocytes with a combination of TNFα and MG132, which led to the production of 68 and 41 kDa RT fragments and disappearance of the 125 kDa ERVK polyprotein band (**Publication 4, Figure 2A**). These non-prototypic fragments may represent degraded and inactive ERVK RT, although this is yet to be validated experimentally.

MG132 is a peptide aldehyde that reversibly inhibits the peptidase activities of the chymotrypsin-like and caspase-like sites in the 20S subunit of the proteasome[142]. Thus, MG132 treatment leads to proteasome inhibtion and protein accumulation in cell cultures[143], yet cleavage of ERVK polyprotein was observed in the presence of MG132, suggesting that other protein degradation pathways may be responsible for homeostatic clearance of ERVK protein products (**Publication 4, Figure 2A**). Another key protein degradation pathway is the autophagy system, which has previously been shown to degrade viral proteins[144]. Proteasome inhibition has also been demonstrated to enhance autophagic activity as a compensatory mechanism[145]. Accordingly, in astrocytes, we observed enhanced expression of the autophagy marker, LC3B-II[144], in the presence of MG132, which is indicative of an ongoing autophagy to clear ERVK expression (**Publication 4, Figures 2A and 6B**). Complete clearance of ERVK polyprotein associated with a decrease in LC3B-II levels in TNFα and MG132 treated astrocytes, suggesting resolution of an autophagic response following ERVK protein degradation (**Publication 4, Figures 2A and 6B**). In addition, confocal microscopy revealed large autophagolysosome-like structures with partial clearance of ERVK polyprotein/RT in astrocytes (**Publication 4, Figure 6B**). These findings suggest that autophagy may be a homeostatic mechanism to degrade ERVK proteins and regulate ERVK activity in human cells. In fact, many cellular enzymes, including caspases, calpains, and cathepsins are known to cleave and degrade viral proteins. For instance, ERVK-10 gag protein is known to be cleaved by caspase, and is associated with the apoptosis of ERVK expressing cells[146]. HIV-1 gp120 protein is also cleaved by caspases and cathepsins[147,148]. Caspase-3 proteolytic activity has been observed in cortical neurons exposed to HIV gp120[148]. Thus, ERVK accumulation may trigger protein degradation pathways to quench viral activity as a protective cellular response; unfortunately, the inherent nature of this response may lead to enhanced cellular damage and death.

4.4 | ERVK proteinopathy in neurological disease

4.4.1 | ERVK RT aggregation is enhanced in human cell line models of pro-inflammatory cytokine-mediated neuroinflammation

ERVK-associated neurological disorders, including ALS, are increasingly being recognized to have common pathological mechanisms, particularly aggregation of cellular proteins and inclusion body formation. Aggregation of wild type and mutant TDP-43, as well as other host proteins, is a hallmark of ALS. The role of these protein aggregates in the pathology of associated neurodegenerative disorders has been extensively studied (and reviewed elsewhere[47,149]. However, ERVK protein aggregation has never been examined in ALS or in other ERVK-associated neurological diseases, despite the fact that ERVK protein levels are significantly enhanced in these pathologies.

For the first time, we have described the formation of ERVK RT aggregates and their cellular localization in human cell line models of neurological disease. First of all, we have utilized confocal microscopy to demonstrate that pro-inflammatory cytokines enhance cytosolic ERVK RT aggregation in astrocytes and neurons. Interestingly, cytokine-induced ERVK expression always resulted in the formation of a large ERVK RT aggregate proximal to the nucleus, along with the formation of a perinuclear ring (**Publication 2, Figure 2; Publication 3, Figure 1B**). Nuclear ERVK RT expression was also enhanced upon cytokine stimulation, and exhibited a speckled pattern (**Publication 2, Figure 2; Publication 3, Figure 1B**). This ERVK staining pattern is consistent with that observed for HIV-1 RT in reverse transcription complexes (RTCs) formed during viral replication[150]. HIV-1 RTCs have been demonstrated to accumulate proximal to the nucleus and form a perinuclear ring before nuclear import of RTCs occurs[150]. The formation of RTCs and their nuclear import is a critical step in retroviral life cycle, as it allows reverse transcription of viral RNA genome into DNA, which is then imported into the nucleus for integration into the host cell genome. The ERVK RT staining pattern suggests the formation of ERVK RTCs in cytokine-stimulated cells, which may be imported into the nucleus as indicated by speckled nuclear RT staining. The nuclear transport of ERVK RTCs may lead to ERVK re-integration into the host cell genome in the presence of an active viral integrase enzyme.

Nonetheless, it remains to be confirmed whether the observed ERVK RT aggregates are truly RTCs, whether they are imported into the nucleus, and which cellular factors promote their nuclear import. Exogenous retroviral RTCs, including those for HIV, have previously been purified from infected cells and visualized by fluorescent confocal microscopy[150]. The identity of ERVK RTCs can be confirmed by utilizing these already existing techniques and modifying them if necessary, to yield functional ERVK RTCs. In addition, nuclear import assays have been used to study the nuclear translocation of HIV RTCs[150], and can also be employed to determine whether ERVK RTCs are imported into the nucleus. Nuclear import of proteins is mainly mediated by a large superfamily of importin factors. Importin 7 is known to shuttle HIV RTCs from the cytosol into the nucleus[150]. Similarly, importin 7 or other related factors may be able to transport ERVK RTCs into the nuclei of host cells. This can be determined using nuclear import assays in importin knockout cells and cultured cells reconstituted with specific transport receptors[150].

The enhanced expression and activity of ERVK RT, formation of viral RTCs, and the subsequent production and nuclear import of ERVK genomic DNA under neuroinflammatory conditions has important implications for ERVK-associated neurological diseases. The nuclear transport of ERVK RTCs and subsequent ERVK re-integration into the host cell genome may disrupt the expression and function of critical host genes, contributing towards the pathology of neurological diseases. Interestingly, these effects may partially explain differences in disease progression and symptom development in individuals with a given disease, as re-integration cycles are likely to generate random and un-identical cellular outcomes in each affected cell.

In addition, the staining pattern of ERVK RT aggregates resembles that of the specialized inclusion bodies called viroplasms, which comprise the viral replication machinery[151,152]. This suggests the formation of putative ERVK viral factories in cytokine-stimulated cells. These findings are consistent with the detection of ERVK RT staining in the axons of cortical neurons from patients with ALS (**Publication 3, Figure 5**). Neurotropic viruses are known to transmit progeny virions or virion components within neuronal axons, which allows the viral infection to spread while escaping extracellular immune responses[153]. Similarly, speckled ERVK staining in neuronal axons may reflect ERVK's attempt to spread from one neuron to another. The morphology of ERVK viroplasms also resembles that of the aggresomes, which are compartments that sequester unwanted proteins in specialized inclusions and facilitate their clearance by autophagy, thereby dissipating the cytotoxic effects of protein aggregates[154,155]. Likewise, the formation of ERVK RT aggresomes may be a cellular response to protect against toxic ERVK protein accumulation. Unfortunately, the appearance of aggresomes and inclusion bodies can impair vital cellular functions, including inactivation of the proteasomal pathway responsible for clearing protein aggregates[155]. Interestingly, protein clearance pathways, such as the proteasome system and autophagy, are dysregulated in ERVK-associated neurological diseases including ALS[156]. In the absence of functional protein degradation pathways, inflammation-induced ERVK viroplasms or aggresomes may persist and perpetuate chronic neuronal damage.

4.4.2 | Failure of the proteasomal and autophagic protein clearance pathways promotes ERVK proteinopathy

Dysfunction of the proteasomal and autophagy pathways, leading to aggregation of cellular proteins, has been extensively documented in ALS[156]. Recent studies have enlarged our understanding of the molecular composition of the protein aggregates resulting from proteasome and autophagy disruption. For instance, the proteasome and autophagy system are known to clear endogenous TDP-43 accumulation, and disruption of these pathways in ALS associates with TDP-43 proteinopathy[149,157]. However, the influence of these pathways on regulation of ERVK expression has remained unexplored. Therefore, we sought to determine whether inhibiting the function of the proteasomal pathway results in ERVK RT aggregation in our cell line models of neurological disease.

As discussed previously, MG132 treatment of cells is well known to abolish the function of the proteasome. MG132 treatment enhanced TDP-43-induced ERVK gene transcription, as well as polyprotein and RT accumulation in astrocytes (**Publication 4, Figures 2 and 4**). Confocal microscopy further revealed large ERVK RT aggregates proximal to the nucleus with the formation of a perinuclear ring; nuclear aggregation of RT was also observed (**Publication 4, Figure 2A**). This staining pattern resembles that of inclusion bodies seen in a variety of proteinopathies. Thus, proteasome inhibition in ALS may trigger ERVK proteinopathy through increased TDP-43 mediated ERVK transcription, as well as aggregation of newly expressed ERVK proteins which are normally cleared by the proteasome. However, the causative link between proteasome dysfunction and ERVK RT aggregation in neurological diseases remains to be validated *in vivo*.

Although in our studies we did not interefere with the autophagy system, our observation of cell-type specific functionality of this pathway sheds light on the influence of successful *versus* incomplete autophagy on ERVK protein levels in CNS cells. In contrast to astrocytes, TNFα and MG132 treatment of neurons did not culminate in complete degradation of the ERVK polyprotein to generate non-prototypic ERVK RT fragments (**Publication 4, Figure 2C**). Yet, there was enhanced expression of LC3B-II in the presence of MG132, suggesting that autophagy was occuring (**Publication 4, Figure 2C**). The inability of neurons to clear ERVK despite an ongoing autophagic response may be explained by failure or active inhibition of autophagy. In line with this, unlike in astrocytes, TNFα and MG132 treatment of neurons did not lead to resolution of autophagy, as LC3B-II protein levels remained high and not all TDP-43 was cleaved (**Publication 4, Figure 2C**). The function of other key proteins involved in the autophagy pathway, such as optineurin, may have been disrupted by MG132 treatment of neurons, and may account for an unsuccessful autophagic response. Alternatively, neurons may not be able to clear ERVK proteins unlike astrocytes.

Recently, impaired autophagic response has been linked to ALS pathology and enhanced neuronal death[158,159–161]. Autophagic dysfunction in ALS is known to stem from mutations in a variety of critical proteins involved in this pathway, such as optineurin, ubiquilin 2, sequestosome 1, phosphoinositide 5-phosphatase, and charged multivesicular body protein 2B[161,162,163]. Mutations in the latter two proteins have previously been shown to cause LC3B-II accumulation as their dysfunction prevents completion of autophagy[161,164]. Overall, the inability of CNS cells to degrade ERVK proteins, as a consequence of impaired autophagic response and/or proteasomal system, may trigger neurocognitive impairment through various mechanisms including viral protein-mediated synaptic dysfunction, excitotoxicity, and loss of neuronal plasticity, as seen with other neurotropic retroviruses[165].

Enhanced ERVK protein expression may further interfere with protein degradation pathways. A number of viruses have evolved strategies to interfere with autophagy by disrupting autophagosome formation or maturation. HTLV-1 tax protein recruits autophagic molecules to lipid rafts and thus deregulates autophagy[166]. Coxsackie virus B3 protease can cleave sequestosome 1, rendering it unable to bind ubiquitinated cargo and form autophagosomes[167]. Likewise, ERVK protease may cleave autophagy sensors and adaptor molecules, leading to failure of an autophagic response and persistence of viral expression. A striking number of viruses also target beclin 1, a protein required for initiation of the formation of autophagosome, to disrupt autophagosome biogenesis. Human cytomegalovirus TRS1 protein, African swine fever virus A179L protein, herpes simplex virus type 1 ICP34.5 protein, human herpesvirus 8 orf16 protein, and HIV Nef protein all bind beclin 1 and block autophagosome biogenesis (reviewed in[168]). ERVK proteins may also prevent autophagosome formation by interfering with the function of beclin 1 or other proteins involved in autophagy. The interactions of ERVK proteins with autophagic molecules, as well as post translational modifications of ERVK proteins that may facilitate these interactions, have not been studied. Thus, the influence of ERVK expression on autophagic response is an interesting avenue of research that clearly needs further exploration.

4.4.3 | TDP-43 overexpression promotes ERVK proteinopathy

There is evidence that TDP-43 mediates transcriptional repression of the HIV-1 promoter[83]. In contrast, TDP-43 overexpression has been associated with enhanced ERVK transcription and protein expression ALS[31]. In our studies, we have confirmed that TDP-43 accumulation enhances ERVK re-activation at the transcriptional level. Here we showcase that TDP-43 overexpression also promotes ERVK proteinopathy in human cell line models of neurological disease. We have utilized confocal microscopy to evaluate TDP-43 and ERVK RT aggregation in astrocytes transfected with constructs encoding wild-type and mutant TDP-43. Overexpression of wild-type as well as mutant TDP-43 in this cell line recapitulated cytosolic TDP-43 aggregation (**Publication 4, Figure 5E-F**). Enhanced ERVK RT aggregation was observed in these cells, with mutant TDP-43 forms generating significantly higher percentage of aggregates as compared to wild type TDP-43 (**Publication 4, Figure 5F**). In addition, overexpression of wild type TDP-43 enhanced overall ERVK polyprotein expression, whereas overexpression of the truncated TDP-43 reduced ERVK expression in astrocytes (**Publication 4, Figure 5A-B**). This clearance of ERVK polyprotein, despite the absence of any external signals to stimulate degradative pathways, may reflect the activity of anti-ERVK endogenous mechanisms in astrocytes. In stark contrast, wild type TDP-43 promoted insoluble ERVK polyprotein accumulation, and mutant TDP-43 enhanced the availability of soluble ERVK polyprotein in neurons (**Publication 4, Figure 5C-D**). This suggests that neurons are intrinsically unable to fully clear ERVK protein accumulation. This may be particularly devastating for individuals carrying heterozygous TDP-43 mutations in ALS[169,170,171], as wild-type TDP-43 will promote insoluble ERVK protein accumulation and mutated TDP-43 will revert it back to the cytosol, culminating in neuronal ERVK persistence.

Overexpression of TDP-43 in astrocytes and neurons also led to the production of 50 kDa TDP-43, which may represent a hyperphosphorylated form of this protein (**Publication 4, Figure 5**). Phosphorylated TDP-43 isoforms have been detected in pathological inclusions in ALS[172]. As with majority of other transcription factors, phosphorylation of TDP-43 may facilitate its interaction with the ERVK LTR and promote ERVK transcription. In addition, phosphorylated TDP-43 aggregates can inhibit proteasome activity[173]; together, phospho TDP-43 – mediated transcriptional re-activation of ERVK and inhibition of proteasomal clearance of ERVK proteins may significantly augment retroviral proteinopathy in ALS.

Interestingly, ERVK RT co-localized with TDP-43 aggregates in our TDP-43 overexpression models in astrocytes (**Publication 4, Figure 5E**). The ERVK RT and TDP-43 staining pattern in cells overexpressing these proteins resembled that of stress granules (SGs)[174]. SGs are nuclear or cytoplasmic aggregates comprised of proteins and RNA molecules that form upon cellular stress. TDP-43 has been shown to promote stress granule assembly, and formation of stress granules has increasingly been implicated in ALS pathology[174,175]. TDP-43 has previously been shown to colocalize with stress granule markers, and overexpression of wild type as well as mutant TDP-43 can enhance the formation of stress granules, although TDP-43 mutants result in more stress granules formed per cell[176]. But, the localization of ERVK proteins to stress granules has not been examined. Herein, we have demonstrated that ERVK RT also co-localizes with the stress granule marker G3BP1 in response to TNFα and MG132 stimulation of astrocytes (**Publication 4, Figure 6A**), suggesting that ERVK proteins can be recruited to stress granules. G3BP1 expression was also significantly enhanced in ERVK+ cortical neurons of patients with ALS as compared to neuro-normal controls, but no co-localization between ERVK RT and G3BP1 was observed (**Publication 4, Figure 6C**). Exclusion of ERVK RT from stress granules in neurons may reflect a viral evasion strategy to escape degradative processes. As TDP-43 is an RNA binding protein, it may bind ERVK nucleic acids and shuttle them to SGs as an antiviral mechanism, to impede viral RNA translation. Despite the fact that TDP-43 mutants have an increased SG association, they may not be able to interact with ERVK RNA. In ALS, TDP-43 mutations or cleavage may result in a failure to traffic viral RNA to SGs, allowing the translation of ERVK polyprotein in neurons.

One of the mechanisms that cells use to counteract viral infection is inhibition of global protein synthesis and recruitment of mRNAs into SGs, where the fate of viral as well as cellular mRNAs is determined[177]. Normally, SG formation is followed by targeting viral mRNAs for degradation and cellular mRNAs for translation[177]. However, a striking number of viruses have developed strategies to escape SGs or to take advantage of them for their replication, and retroviruses are no exception. Cytosolic expression of HTLV-1 Tax protein inhibits the formation of SGs, allowing synthesis of HTLV-1 proteins[177]. HIV-1 is also known to interfere with SG assembly by redistributing staufen-1 from SGs to distinct cytoplasmic granules, where staufen-1 is exploited to aid in the assembly of HIV-1 virions[178–180]. In contrast HIV-2 induces the formation of SGs to allow gag protein synthesis and virion production[181]. Similar to these exogenous viruses, ERVK RNAs may escape SGs formed in response to TDP-43 over-expression, or alternatively ERVK may exploit SG formation for its protein synthesis – both of these processes have the potential to facilitate ERVK proteinopathy.

Overall, we have comprehensively described several mechanisms behind aberrant ERVK transcription and protein aggregation in neuroinflammatory diseases, particularly ALS and HAND. In the studies outlined in this thesis, we have linked pro-inflammatory stimuli, impairment of proteasomal and autophagic protein degradation pathways, as well as TDP-43 dysregulation with ERVK re-activation in ALS and HAND. Thus, ERVK is a novel marker that may define the pathophysiology of neuronal loss in these conditions. Consequently, controlling ERVK activity in the associated neurological diseases may be beneficial in managing disease symptoms and in reverting retrovirus-mediated neuropathology.

5. GLOBAL SUMMARY AND SIGNIFICANCE

In this research work, we have established human cell line models of ERVK-associated neurological disease which have allowed us to expand our understanding of the mechanisms behind transcriptional re-activation of ERVK in the context of neuroinflammation (summarized in **Figure 9**). We are the first to demonstrate that inflammation is a key trigger of ERVK activity in associated neurological conditions. Pro-inflammatory cytokines that lead to IRF1 and NF-κB activation augment ERVK expression in astrocytes and neurons in a cell-type specific manner. This is achieved via binding of IRF1 and NF-κB to funcitonal ISREs in the ERVK promoter. We have also shown for the first time that TDP-43 is a transcriptional activator of ERVK, and that TDP-43 overexpression and aggregation can promote ERVK proteinopathy. In addition, we showcase that ERVK protein accumulation can be cleared via the proteasome and autophagy systems, and that diruption of these pathways promotes ERVK proteinopathy. Overall, simultaneous de-regulation of pro-inflammatory cytokine signaling, TDP-43 function, and protein clearance pathways has the potential to significantly enhance ERVK proteinopathy – a novel pathological mechanism in ERVK-associated neurological diseases.

The findings generated from this work have significantly changed our views on neuropathogenesis of HAND and ALS, by including ERVK proteinopathy as a novel mechanism of neuronal damage in these diseases. Thus, ERVK is a novel marker of neurodegeneration in HAND and ALS. Consequently, quenching ERVK activity in these conditions may improve disease symptoms, and reverse virus-mediated pathology. This may be achieved through the use of anti-retroviral drugs, such as viral protease and RT inhibitors. HIV-1 protease inhibitors have been used in the past to target ERVK protease activity; however, ERVK protease is highly resistant to these drugs[182]. This is likely because ERVK and HIV-1 proteases, and similarly RT, vary significantly at their structural levels. Thus, new ERVK-specific anti-retroviral drugs will be required to control ERVK activity in the associated diseases. Alternatively, drugs that trigger broad-spectrum anti-viral innate immune responses, such as GSK984, may be able to quench ERVK activity in the context of inflammation by stimulating the global production of viral restriction factors[183].

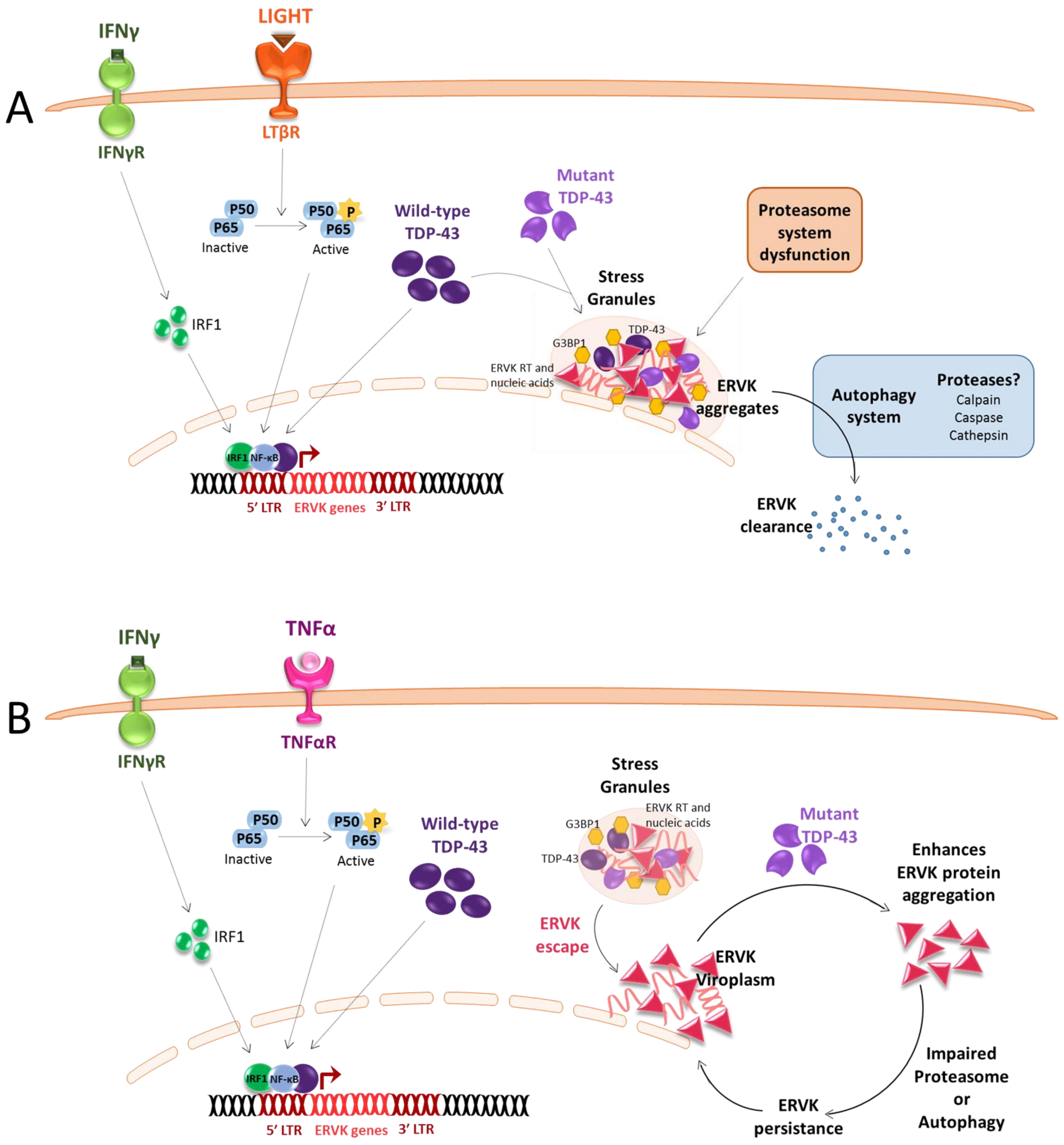

FIGURE 9. Summary of mechanisms behind (A) astrocytic and (B) neuronal ERVK proteinopathy and the cell-type specific responses to clear ERVK aggregation. Artwork by M. Manghera.

ERVK protein aggregation can also be targeted in novel therapeutics. Viroplasm inhibitors, such as the thiazolide class of antivirals, have been used in the past to prevent replication of certain types of viruses[184]. Such inhibitors may also be beneficial in preventing

ERVK viroplasm formation. Small-molecule autophagy enhancers can also be exploited to facilitate the degradation of ERVK protein aggregates. Trehalose is an excellent candidate for this purpose, as it has previously been demonstrated to increase neuronal survival by promoting autophagic clearance of protein aggregates including tau, huntingtin, and SOD1 inclusions *in vitro* and in mouse models of neurological disease[185].

Immunomodulatory regimens, such as anti-TNFα therapy[186], may further lend benefit to managing the clinical symptoms of ERVK-associated diseases such as ALS and HAND. Since NF-κB plays a crucial role in ERVK transcriptional re-activation, it is desirable to target the activity of this pro-inflammatory TF in neurological disease. However, the use of NF-κB inhibitors is complicated by the fact that NF-κB is crucial for proper neuronal functioning, such as synaptic signaling and plasticity; and thus, inhibiting NF-κB activity may actually exacerbate neurodegenerative processes[187]. Nonetheless, novel ERVK-targeting therapeutics will give hope to thousands of Canadians currently living with ALS and HAND, but without an effective treatment or a cure.

6. FUTURE DIRECTIONS

Many interesting avenues of future ERVK research have stemmed from this work. First of all, we observed prototypical cleavage of the ERVK polyprotein to generate mature RT isoforms under a variety of inducible conditions. This raises the question as to which protease – cellular or viral – is responsible for cleaving the ERVK polyprotein. Libraries of human and retroviral protease inhibitors can be screened in our inducible ERVK models to pinpoint the involved protease(s). The findings can be used to inform novel therapeutics, such as the use of protease inhibitors to prevent the formation of functional ERVK proteins. This approach can also be extended to screen and identify novel ERVK RT inhibitors for therapeutic use.

Another interesting research direction stemming from this work is to elucidate which autophagic sensors are responsible for detecting ERVK proteins and targeting them for degradation in the CNS. Our preliminary findings support the potential role of optineurin, a critical component of the autophagy system, in targeting ERVK elements for autophagic degradation. Interestingly, optineurin is mutated in some ALS cases, and an impaired autophagic response has been linked to ALS pathology and enhanced neuronal death[159,163]. Future studies will focus on determining whether optineurin is required for homeostatic clearance of ERVK in astrocytes and neurons, and whether ALS-associated mutations in this sensor enhance ERVK proteinopathy in ALS.

Although in this work we have demonstrated several mechanisms that promote ERVK re-activation in the context of neuroinflammation, it remains to be determined whether ERVK nucleic acid and protein accumulation further leads to immune activation and contributes to neuronal injury. Future studies should aim to determine which innate immune sensors recognize ERVK elements in the CNS, and what the subsequent consequences are, including characterization of the resulting immune responses and their influence on neuronal survival and tissue integrity. Human "mini brains" or cerebral organoids will prove to be useful tools for this purpose. This research direction will help us link ERVK re-activation with immunopathology of neurodegenerative diseases, such as ALS. In addition, it would be interesting to study the influence of functional polymorphisms in molecules such as NF-κB, IRF1, TNFα, and innate immune sensors on ERVK biology in the context of neuroinflammatory disease.

7. CONCLUSIONS

- The ERVK promoter contains functional ISREs.
- Pro-inflammatory cytokines stimulate ERVK transcription in a cell- type specific manner by enhancing the interactions of pro-inflammatory transcription factors NF-κB (p50 and p65) and IRF1 with the ISREs on the ERVK promoter.
- Pro-inflammatory cytokine signaling leads to enhanced cleavage of the ERVK polyprotein to yield mature RT isoforms.
- TDP-43 over-expression leads to enhanced ERVK transcription.
- ALS-associated TDP-43 mutants promote ERVK protein aggregation.
- Proteasome and autophagy are homeostatic mechanisms to clear ERVK expression, and disruption of these protein degradation pathways causes ERVK polyprotein/RT aggregation.
- Transcriptional re-activation of ERVK and viral polyprotein/RT aggregation culminates in ERVK proteinopathy – a novel pathological mechanism in ERVK-associated neurodegenerative diseases.

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