Chapter 4

The MOV10 Helicase Restricts Human LINE-1 Retrotransposition

MOV10 was initially identified as an ORF1p-interacting protein in Chapter 3. The data presented in this Chapter extend the data from Chapter 3 and suggest that the RNA helicase MOV10 inhibits L1 retrotransposition in cultured cells. It should be noted that while I was working on MOV10, several studies were published that also showed that human MOV10 restricts human L1 retrotransposition (Arjan-Odedra et al. 2012, Goodier et al. 2012, Li et al. 2013). For this reason, I stopped working on MOV10 and changed the focus of my thesis research to study the effect of ZAP on L1 retrotransposition. Thus, some of the data pertaining to MOV10 should be considered as preliminary data. I designed and carried out all experiments described in this chapter.

Abstract

Long INterspersed Element-1 (LINE-1 or L1) is the only active autonomous retrotransposon in the human genome. L1s comprise ~17% of human DNA and it is estimated that an average human genome has ~80-100 active L1s. L1 moves throughout the genome via a “copy-and-paste” mechanism known as retrotransposition. L1 retrotransposition is known to cause mutations; thus, it stands to reason that the host cell has evolved mechanisms to protect the cell from unabated retrotransposition. Here, we demonstrate that the RNA helicase MOV10 inhibits the retrotransposition of human L1 and Alu retrotransposons, as well as related retrotransposons from mice and zebrafish. Biochemical, genetic, and immunofluorescence microscopy data suggest that

MOV10 interacts with L1 RNA and inhibits the expression of full-length L1 RNA providing insight into a possible mechanism for how MOV10 restricts retrotransposition.

Introduction

The Long Interspersed Nuclear Element-1 (LINE-1 also known as L1) comprises ~17% of the genome and is the only active autonomous transposable element in humans (Lander et al. 2001). L1 mobilizes via an RNA intermediate by the process of retrotransposition (Boeke et al. 1985), which disperses new L1 copies throughout the genome. Most genomic L1 sequences are not capable of retrotransposition due to mutations (Grimaldi et al. 1984, Lander et al. 2001); however, it is currently estimated that each human genome does contain between 80 - 100 L1 copies that are capable of retrotransposition (Sassaman et al. 1997, Brouha et al. 2003). L1 retrotransposition has caused mutations that have led to nearly 100 cases of human genetic diseases (Hancks and Kazazian 2012) such as hemophilia A (Kazazian et al. 1988) and cancer (Miki et al. 1992). The process of L1 retrotransposition disseminates additional L1 sequences throughout the genome generating additional sequence diversity, which contributes to inter- and intra-genomic variation in the human population (Cordaux and Batzer 2009, Beck et al. 2010, Beck et al. 2011).

An active L1 element is approximately 6 kb in length and encodes a 5' UTR that promotes L1 transcription (Swergold 1990, Becker et al. 1993, Athanikar et al. 2004). Immediately following the L1 5' UTR are two open reading frames (ORF) that are separated by a short 63 bp intergenic spacer (Scott et al. 1987, Alisch et al. 2006). The first L1 ORF (ORF1) encodes ORF1p, an ~40 kDa nucleic acid binding protein (Martin 1991, Holmes et al. 1992, Hohjoh and Singer 1996, Hohjoh and Singer 1997) with demonstrated nucleic acid chaperone activity (Martin and Bushman 2001, Khazina and Weichenrieder 2009, Callahan et al. 2012). The second L1 ORF (ORF2) encodes an ~150 kDa protein (Ergun et al. 2004, Doucet et al. 2010, Goodier et al. 2010) with reverse transcriptase (RT)

(Mathias et al. 1991, Dombroski et al. 1994) and endonuclease (EN) (Feng et al. 1996) activity. The activity of both L1-encoded proteins is required for L1 retrotransposition (Feng et al. 1996, Moran et al. 1996).

The L1 retrotransposition cycle likely begins with the transcription of a full-length genomic L1 element. The resulting bi-cistronic L1 transcript is then translated in the cytoplasm by an unconventional cap-dependent translation mechanism (Leibold et al. 1990, McMillan and Singer 1993, Alisch et al. 2006, Dmitriev et al. 2007). During human L1 translation, ribosomes are thought to continue scanning past the stop codon in ORF1 until reaching the first start codon in ORF2 before reinitiating translation (Alisch et al. 2006). After translation, the L1-encoded proteins are then thought to preferentially bind back to their encoding L1 RNA, a phenomenon termed *cis*-preference (Esnault et al. 2000, Wei et al. 2001). The complex of L1 RNA and proteins forms an L1 ribonucleoprotein complex (RNP), which is a critical L1 intermediate (Martin 1991, Hohjoh and Singer 1996, Kulpa and Moran 2005, Kulpa and Moran 2006). Components of the L1 RNP enter the nucleus (Kubo et al. 2006), where an L1 cDNA copy is synthesized by the L1-encoded RT using L1 RNA as a template, and then inserted into a new genomic location by a process termed target site primed reverse transcription (TPRT) (Luan et al. 1993).

The L1 retrotransposition process is inherently mutagenic and thus threatens the stability and/or fidelity of genomic DNA. It is therefore reasonable to hypothesize that certain host cell mechanisms may have arisen in response to these intracellular selfish DNA entities. Several mechanisms that are thought to restrict L1 retrotransposition have been described. For example, methylation of genomic L1 sequences are though to silence L1 transcription in most cells (Yoder et al. 1997, Levin and Moran 2011). The PIWI-interacting RNA (piRNA) pathway, a small silencing RNA pathway in the germline of mice and flies, has been demonstrated to silence transposable element expression and to destroy actively expressed transposable element transcripts in germline tissues (Aravin et al. 2007, Siomi et al. 2011). Several host proteins have also been demonstrated to inhibit L1 retrotransposition, including APOBEC3 protein family

members (Bogerd et al. 2006, Chen et al. 2006, Muckenfuss et al. 2006, Wissing et al. 2011, Horn et al. 2013, Richardson et al. 2014), TREX1 (Stetson et al. 2008), hnRNPL (Peddigari et al. 2013), SAMHD1 (Zhao et al. 2013), and RNase L (Zhang et al. 2014).

In Chapter 3 the MOV10 protein was identified as an ORF1p-interacting protein (Figure 3.1 and Table 3.1). MOV10 is an RNA helicase that exhibits RNA binding and 5' to 3' RNA unwinding activity (Gregersen et al. 2014). Recent studies suggest that MOV10 may function as a cellular antiviral protein as MOV10 has been demonstrated to inhibit the activity of several retroviruses including HIV-1 (Zheng et al. 2012). Although the mechanism explaining how MOV10 inhibits retroviruses is not yet completely understood, MOV10 has been shown to restrict the expression of HIV-1 proteins and to interact directly with the HIV-1 gag protein to become packaged into HIV-1 virions (Burdick et al. 2010, Furtak et al. 2010, Wang et al. 2010, Abudu et al. 2012).

Since a number of host cell antiviral proteins have also been demonstrated to inhibit L1 retrotransposition (*i.e.*, APOBEC3 proteins, TREX1, SAMHD1, RNase L) (Richardson et al. 2015), we hypothesized that the MOV10 might also be able to restrict L1 retrotransposition. In this study we tested the effect of MOV10 on human L1 retrotransposition. We report that MOV10 efficiently inhibits the retrotransposition of human L1 and Alu elements as well as LINE elements from mice and zebrafish. Genetic and biochemical analyses suggest that MOV10 associates with L1 ORF1p via an RNA bridge and inhibits L1 retrotransposition by promoting the destruction of L1 RNA.

Results

MOV10 associates with L1 ORF1p

In Chapter 3 we utilized co-immunoprecipitation experiments to show that the transfected L1 ORF1p from pJM101/L1.3FLAG (see Chapter 3 and Figure 3.1) interacts with endogenous MOV10 in HeLa cells (see Chapter 3 and Figure

3.1D and Table 3.1). Additional co-immunoprecipitation experiments conducted in the presence and/or absence of RNase A revealed that MOV10 interacts with ORF1p via an RNA bridge (Figure 3.1D). Thus, the data suggest that MOV10 associates with L1 ORF1p by binding to L1 mRNA.

To further analyze the interaction between MOV10 and ORF1p *in vitro*, we tested if transfected ORF1p interacts with purified carboxyl-terminal FLAG-tagged recombinant MOV10 (FLAG-rMOV10). HeLa cells were transfected with the L1 plasmid, pJM101/L1.3, and transfected cell lysates were incubated with the FLAG-rMOV10 and anti-FLAG beads. As controls, transfected cell lysates were incubated with either anti-FLAG antibody-coated beads alone, or anti-FLAG antibody-coated beads plus a FLAG-tagged bacterial alkaline phosphatase (FL-BAP) control peptide. Immunoprecipitation reactions were then analyzed using SDS-PAGE. Analysis of SDS-PAGE gels revealed an enrichment of transfected ORF1p in immunoprecipitation reactions that contained FLAG-rMOV10 when compared to control immunoprecipitation reaction with either FL-BAP plus beads or beads alone (Figure 4.1A: blue arrow). Thus, the data suggest that L1 ORF1p associates with endogenous and FLAG-tagged recombinant MOV10 proteins. Note that experiments in Figure 4.1A were performed only once, thus these experiments represent preliminary data.

MOV10 co-localizes with ORF1p in HeLa cells

L1 ORF1p co-localizes with L1 ORF2p and L1 RNA in cytoplasmic foci that can be visualized using fluorescence microscopy (Goodier et al. 2007, Doucet et al. 2010, Goodier et al. 2010). L1 foci associate with a multitude of RNA binding proteins including markers of cytoplasmic stress granules (SG) and processing bodies (PB) (Goodier et al. 2007). To determine if ORF1p associates with MOV10 in the cytoplasm, HeLa cells were transfected with pAD2TE1, which expresses an L1.3 element marked with a T7 *gene10* epitope tag on the carboxyl terminus of ORF1. Confocal microscopy revealed that T7-tagged ORF1p co-localized with endogenous MOV10 in cytoplasmic foci in HeLa cells (Figure 4.1B). Thus, the data suggest that ORF1p and MOV10 partition to the same

region of the cellular cytoplasm. Note that experiments in Figure 4.1B were performed twice with similar results and thus should be repeated to confirm these results.

MOV10 inhibits L1 retrotransposition in HeLa cells

In order to monitor the effect of MOV10 on L1 retrotransposition we over-expressed MOV10 in a cell-culture-based retrotransposition assay (see Chapter 3 and Figures 3.2A and 3.2B for explanation of retrotransposition assays) (Moran et al. 1996, Wei et al. 2000). MOV10 over-expression inhibited pJJ101/L1.3 retrotransposition activity to ~10% of pCEP4 control levels (Figure 4.2A; black bars). Notably, overexpression of MOV10 in pcDNA6 control assays (see Chapter 3 Figures 3.2A and 3.2B for explanation of pcDNA6 control assays) did not adversely affect the ability of HeLa cells to form blasticidin-resistant colonies (Figure 4.2A; white bars). Thus, the data suggest that MOV10 inhibits L1 retrotransposition in cultured cells.

MOV10 orthologs exist in a variety of other organisms including insects and plants (Meister et al. 2005); thus, it is unlikely that MOV10-mediated restriction of non-LTR retrotransposons is specific to human MOV10. To test this hypothesis we co-transfected HeLa cells with pJJ101/L1.3 and MOV10 from mouse (mMOV10), which shares 91% amino acid identity with human MOV10 (Wang et al. 2010). Mouse MOV10 restricted pJJ101/L1.3 retrotransposition to ~30% of control levels (Figure 4.2A; last set of black bars). Control experiments showed that mMOV10 did not adversely affect the ability of HeLa cells to form blasticidin-resistant colonies (Figure 4.2A; last set of white bars). Thus, the data suggest that MOV10-mediated restriction is a conserved feature of mammalian MOV10.

MOV10 domains outside of the core helicase domain are required for L1 restriction

MOV10 is a superfamily-1 (SF1) RNA helicase that exhibits 5' to 3' RNA unwinding activity (Gregersen et al. 2014). Previous studies have demonstrated the MOV10 helicase domain is required for RNA unwinding activity and anti HIV-

1 activity (Abudu et al. 2012, Gregersen et al. 2014). In addition to the core helicase domain, MOV10 also contains a domain rich in cysteine and histidine (CH) residues (Abudu et al. 2012), and a carboxyl terminal domain that mediates interactions with APOBEC3G (A3G) and AGO2 (Liu et al. 2012) (Figure 2A; top panel).

To determine whether the MOV10 CH and carboxyl terminus domains were required for L1 restriction, we tested a MOV10 mutant that lacks the MOV10 cysteine-histidine (CH) domain (MOV10/Δ100-355; lacking amino acids 100-355) and a MOV10 mutant that lacks the C-terminal region of MOV10 (MOV10/Δ912-1003; lacking amino acids 912-1003) in pJJ101/L1.3 retrotransposition assays (Figure 4.2A; top panel). Compared to wild type MOV10, MOV10/Δ100-355 and MOV10/Δ912-1003 had little affect on L1 retrotransposition (~82% and ~83% of control levels, respectively) (Figure 4.2A; black bars). Notably, the MOV10 mutants did not affect the ability of HeLa cells to form blasticidin resistant colonies in pcDNA6/TR control assays (Figure 4.2A; white bars). Western blots confirmed similar expression levels of ectopic wild type MOV10 and the MOV10 mutant proteins as well as endogenous MOV10 (Figure 4.2B) in HeLa cell lysates 48 hours after transfection. These data suggest that the MOV10 carboxyl terminus domain and the CH domains contain amino acid residues that are required for MOV10-mediated inhibition of L1 retrotransposition.

The effect of other ORF1p-associated RNA helicases on L1 retrotransposition.

MOV10 is not the only helicase that associates with L1 RNPs as we previously identified several other helicases that co-immunoprecipitated with L1 RNPs including UPF1, DHX9, and DDX21 (Table 3.1). To test that inhibition of L1 retrotransposition is not a general function of RNA helicases, we analyzed two other L1 RNP associated helicases in pJJ101/L1.3 retrotransposition assays, UPF1 and DDX21. UPF1 is a SF1 helicase that is involved in nonsense-mediated decay (NMD) (Kervestin and Jacobson 2012) and DDX21 is a DEAD-

box helicase belonging to RNA helicase super-family-2 (SF2). Neither UPF1 nor DDX21 had any appreciable effect on pJJ101/L1.3 retrotransposition (Figure 4.2A; black bars). We also tested the MOV10 paralog, MOV10L1, which participates in the piRNA-mediated silencing of transposable elements in the reproductive cells of male mice (Frost et al. 2010, Zheng et al. 2010). Like DDX21 and UPF1, MOV10L1 did not affect pJJ101/L1.3 activity (Figure 4.2A; black bars). Thus, RNA helicases in general do not restrict L1 retrotransposition.

MOV10 inhibits other non-LTR retrotransposons from humans, mice, and zebrafish

To determine if MOV10 could inhibit other non-LTR retrotransposons, we tested whether MOV10 could restrict human Alu retrotransposition. Alu is a short interspersed nuclear element (SINE) derived from 7SL RNA (Ullu and Tschudi 1984). Alu does not encode its own protein(s) and thus requires L1 ORF2p to be supplied *in trans* by L1 in order to retrotranspose (Dewannieux et al. 2003). Briefly, HeLa cells were co-transfected with a full-length L1 element (JM101/L1.3Δneo), an Alu retrotransposition reporter plasmid (pAlu*neo*^{Tet}) (Dewannieux et al. 2003), and a MOV10 expression plasmid. MOV10 completely abolished Alu retrotransposition (Figure 4.2C). Notably, deletion of the MOV10 carboxyl terminal domain partially relieved MOV10-mediated Alu restriction, whereas the expression of the L1 restriction-deficient MOV10/Δ912-1003 inhibited Alu retrotransposition to ~20% of control levels. MOV10/Δ100-355 did not significantly affect Alu retrotransposition (~68% of control levels) (Figure 4.2C). These data suggest that the MOV10 carboxyl terminus and the MOV10 CH domains are important for Alu restriction. That MOV10/Δ912-1003 more efficiently restricts Alu retrotransposition than MOV10/Δ100-355 could suggest a functional separation between the MOV10 CH and the MOV10 carboxyl terminal domains. Notably, the MOV10 CH domain is important for mediating interactions between MOV10 and HIV-1 gag protein and for MOV10 packaging into HIV-1 virions (Abudu et al. 2012), while the MOV10 carboxyl terminal domain is required for anti-HIV-1 activity (Abudu et al. 2012) and has also been shown to be required for interactions with AGO2 and APOBEC3G (Liu et al. 2012). In sum,

the results suggest that MOV10 restricts the two most active transposable elements that populate the human genome.

We next tested if human MOV10 could restrict the retrotransposition of a codon-optimized synthetic mouse L1 (pCEPsmL1) (Han and Boeke 2004), a natural mouse L1 (pG_F21) (Goodier et al. 2001), or a zebrafish LINE-2 (pZfL2-2) (Sugano et al. 2006). Human MOV10 inhibited the retrotransposition of human L1 (pJM101/L1.3; ~18% of control levels), synthetic mouse L1 (pCEPsmL1; ~30% of control levels), natural mouse L1 (pG_F21; ~8% of control levels), and zebrafish LINE-2 (pZfL2-2; ~10% of control levels) (Figure 4.2D). The L1 restriction-defective MOV10 mutant, MOV10/Δ912-1003, did not significantly affect the retrotransposition activity of these retrotransposons (Figure 4.2C). The increased activity of the synthetic mouse L1 compared to the natural mouse L1 in the presence of MOV10 is likely due to the increased RNA and protein expression, and or the increased G/C sequence content reported for the synthetic mouse L1 (Han and Boeke 2004). Thus, the data suggest that MOV10-mediated restriction of retrotransposition is not specific to human non-LTR retrotransposons.

Depletion of endogenous MOV10 enhances L1 retrotransposition

To test if endogenous MOV10 is capable of modulating L1 retrotransposition activity, we used small interfering RNA (siRNA) to deplete endogenous MOV10 from HeLa cells. Following siRNA treatment, cells were transfected with an L1 plasmid (pLRE3-*mEGFPI*) tagged with an *EGFP* indicator cassette (*mEGFPI*), which allows retrotransposition activity to be detected by EGFP fluorescence (Ostertag et al. 2000). As a negative control, HeLa cells were transfected with the L1 retrotransposition-defective plasmid pJM111-LRE3-*mEGFPI*, which carries two missense mutations that adversely affect ORF1p RNA binding (Moran et al. 1996, Martin et al. 2005, Khazina and Weichenrieder 2009). Treatment of HeLa cells with an siRNA pool against MOV10 resulted in an ~85% reduction of MOV10 protein levels in HeLa cell lysates when compared to HeLa cells treated with a non-targeting control siRNA pool (Figure 4.3A; left

panel). MOV10 siRNA treatment led to an approximately two-fold increase in pLRE3-*mEGFPI* retrotransposition activity when compared to assays conducted in the presence of a control siRNA (Figure 4.3A; right panel and Figure 4.3B). Notably, similar increases in L1 retrotransposition in response to knockdown of endogenous MOV10 have been reported elsewhere (Arjan-Odedra et al. 2012, Goodier et al. 2012). Thus, the data suggest that endogenous levels of MOV10 can affect L1 retrotransposition activity.

The effect of MOV10 on the expression of L1 RNA

To investigate how MOV10 inhibits L1 retrotransposition, we analyzed the effect of MOV10 overexpression on L1 RNA expression. HeLa cells were co-transfected with pJM101/L1.3Δneo and either MOV10 or MOV10/Δ912-1003 (Figure 4.4A). Polyadenylated RNA from whole cell extracts then was analyzed by northern blot using RNA probes complementary to sequences within the L1.3 5' UTR (5UTR99) and ORF2 (ORF2_5804) (Figure 4.4A). Co-transfection of pJM101/L1.3Δneo with MOV10 resulted in a reduction of full-length polyadenylated L1 RNA levels (~39% of control levels) compared to cells co-transfected with an empty pcDNA3 control vector (Figure 4.4B; blue arrow). Notably, co-transfection with the restriction-defective MOV10/Δ912-1003 also resulted in a similar reduction in full-length L1 RNA (~43% of control levels). Both MOV10 and MOV10/Δ912-1003 also had a pronounced effect on the accumulation of the smaller L1 RNA species that may have resulted from cryptic splicing and/or premature polyadenylation (Figure 4.4B; green and orange arrows) (Perepelitsa-Belancio and Deininger 2003, Belancio et al. 2006, Belancio et al. 2008). MOV10 and MOV10/Δ912-1003 reduced the accumulation of the smallest ~1.5 kb L1 RNA species to ~21% and 20% respectively (Figure 4.4B; orange arrow) while the ~2.3 kb L1 RNA species remained unaffected by either MOV10 construct. Control experiments revealed that ectopic MOV10 expression did not affect endogenous actin RNA levels (Figure 4.4B). Thus, the expression of MOV10 and the restriction-deficient MOV10/Δ912-1003 reduces the accumulation of L1 RNA in HeLa cells.

MOV10 affects the expression of L1 ORF1p and ORF2p

We next examined the effect of MOV10 expression on the expression of the L1-encoded proteins, ORF1p and ORF2p. HeLa cells were co-transfected with either MOV10 or MOV10/Δ912-1003 and the L1 plasmid, pJBM2TE1, which expresses an L1.3 element marked with a T7 *gene10* epitope tag on the carboxyl-terminus of ORF1p and a TAP epitope-tag on the carboxyl-terminus of ORF2p (Figure 4.5A). Following co-transfection, HeLa cells were treated with puromycin to select for cells expressing pJBM2TE1. Both whole cell lysates (WCL) and RNP fractions were collected 5 days post-transfection and subjected to western blot analyses to monitor ORF1p and ORF2p expression levels.

Expression of both MOV10 and MOV10/Δ912-1003 led to a marked decrease in the level of ORF1p and ORF2p in both WCL and RNP fractions compared to cells co-transfected with an empty pcDNA3 control vector (Figure 4.5B). Quantification of protein expression in the RNP fraction revealed that MOV10 reduced ORF1p expression to ~19% of control levels and reduced ORF2p expression to ~17% of control levels (Figures 4.5B and 4.5C). MOV10/Δ912-1003 had slightly less of an effect reducing ORF1p expression to ~30% of control levels and ORF2p to ~43% of control levels in RNP fractions (Figures 4.5B and 4.5C). Notably, the reduction in ORF1p and ORF2p appeared most evident in the RNP fraction, likely because both ORF1p and ORF2p are enriched in RNPs (Hohjoh and Singer 1996, Hohjoh and Singer 1997, Kulpa and Moran 2005, Kulpa and Moran 2006, Doucet et al. 2010). Control experiments revealed that MOV10 expression did not affect the level of endogenous eIF3 protein (Figure 4.5B). Additional control experiments verified the overexpression of MOV10 in cells transfected with the MOV10 plasmid (~12% higher MOV10 expression in MOV10-transfected cells compared to untransfected controls) and the mutant MOV10/Δ912-1003 protein in cells transfected with the MOV10/Δ912-1003 plasmid.

To determine if MOV10 affects the expression of non-L1 proteins, we examined the effect of MOV10 on EGFP expression. Briefly, HeLa cells were co-

transfected with MOV10 and an L1 plasmid (pLRE3-EF1-*mEGFP*ΔIntron) (Wissing et al. 2011) that expresses both LRE3 and EGFP from convergent promoters (Figure 4.5C). Forty-eight hours post-transfection, flow cytometry was used to isolate EGFP-positive cells (*i.e.,* cells expressing pLRE3-EF1-*mEGFP*ΔIntron) (Figure 4.5E). Western blotting demonstrated similar reductions in ORF1p and EGFP levels in cells that were co-transfected with MOV10 or MOV10/Δ912-1003 (Figure 4.5D) compared to cells co-transfected with the empty pCEP4 vector. Control experiments revealed that MOV10 did not affect endogenous tubulin protein levels (Figure 4.5D) and that MOV10 and MOV10/Δ912-1003 were expressed at similar levels in whole cell lysates. Thus, the data suggest that MOV10 and the restriction-defective MOV10/Δ912-1003 mutant perturb the accumulation of L1 proteins and possibly other cellular proteins.

The Effect of MOV10 on L1 LEAP activity

MOV10 RNA helicase activity is thought to remodel RNP complexes (Gregersen et al. 2014). Thus, we thought that MOV10 could interfere with L1 RNP function. In order to determine if MOV10 affected L1 RNP function, we used the LINE-1 element amplification protocol (LEAP) (Kulpa and Moran 2006) to test the effect of MOV10 on L1 ORF2p reverse transcriptase activity. Briefly, HeLa cells were transfected with the L1-expressing plasmid, pJM101/L1.3Δneo, and ultracentrifugation was used to isolate L1 RNPs from transfected HeLa cell lysates. The LEAP assay was then used to test the effect of recombinant MOV10 protein (FLAG-rMOV10) on ORF2p reverse transcriptase activity (Figure 4.6A). In control pJM101/L1.3Δneo LEAP reactions that did not contain FLAG-rMOV10, a band of ~220 bp (the expected size of the PCR product amplified by the LEAP PCR primers) was detected in agarose gels (Figure 4.6B). The addition of increasing amounts of FLAG-rMOV10 protein (up to 2 micrograms) or heat-inactivated FLAG-rMOV10 to pJM101/L1.3Δneo LEAP reactions did not affect LEAP activity (Figure 4.6B). Thus, these data suggests that FLAG-rMOV10 does not affect L1 LEAP activity. Alternatively, it is possible that the FLAG-rMOV10 used in this assay was not biologically active (*e.g.*, did not possess RNA helicase

activity). Additional experiments will be required to determine if MOV10 affects L1 LEAP activity.

Discussion

We demonstrated that the RNA helicase, MOV10 inhibits human L1 and Alu retrotransposition, as well as the retrotransposition of LINE elements from mice and zebrafish. It is worth mentioning that during the course of this work several studies were published that also showed that human MOV10 restricts human L1 retrotransposition (Arjan-Odedra et al. 2012, Goodier et al. 2012, Li et al. 2013). Our data are consistent with these analyses and demonstrates that human MOV10 also inhibits the retrotransposition of non-human LINE elements from mouse and zebrafish, and that mouse MOV10 was able to restrict the activity of a human L1. Thus MOV10-mediated restriction of non-LTR retrotransposons is a conserved function of MOV10.

Our data suggest that MOV10 associates with L1 ORF1p by binding to L1 RNA. Immunofluorescence microscopy showed that MOV10 co-localizes with L1 ORF1p in the cytoplasm (Figure 4.1B) and co-immunoprecipitation experiments revealed that the interaction between L1 ORF1p and MOV10 is dependent on RNA (Figures 3.1D and 4.1A). Recent studies have demonstrated that MOV10 co-localizes with ORF1p in the cytoplasm and that association between MOV10 and ORF1p are dependent on RNA (Goodier et al. 2012, Goodier et al. 2013). Critically, a recent study that used PAR-CLIP (Photoactivatable Ribonucleoside Enhanced-Crosslinking and Immunoprecipitation) experiments to identify cellular RNAs that bind to MOV10 demonstrated that MOV10 binds directly to endogenous L1 RNA in cultured human cell lines (Goodier et al. 2012, Goodier et al. 2013, Gregersen et al. 2014). Thus, these data indicate that MOV10 likely binds to L1 RNA in order to mediate L1 restriction.

Previous studies have demonstrated that the MOV10 helicase domain is required to restrict L1 retrotransposition (Goodier et al. 2012, Li et al. 2013). Notably, RNA helicases also may contain terminal accessory domains that

provide additional functions, such as protein binding (Jankowsky 2011). Herein, we showed that MOV10 mutants that lacked either the MOV10 CH domain (MOV10/Δ100-355) or the MOV10 carboxyl terminal domain (MOV10/Δ912-1003) were unable to efficiently restrict human L1 retrotransposition, suggesting that MOV10 domains outside of the MOV10 core helicase domain are required to restrict L1 retrotransposition. Recent evidence suggests that the MOV10 CH domain is necessary for MOV10 anti-HIV-1 activity, and that the CH domain is required for interactions with HIV-1 gag protein and for MOV10 packaging into HIV-1 virions (Abudu et al. 2012). Additionally, the carboxyl region of MOV10 is important for anti-HIV-1 activity (Abudu et al. 2012) and has been demonstrated to mediate protein-protein interactions with APOBEC3G and AGO2 (Liu et al. 2012). We also tested the effects of other RNA helicases that interact with L1 ORF1p on L1 retrotransposition and found that none of these other ORF1p-interacting helicases were able to restrict L1 retrotransposition. These data imply that RNA helicase activity by itself is not sufficient to restrict L1 activity and further suggest that the MOV10 CH and carboxyl terminal domains are required for efficient L1 restriction.

How does MOV10 inhibit retrotransposition? Our data demonstrated that MOV10 restricts the accumulation of L1 RNA (Figure 4.4B) and L1 proteins (Figures 4.5B, 4.5C, and 4.5D). Indeed, recent studies have shown that MOV10 prevents the accumulation of L1 RNA and L1-encoded proteins (Goodier et al. 2012, Li et al. 2013) and that MOV10 contributes to the post-transcriptional degradation of MOV10-targeted cellular mRNAs (Gregersen et al. 2014). These data suggest that MOV10 prevents the accumulation of the L1 RNA and/or L1-encoded proteins. The mechanism by which MOV10 prevents the accumulation of L1 RNA and/or the L1-encoded proteins, however, requires further investigation.

Surprisingly, the MOV10 mutant lacking the MOV10 carboxyl terminal region (MOV10/Δ912-1003) inhibited the expression of L1 RNA (Figure 4.4B) and the L1-encoded proteins (Figures 4.5B, 4.5C, and 4.5D) just as effectively as wild type MOV10. These data suggest that the MOV10 carboxyl terminal region

may not be required to restrict the expression of L1 RNA and/or L1-encoded proteins. Notably, these results demonstrate the importance of using mutant proteins (*i.e.*, MOV10/Δ912-1003) with a defined phenotype in comparative biochemical analyses, as opposed to an empty vector control (*e.g.,* pcDNA3). Whereas MOV10/Δ912-1003 was unable to efficiently inhibit L1 retrotransposition, MOV10/Δ912-1003 inhibited Alu retrotransposition to ~20% of control levels (Figure 4.2C). These data suggests that the MOV10 carboxyl terminal region may not be required for efficient inhibition of Alu retrotransposition. That MOV10/Δ912-1003 restricts the expression of L1 RNA and/or proteins also is consistent with the ability of MOV10/Δ912-1003 to inhibit Alu retrotransposition, as the L1 proteins are required for Alu retrotransposition. In sum, the above data suggest that MOV10-mediated inhibition of L1 RNA and L1-encoded protein expression *per se* is insufficient to explain how MOV10 restricts L1 retrotransposition and suggest the possibility that the MOV10 carboxyl terminal region provides an additional function independent of the reduction of L1 RNA and/or proteins that is necessary to inhibit L1 retrotransposition.

As a possible explanation for how MOV10 restricts L1 retrotransposition, I propose that MOV10 associates with L1 RNA and sequesters the L1 RNA and/or L1-encoded proteins (*i.e.,* L1 RNPs) in the cytoplasm, thereby preventing L1 RNPs from accessing genomic DNA. A similar hypothesis has been proposed to explain APOBEC3G-mediated Alu restriction wherein, A3G is thought to sequester Alu RNA into high molecular mass cytoplasmic RNA complexes that contain A3G, MOV10 and a variety of other RNA binding proteins (Chiu et al. 2006). Relevant to this possibility, the MOV10 carboxyl terminal domain is required to inhibit HIV-1 activity (Abudu et al. 2012). Additionally, the MOV10 carboxyl terminal domain has been shown to mediate interactions with other cellular proteins such as AGO2 and APOBEC3G (A3G) (Liu et al. 2012) and MOV10 has recently been shown to interact directly with UPF1 (Gregersen et al. 2014). AGO2 and UPF1 interact with cytoplasmic SGs, which are cytoplasmic RNA complexes that are involved in mRNA metabolism (Buchan and Parker

2009, Decker and Parker 2012). Notably, SGs also have been hypothesized as a defense mechanism against L1 retrotransposition (Goodier et al. 2007). Thus, it is conceivable that the MOV10 carboxyl terminal domain could be important for mediating interactions with other cellular factors that help sequester L1 RNPs and/or Alu RNA in larger RNP complexes such as SGs to prevent L1 RNPs from entering the nucleus (Figure 4.7).

Methods

Cell Lines and Culture Conditions

HeLa-JVM cells were grown in high-glucose DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin-streptomycin (Invitrogen), and 0.29mg/mL L-glutamine (Gibco). HeLa-HA cells were grown in MEM (Gibco) with 10% FBS, 100 U/mL penicillin-streptomycin, 0.29 mg/mL L-glutamine, and 0.1 mM nonessential amino acids (Gibco). Cell lines were maintained at 37°C with 7% CO_2.

Plasmids

Oligonucleotide sequences used in this study and cloning strategies are available upon request. All human L1 plasmids contain the indicated fragments of L.3 (accession no. L19088) DNA cloned into pCEP4 (Invitrogen) unless otherwise indicated. All L1 and cDNA plasmids contain the CMV promoter unless otherwise noted. All plasmid DNA was prepared with a Midiprep Plasmid DNA Kit (QIAGEN).

The following cDNA plasmids were obtained from OriGene: DDX21 (SC108813); MOV10 (SC126015); MOV10L1 (SC304649); UPF1 (SC118343); USP10 (SC111097); MOV10 mouse (MC206654).

pJM101/L1.3, is a pCEP4-based plasmid that contains an active human L1 (L1.3) equipped with an *mneoI* retrotransposition indicator cassette (Dombroski et al. 1993, Moran et al. 1996, Sassaman et al. 1997)

pJM105/L1.3, is derivative of pJM101/L1.3 that contains a D702A (GAC to GCU) missense mutation in the RT active site of L1.3 ORF2 (Wei et al. 2001).

pCEP/GFP, is a pCEP4-based plasmid that contains the humanized enhanced green fluorescent protein (hrGFP) coding sequence from phrGFP-C (Stratagene) under the control of the pCEP4 CMV promoter.

pJJ101/L1.3, is a pCEP4-based plasmid that contains an active human L1 (L1.3) equipped with an *mblastI* retrotransposition indicator cassette (Kopera et al. 2011).

pJJ105/L1.3, is a derivative of pJJ101/L1.3 that contains a D702A missense mutation in the RT active site of L1.3 ORF2 (Kopera et al. 2011).

pJM101/L1.3Δneo, is a pCEP4-based plasmid that contains an active human L1 (L1.3) (Wei et al. 2001).

pJM105/L1.3Δneo, is derivative of pJM101/L1.3Δneo that contains a D702A missense mutation in the RT active site of L1.3 ORF2.

pCEPsmL1, is a pCEP-based plasmid that expresses a codon optimized full-length mouse element (derived from L1spa) that contains the *mneoI* indicator cassette (Han and Boeke 2004).

pAD2TE1: is similar to pJM101/L1.3 except that it was modified to contain a *T7 gene10* epitope-tag on the carboxyl-terminus of ORF1p and a TAP epitope-tag on the carboxyl-terminus of ORF2p. The 3′-UTR contains the *mneoI* retrotransposition indicator cassette.

pJBM2TE1: is similar to pAD2TE1 except that the pCEP4 backbone was modified to contain the puromycin resistance (PURO) gene in place of the hygromycin resistance gene.

pLRE3-*mEGFPI*: is a pCEP4-based plasmid that contains an active human L1 (LRE3) equipped with an *mEGFPI* retrotransposition indicator cassette. The pCEP4 backbone was modified to contain a puromycin resistance (PURO) gene in place of the hygromycin resistance gene. The CMV promoter also was deleted from the vector; thus, L1 expression is driven only by the native 5′ UTR.

pJM111-LRE3-*mEGFPI*: is identical to pLRE3-*mEGFPI* except that it contains two missense mutations in ORF1 (RR261-262AA), which render the L1 retrotransposition-defective. Mr. William Giblin (University of Michigan Medical School) constructed the plasmid.

pZfL2-2: is a pCEP4 based plasmid that contains the ZfL2-2 ORF (ZL15, accession no. AB211150) cloned upstream of the *mneoI* indicator cassette.

pLRE3-EF1-*mEGFP*ΔIntron: is a pBSKS-II+ based plasmid that expresses an active human L1 (LRE3) that is tagged with an *EGFP* cassette (*mEGFPI*) containing an antisense, intronless copy of the EGFP gene. A UbC promoter drives EGFP expression. An EF1α promoter drives L1 expression (Wissing et al. 2011).

pG_F21, contains a 8.8-kb fragment containing a full length mouse TGf21 L1 element that contains the *mneoI* indicator cassette (Goodier et al. 2001).

MOV10/Δ912-1003, was derived by deleting the *Sma*I-*Sma*I fragment from MOV10 (OriGene, SC126015).

MOV10/Δ100-355, was derived by deleting the *Eco*RV-*Eco*RV fragment from MOV10 (OriGene, SC126015).

L1 Retrotransposition Assays

The cultured cell retrotransposition assay was carried out essentially as described (Moran et al. 1996, Wei et al. 2000). For retrotransposition assays with L1 constructs tagged with *mblastI*, HeLa-JVM cells were seeded at 1×10^4 cells/well in a 6-well plate (BD Falcon™). Within 24 hours, each well was transfected with 1 µg of plasmid DNA (0.5 µg L1 plasmid + 0.5 µg cDNA plasmid or pCEP4) using 3 µL of FuGENE® 6 transfection reagent (Promega). Four days post-transfection, media containing blasticidin (EMD Millipore) (10 µg/mL) was added to cells to select for retrotransposition events. Media was changed every two days. After ~8 days of selection, cells were washed with PBS, fixed, and then stained with crystal violet to visualize colonies. To control for transfection efficiency and off-target effects of cDNA plasmids, in parallel to retrotransposition

assays, HeLa-JVM cells were plated in 6-well dishes at 500 cells/well and transfected with 0.5 µg pcDNA6T/R (Invitrogen) plasmid + 0.5 µg cDNA plasmid using 3 µL of FuGENE® 6 transfection reagent (Promega). The pcDNA6T/R control assays were treated with blasticidin in the same manner as for retrotransposition assays.

For retrotransposition assays with L1 constructs tagged with *mneoI*, HeLa-JVM cells were transfected as described above. Two days after transfection, cells were treated with media supplemented with G418 (Gibco) (500µg/mL) for ~10-12 days. As a control, HeLa cells were plated at 1×10^4 cells/well in a 6-well plate and transfected with 0.5 µg pcDNA3 (Invitrogen) plasmid + 0.5 µg cDNA plasmid using 3 µL of FuGENE® 6 transfection reagent (Promega). The pcDNA3 control assays were treated with G418 in the same manner as for retrotransposition assays.

Alu Retrotransposition Assays

For Alu retrotransposition assays, ~4×10^5 HeLa-HA cells were plated per well of a 6-well plate (BD Falcon™) and transfected with 0.67 µg of pJM101/L1.3Δneo + 0.67 µg of pAlu*neo*^{Tet} + 0.67 µg of cDNA plasmid using 6 µL FuGENE® HD (Promega). Three days post-transfection, cells were grown in the presence of G418 (500µg/mL) to select for Alu retrotransposition events. As a control, HeLa-HA cells were plated at ~4×10^5 cells/well in a 6-well plate and transfected with 0.67 µg of pcDNA3 (Invitrogen) + 0.67 µg of pAlu*neo*^{Tet} + 0.67 µg of cDNA plasmid using 6 µL of FuGENE® HD (Promega). The pcDNA3 control assays were treated with G418 in the same manner as for Alu retrotransposition assays.

siRNA knockdown and pLRE3-*mEGFPI* retrotransposition assays

In experiments to study the effect of endogenous proteins on L1 retrotransposition, HeLa cells (~8×10^5 cells) were plated in 60 mm tissue culture dishes (BD Falcon™). The next day, the cells were transfected with 50 nM of a control siRNA pool (D-001810-10, ON-TARGETplus Non-targeting Pool, Thermo Scientific) or siRNA against MOV10 (L-014162-00-0005, ON-TARGETplus

Human MOV10 (4343) siRNA - SMARTpool, Thermo Scientific) using the DharmaFECT 1 transfection reagent (Thermo Scientific). Twenty-four hours after siRNA treatment, cells were transfected with pLRE3-*mEGFPI* or pJM111-LRE3-*mEGFPI* (5 µg), using 15 µL of FuGENE® HD transfection reagent (Roche). After 48 hours, cells were trypsinized and an aliquot of the cells (~2×10^6 cells) was used to monitor endogenous protein levels (72 hours after siRNA treatment) by western blot analysis (see below for list of primary antibodies). Blots were analyzed using an Odyssey® CLx (LI-COR) with the following secondary antibodies: IRDye 800CW Donkey anti-Rabbit IgG (1:10,000) (LI-COR) and IRDye 680RD Donkey anti-Mouse IgG (1:10,000) (LI-COR). Knockdown efficiencies were calculated using LI-COR Image Studio Software (v3.1.4) and are the average of three independent experiments. Endogenous tubulin was used as the normalization control. The remaining cells were re-plated at ~2×10^5 cells/well of a 6-well plate and cultured in medium supplemented with puromycin (5 µg/ml, Gibco/Life Technologies) to select for cells transfected with pLRE3-*mEGFPI*. After 4 days of puromycin selection, the percentage of GFP positive cells was determined by flow cytometry using an Accuri™ C6 flow cytometer (BD Biosciences).

RNP Isolation

RNPs were isolated as previously described (Kulpa and Moran 2006). Briefly, HeLa-JVM cells were seeded onto 60 mm tissue culture dishes (BD Falcon™) and 24 hours later cells were co-transfected with 2.5 µg of pJBM2TE1 and 2.5 µg of the indicated cDNA plasmid using 15 µL of FuGENE® HD (Promega). Approximately two days after transfection, puromycin (5 µg/mL) was added to culture medium to select for cells transfected with pJBM2TE1. After ~3 days of puromycin selection (5 days after transfection), cells were lysed in RNP lysis buffer (150 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 1mM DTT, 0.1% NP-40, and 1x complete EDTA-free protease inhibitor cocktail (Roche)). Following lysis, whole cell lysates were centrifuged at 12,000xg for 10 minutes at 4°C, and then the cleared lysate was layered onto a sucrose cushion (8.5% and 17% sucrose) and subjected to ultracentrifugation at 4°C for 2 hours

at 178,000xg. The supernatant was discarded and the resulting pellet was resuspended in water supplemented with 1x complete EDTA-free protease inhibitor cocktail (Roche). Approximately 20 µg (total protein) of the RNP sample or ~30 µg (total protein) of the cleared whole cell lysate (supernatant post 12,000xg centrifugation) were then analyzed by western blot. Blots were analyzed using an Odyssey® CLx (LI-COR) with the following secondary antibodies: IRDye 800CW Donkey anti-Rabbit IgG (1:10,000) (LI-COR) and IRDye 680RD Donkey anti-Mouse IgG (1:10,000) (LI-COR).

To simultaneously analyze the effects of MOV10 on ORF1p and EGFP protein expression, HeLa-JVM cells were seeded onto 10 cm dishes (~2.7×10^6 cells/dish) (BD Falcon™) and transfected with 10 µg of plasmid DNA (5.0 µg pLRE3-EF1A-*mEGFP*ΔIntron + 5.0 µg cDNA plasmid or pCEP4) using 30 µL of FuGENE® HD. After 48 hours, cells were harvested with trypsin and then subjected to flow cytometry to isolate GFP expressing cells. Approximately 1.2-1.7×10^6 GFP positive cells were collected for each transfection condition using a MoFlo® Astrios™ cell sorter (Beckman Coulter). The GFP gate was set using untransfected HeLa-JVM cells. The sorted cells were lysed as described in the IP procedure and lysates were then subjected to western blotting using standard procedures. For all other protein expression analyses, HeLa-JVM cells were seeded at ~4×10^5 cells/well in 6-well plates and transfected with 2 µg of plasmid DNA with 6 µL of FuGENE® HD. Cells were collected 48 hours after transfection using a rubber policeman and lysates were prepared as described above. Western blots were visualized using either the SuperSignal West Femto Chemiluminescent Substrate (Pierce) or SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Hyperfilm ECL (GE Healthcare).

Northern Blots

HeLa-JVM cells were seeded in T-175 flasks (BD Falcon™) and transfected with 20 µg of plasmid DNA (10 µg pJM101/L.13Δneo + 10 µg cDNA plasmid) using 60 µL FuGENE® HD. Two days after transfection, cell pellets were collected and frozen at -80°C. Frozen cell pellets were then thawed and

total RNA was extracted with TRIzol® reagent (Ambion), and then poly(A)+ RNA was prepared from total RNA using an Oligotex mRNA kit (Qiagen). Each sample (~1.5 µg of poly(A)+ RNA) was subjected to glyoxal gel electrophoresis and northern blotting using the NorthernMax®-Gly Kit (Ambion) according to the manufacturer's protocol. Following electrophoresis, RNA was transferred to BrightStar® Nylon membranes (Invitrogen) and then cross-linked using UV light. For northern blot detection, membranes were prehybridized for ~4 hours at 68°C in NorthernMax® Prehybridization/Hybridization Buffer (Ambion), and then incubated with a strand specific RNA probe (final concentration of probe ~3×10^6 cpm ml^{-1}) overnight at 68°C.

Strand-specific RNA probes were generated using the MAXIscript® T3 system (Invitrogen). The 5UTR99 probe corresponds to bases 7-99 of the L1.3 5' UTR and the ORF2_5804 probe corresponds to nucleotides 5560-5804 of the L1.3 sequence. RNA probe templates for T3 reactions were generated by PCR using pJM101/L1.3Δneo as a PCR template with the following primers:

5UTR99 (Forward): 5'-GGAGCCAAGATGGCCGAATAGGAACAGCT-3'

5UTR99 (Reverse): 5'-<u>AATTAACCCTCAAAGGG</u>ACCTCAGATGGAAATGCAG-3'

ORF2_5804 (Forward): 5'-GACACATGCACACGTATGTTTATT-3'

ORF2_5804 (Reverse): 5'-<u>AATTAACCCTCACTAAAGGG</u>TGAGTGAGAATATGCGGTGTTT-3'

The T3 promoter sequence (<u>underlined</u>) was added to the reverse primer of each primer pair. The pTRI-β-actin-125-Human Antisense Control Template (Applied Biosystems) was used in T3 reactions as a template to generate the β-actin RNA probe. Each northern blot experiment was independently repeated three times with similar results.

Immunofluorescence Microscopy

Cells were plated on round glass cover slips (Fisher) in a 12-well plate or into 4-well chambered glass slides (Fisher) and transfected ~24 hours later with

0.5 µg of plasmid DNA using 1.5 µL of FuGENE® 6 transfection reagent. To visualize proteins, approximately 48 hours post-transfection cells were washed with 1x PBS, fixed with 4% paraformaldehyde for 10 minutes and then treated with ice-cold methanol for 1 minute. Next, cells were incubated for 30 minutes at 37°C in 1x PBS + 3% BSA. Cells were then incubated with primary antibodies in 1x PBS + 3% BSA for 1 hour at 37°C. Cells were then washed thrice with 1x PBS (10 minutes per wash) and then incubated with appropriate fluorescently-labeled secondary antibodies diluted in 1x PBS for 30 minutes at 37°C. The following secondary antibodies were used for indirect immunofluorescence: Alexa Fluor 488 conjugated Goat anti-Mouse and Goat anti-Rabbit (Invitrogen) (1:1000), Alexa Fluor 546 conjugated Goat anti-Mouse and Goat anti-Rabbit IgG (Invitrogen) (1:1000), and Cy5 conjugated Donkey anti-Rabbit IgG (H+L) (Jackson ImmunoResearch) (1:100). To obtain images, a cover slip and/or slide was visually scanned and representative images were captured using a Leica SP5X confocal microscope (63x/1.4 objective; section thickness 1 µm).

L1 Element Amplification Protocol (LEAP) Assay.

The LEAP assay was previously described (Kulpa and Moran 2006). Briefly, approximately 6×10^6 HeLa cells were seeded into T175 flasks (BD Falcon) and transfected with 20 µg pJM101/L1.3 plasmid using FuGENE® HD (Roche) the following day. Selection with 200 µg/mL hygromycin B began 72 hours post-transfection. Approximately 9 days after transfection, cells were lysed [1.5 mM KCl, 2.5 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.5), 1% deoxycholic acid, 1% Triton X-100, and 1× protease inhibitor EDTA-free mixture (Roche)] and subjected to sucrose cushion (8.5% and 17% sucrose) ultracentrifugation at 4°C for 2 hours at 178,000 × g. Following ultracentrifugation, the supernatants were discarded and RNP pellets were resuspended in 1× protease inhibitor EDTA-free mixture (Roche), and protein concentration was determined by the Bradford reagent assay (BioRad). Prior to starting LEAP reactions, 1 µg of L1 RNPs was pre-incubated with up to 2.0 µg recombinant MOV10 (OriGene) in buffer (25 mM Tris-HCl; pH7.3, 100 mM Glycine, 10% glycerol) and incubated at 37°C for 20 minutes. To start the LEAP reaction, the RNP/MOV10 mixture was combined

and incubated with the LEAP reaction mixture [50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl2, 10 mM DTT, 20 U RNasin (Promega), and 0.05% Tween-20] containing HPLC-purified RACE adapters (0.4 μM) and dNTPs (0.2 mM)], and then the LEAP reaction was incubated at 37°C for 1 hour. One microliter of LEAP reaction was then amplified by PCR using primers specific for the transfected L1 (sense: 5′-GGGTTCGAAATCGATAAGCTTGGATCCAGA-3′) and the adapter (RACE: 5′-GCGAGCACAGAATTAATACGACT-3′).

L1 Element Amplification Protocol (LEAP) Assay.

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Recombinant FLAG-tagged MOV10 (FLAG-rMOV10) immunoprecipitation

HeLa-JVM cells were seeded in T-175 flasks (BD Falcon™) at ~6-8×10^6 cells/flask and transfected the next day with 20 µg of pJM101/L1.3 using 60 µL of FuGENE® HD (Promega). Approximately 48 hours post-transfection, hygromycin B (Gibco) (200 µg/mL) was added to the medium to select for transfected cells. After approximately one week of hygromycin selection, cells were washed 3 times with ice cold PBS and collected with a rubber policeman into 50 mL conical tubes (BD Falcon™). Cells were then pelleted at 1,000×g and frozen at -80°C. To produce whole cell lysates (WCL), frozen cell pellets were rapidly thawed and then lysed in ~3 mL (1 mL lysis buffer per 100 mg of cell pellet) of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% IGEPAL CA-630 (Sigma), 1X complete EDTA-free protease inhibitor cocktail (Roche)) on ice for 30 minutes. WCLs were then centrifuged at 15,000×g for 15 minutes at 4°C. Supernatants were transferred to a clean tube and protein concentration was determined using the Bradford reagent assay (BioRad). For immunoprecipitation reactions, ~1 mL of the supernatant (~3 mg total protein) was pre-cleared with ~15 µL (packed gel volume) EZview™ Red ANTI-FLAG® M2 Affinity Gel (Sigma). The pre-cleared lysates were then transferred to a fresh tube to which was added either 2 µg of FLAG-rMOV10 (OriGene) or 2 µg of FL-BAP (Sigma), and ~15 µL (packed gel volume) EZview™ Red ANTI-FLAG® M2 Affinity Gel. Immunoprecipitation reactions were incubated overnight at 4°C with rotation. The next day, the beads were washed 3 times with lysis buffer and protein complexes were eluted from the beads by boiling for 5 minutes in ~60 µL of 2x Laemmli buffer (Laemmli 1970).

Primary Antibodies

Polyclonal antibodies against peptide sequences 31-49 of L1.3 ORF1p (αORF1-N) were raised in rabbits and affinity-purified (Open Biosystems). αGFP (2955) was obtained from Cell Signaling Technology. αMOV10 (953-1003) (NB100-77314) was obtained from Novus Biologicals. αHA (ab9110) was obtained from Abcam. αMOV10 (111-125) (SAB1100141), αFLAG, and

αTubulin (T9026) were obtained from Sigma. αeIF3 (p110) (sc-28858) was obtained from Santa Cruz Biotechnology. αTAP rabbit polyclonal (CAB1001) was obtained from Thermo Scientific. αT7-Tag mouse monoclonal (69522-3) was obtained from Novagen.

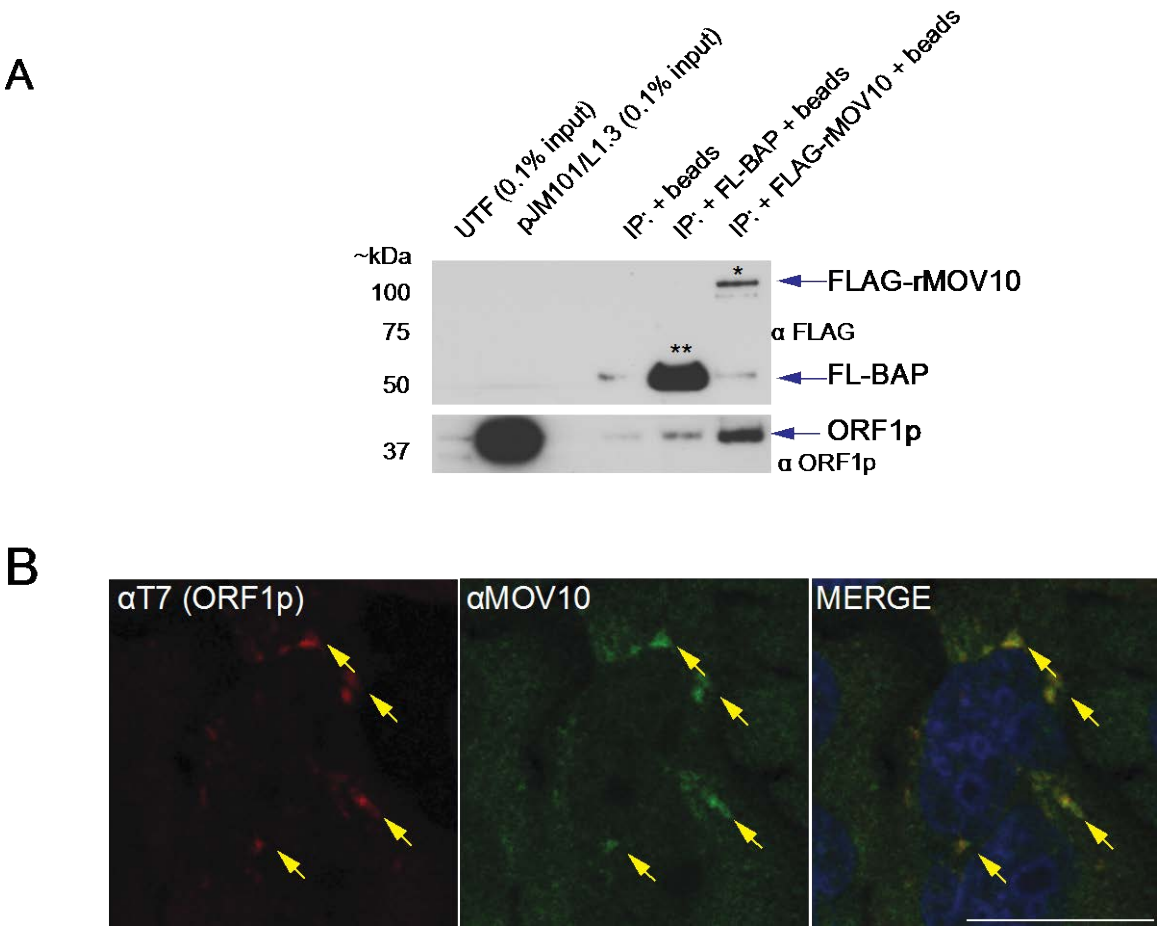

Figure 4.1: MOV10 associates with L1 ORF1p in HeLa cells.

(A) *pJM101/L1.3 immunoprecipitation reactions with FLAG-tagged recombinant MOV10 (FLAG-rMOV10):* HeLa cells were transfected with pJM101/L1.3 and immunoprecipitation (IP) reactions were carried out by incubating whole cell lysates with αFLAG coated magnetic beads plus FLAG-rMOV10 or a negative control FLAG-tagged bacterial alkaline phosphatase (FL-BAP) fusion protein. UTF = untransfected HeLa lysates; pJM101/L1.3 = HeLa cell lysates transfected with pJM101/L1.3 (input); beads = immunoprecipitation reactions (IP) with beads alone. Single asterisk (*) = FLAG-rMOV10 protein band; double asterisk (**) = FL-BAP peptide band (~49.1 kDa). Experiment was performed once (n=1). (B) T7-tagged ORF1p (red) expressed from pAD2TE1 co-localizes with endogenous MOV10 (green) in cytoplasmic foci (yellow arrows) in HeLa cells. αT7-Tag antibodies were used to detect T7-tagged ORF1p (red) and αMOV10 antibodies used to detect endogenous MOV10 (green). Right-most image represents merged image. Nuclei stained with DAPI (blue); scale bar = 25 μM. Experiment was repeated three times with similar results.

Figure 4.2: MOV10 restricts LINE and Alu retrotransposition.

(A) MOV10 inhibits L1 retrotransposition: Top panel: Schematics of MOV10 constructs. Depicted are the relative positions of the cysteine-histidine (CH) domain (light gray rectangles), helicase domain (brown rectangles), and APOBEC3G/AGO2 interaction domains (yellow rectangles). The MOV10/Δ912-1003 construct contains an additional 4 amino acids (VASL) after MOV10 amino acid 911. Middle panel: Results of retrotransposition assays. The X-axis indicates the cDNA co-transfected with pJJ101/L1.3 or pcDNA6/TR. The Y-axis indicates pJJ101/L1.3 retrotransposition activity (black bars), or pcDNA6/TR colony formation activity (white bars). All values have been normalized to the pCEP4 empty vector control (100%). The numbers above the bar graphs indicate the number of independent experiments performed with each cDNA. Error bars represent standard deviations. Bottom panel: A single well of a representative six-well tissue culture plate, displaying blasticidin-resistant colonies from the pJJ101/L1.3 retrotransposition assay (top, black rectangle) and the pcDNA6/TR control assay (bottom, white rectangle). *(B) Transfected MOV10 is expressed in HeLa cells:* Western blots of whole cell lysates transfected with the indicated MOV10 constructs (above blots) ~48 hours post-transfection. UTF indicates untransfected HeLa cell lysates. Blue arrows indicate the approximate locations of the MOV10 proteins. Tubulin serves as a loading control. Molecular weight standards (~kDa) are shown on the left side of the blots. *(C) MOV10 inhibits Alu retrotransposition:* The X-axis indicates the cDNA co-transfected with pJM101/L1.3Δneo and pAlu*neo*^{Tet}. The Y-axis indicates Alu retrotransposition efficiency. All values are normalized to the pCEP4 empty vector control (100%). Control assays using a plasmid that expresses the neomycin phosphotransferase gene (pcDNA3) were conducted similarly to pcDNA6/TR control assays. Representative images of G418-resistant HeLa foci from the Alu retrotransposition assay are shown below the bar graph. The results are the average of three independent experiments. Error bars indicate standard deviations. *(D) MOV10 inhibits the retrotransposition of mouse and zebrafish LINE elements.* The X-axis indicates the cDNA that was co-transfected with human L1 (pJM101/L1.3 (black bars)), synthetic mouse L1 (pCEPsmL1 (grey bars)), mouse L1 (pG_F21 (light grey bars)), or zebrafish LINE-2 (pZfL2-2 (white bars)). The Y-axis indicates the retrotransposition efficiency. Representative images of G418-resistant HeLa cell foci are shown below the bar graph. All values are normalized to the pCEP4 empty vector control (100%). Error bars indicate standard deviations.

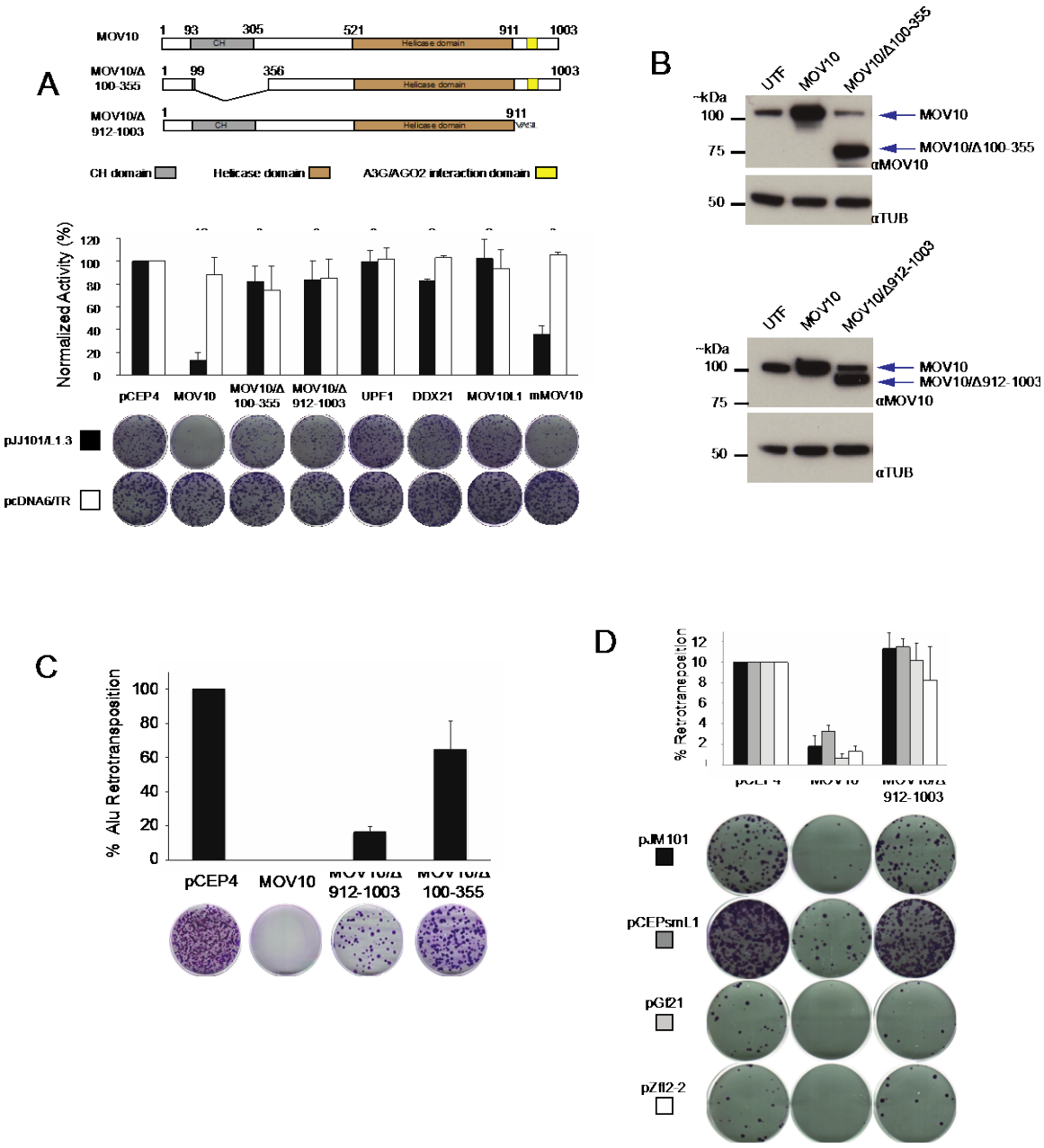

Figure 4.2: MOV10 restricts LINE and Alu retrotransposition.

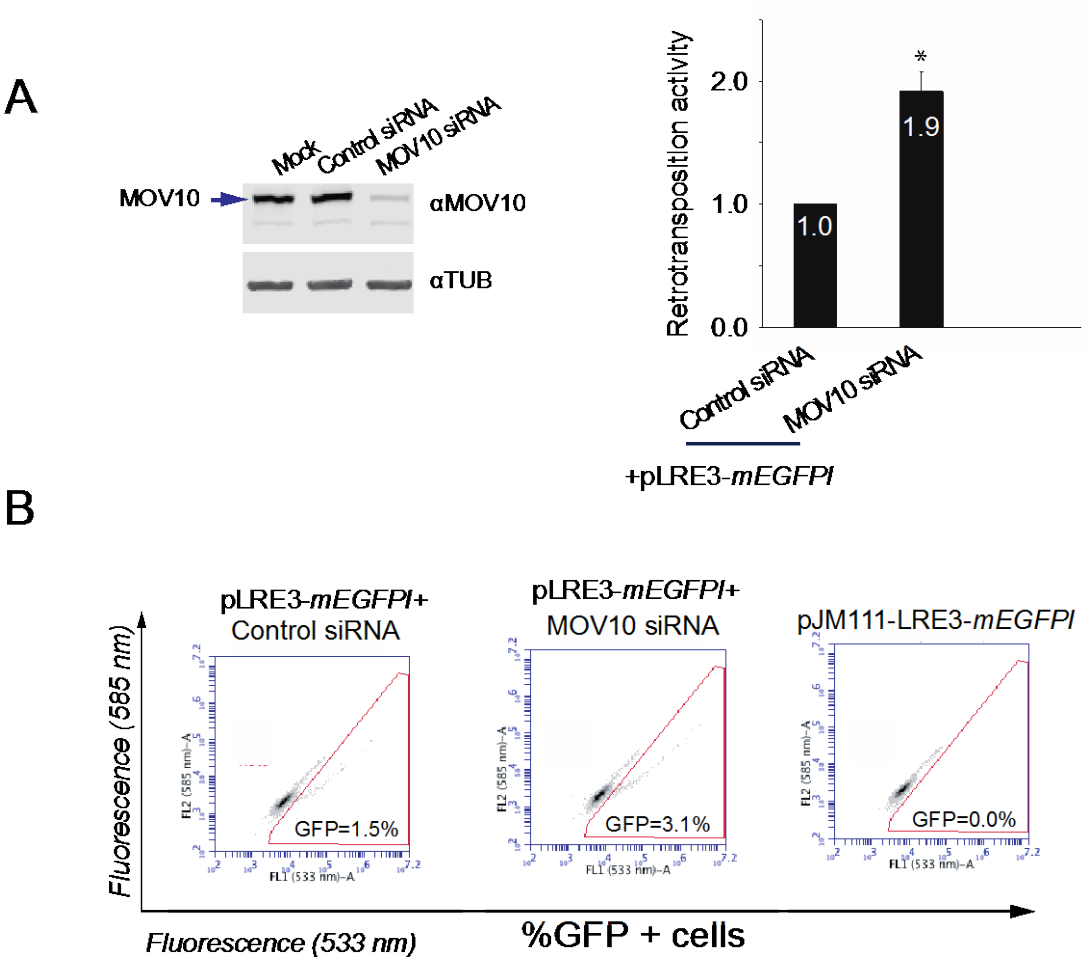

Figure 4.3: Endogenous MOV10 affects L1 retrotransposition.

(A) The depletion of MOV10 enhances L1 retrotransposition: Left panel: Western blots of whole cell lysates from HeLa cells transfected with indicated siRNAs. The blue arrow points to the approximate location of endogenous MOV10. Right panel: The bar graph depicts pLRE-*mEGFP1* retrotransposition activity following siRNA treatment. The X-axis indicates the siRNA. The Y-axis indicates the pLRE-*mEGFP1* retrotransposition efficiency normalized to the control siRNA (set to 1). Retrotransposition efficiency values are reported as the mean from four independent experiments. Error bars indicate the standard deviations. Asterisks indicate statistically significant differences from the control siRNA experiments (two-tailed t test/$p<0.05$). *(B)* Flow cytometry was used to determine the percentage of EGFP-positive, live-gated cells for each siRNA transfection condition (noted above the plots). The X-axis depicts the scattering at 533 nm; the Y-axis depicts the scattering at 585 nm. The EGFP-positive gate was set using the retrotransposition-deficient negative control, pJM111-LRE3-*mEGFPI*.

Figure 4.4: The effect of MOV10 on L1 RNA expression.

(A) Schematic of pJM101/L1.3Δneo: Bold black lines indicate the approximate location of probes (5UTR99 and ORF2_5804) used in the northern blot experiments. pJM101/L1.3Δneo is expressed from a pCEP4 vector. A CMV promoter augments L1 expression *(B) Results of northern blots:* HeLa cells were co-transfected with pJM101/L1.3Δneo and either the indicated MOV10 expression plasmids or an empty pCEP4 vector. Northern blot images depict the effect of MOV10 overexpression on polyadenylated L1 RNA levels. The constructs transfected into HeLa cells are indicated above each lane. UTF indicates untransfected HeLa cells and serves as a negative control. Probes (5UTR99 and ORF2_5804) are indicated in the top left corner of the respective blots. The blue arrow indicates the position of the full-length L1 RNA and green and orange arrows indicate shorter L1 RNA species. The yellow numbers above the northern blot bands (left blot) indicate the normalized band intensities (given in % and normalized to band in the pcDNA3 control lane). Actin served as a loading control. RNA size standards (~kb) are shown at the right of the blot image. MOV10 northern blot experiment was performed twice with three biological replicates.

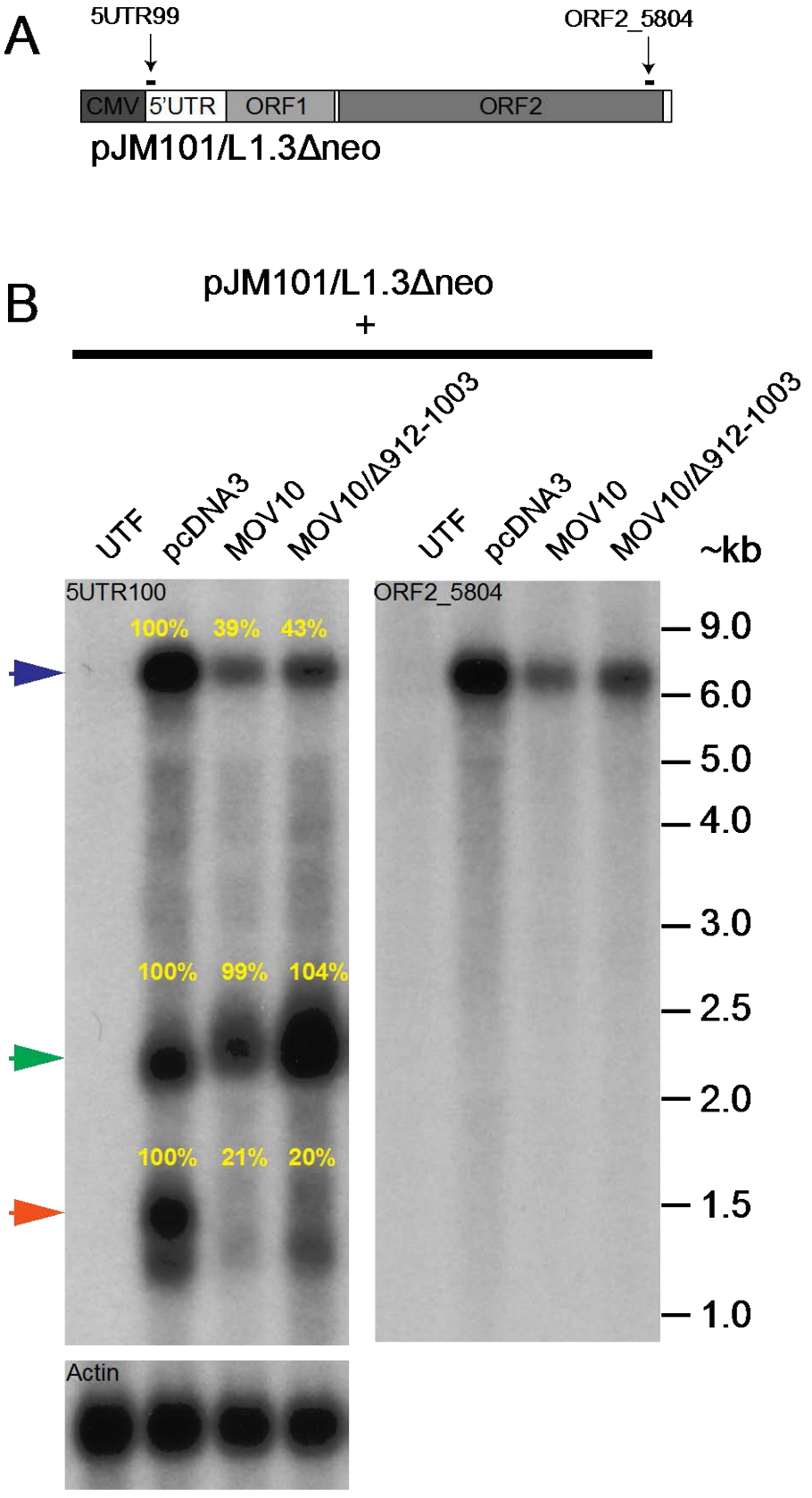

Figure 4.4: The effect of MOV10 on L1 RNA expression.

Figure 4.5: The effect of MOV10 on L1 protein expression.

(A) Schematic of pJBM2TE1: The construct contains a T7 epitope tag on the carboxyl-terminus of ORF1p and a TAP tag on the carboxyl-terminus of ORF2p. An *mneoI* retrotransposition indicator cassette is present in the 3' UTR. pJMB2TE1 is expressed from a pCEP4 backbone, which has been modified to contain a puromycin selectable marker. A CMV promoter augments L1 expression. *(B) MOV10 decreases the accumulation of the L1-encoded proteins*: HeLa cells were co-transfected with pJBM2TE1 and the plasmids indicated above each lane. UTF indicates untransfected HeLa cells and serves as a negative control. Depicted are western blots using whole cell lysates (WCL, top panel) or RNP fractions (RNP, bottom panel). Blue arrows indicate the positions of ORF2p, ORF1p, MOV10, and MOV10/Δ912-1003. The eIF3 protein is used as a loading control. Representative images are shown. The experiments were repeated two times with similar results. (C) Quantification of ORF1p (dark grey bars) and ORF2p (white bars) protein expression from RNP fractions. The X-axis indicates the MOV10 construct co-transfected with pJBM2TE1 and the Y-axis indicates the protein expression level (normalized band intensity) normalized to pcDNA3 controls. The experiment was performed twice with 4 biological replicates. Error bars indicate standard deviations. (D) Top panel: *A schematic of the pLRE3-EF1-mEGFPΔIntron*: pLRE3-EF1-*mEGFP*ΔIntron expresses a human L1 (LRE3) that is tagged with an *mEGFPI* expression cassette that lacks an intron. The human elongation factor-1 alpha (EF1α) promoter (arrow) augments L1 transcription. The ubiquitin C (UbC) promoter (upside down arrow) drives EGFP transcription. Bottom panel: *MOV10 inhibits ORF1p and EGFP expression:* Western blots were conducted using whole cell lysates derived from cells co-transfected with pLRE3-EF1-*mEGFP*ΔIntron and the MOV10 expression plasmid or pCEP4 indicated above each lane. UTF indicates whole cell lysates from untransfected HeLa cell. Antibodies are indicated on the right side of each blot. Blue arrows indicate ORF1p, EGFP, MOV10, and MOV10/Δ912-1003 proteins. Tubulin is used as a loading control. Western blot images depict a representative experiment that was repeated three times with similar results. *(E)* Flow cytometry was used to determine the percentage of EGFP-positive, live-gated cells for each condition. UTF indicates untransfected HeLa cells. The EGFP-positive gate was set using the UTF sample as a negative control. The X-axis depicts the percentage of EGFP positive cells. The Y-axis indicates the side scattering profile (SSC). Approximately 1.2 - 1.7 x 10^6 GFP positive cells were collected and analyzed for each transfection condition.

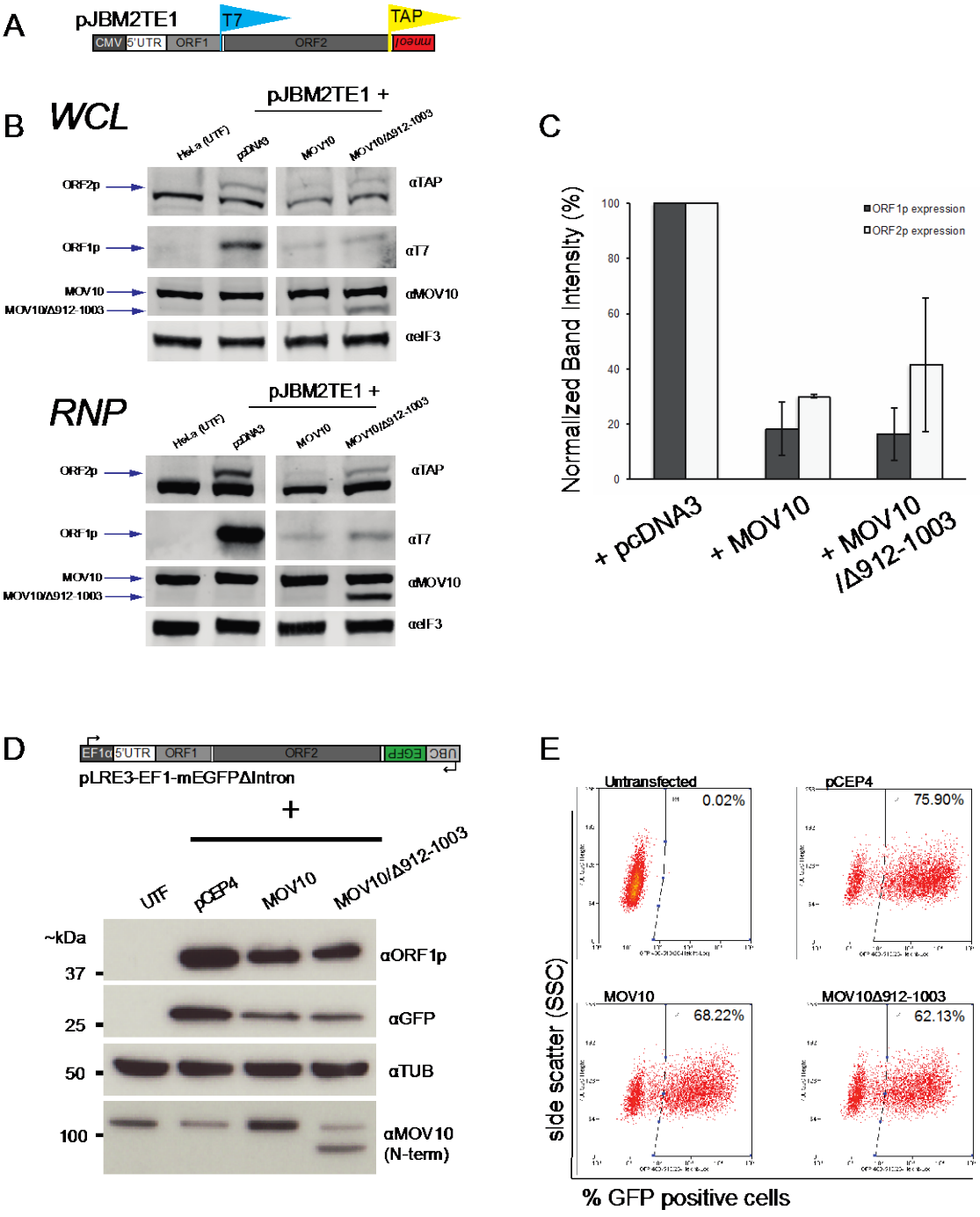

Figure 4.5: The effect of MOV10 on L1 protein expression.

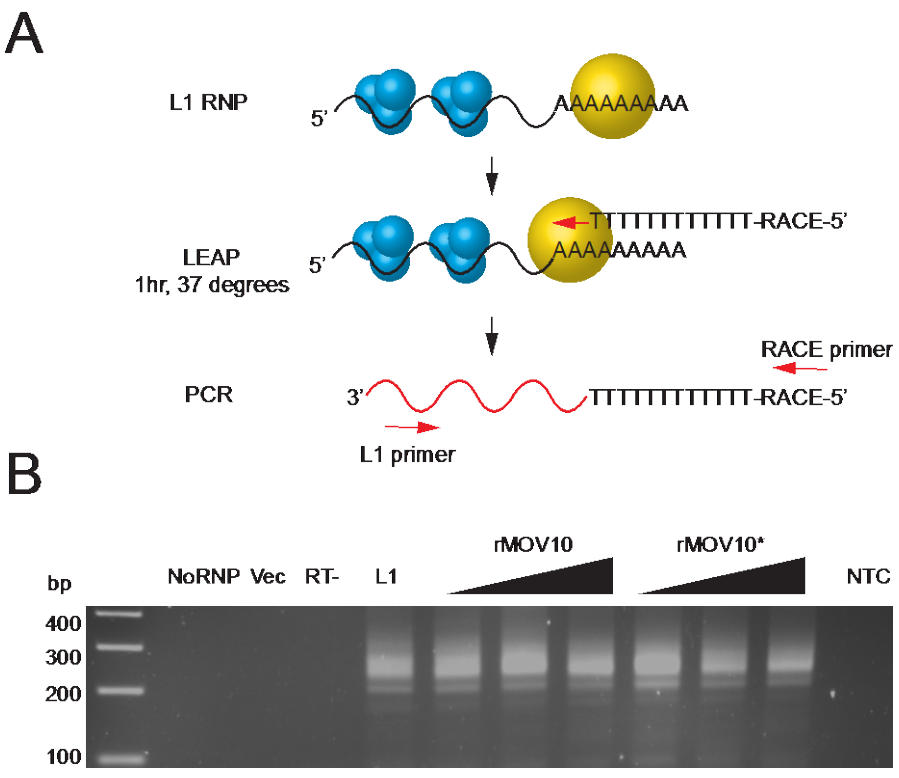

Figure 4.6: The effect of recombinant MOV10 on L1 reverse transcriptase activity.

(A) *LEAP assay description:* L1 reverse transcriptase activity is measured by the LINE-1 element amplification protocol (LEAP). HeLa cells are transfected with L1 plasmid (pJM101/L1.3Δneo) and L1 RNPs are isolated using differential centrifugation. In the schematic, L1 RNPs (ORF1p (blue spheres), ORF2p (yellow spheres) and L1 RNA) are combined with a LEAP adapter which serves as a primer for ORF2p to reverse transcribe L1 cDNA (red) using the L1 mRNA as a template. PCR with a RACE primer and a pJM101/L1.3 specific L1 primer is subsequently used to amplify an L1 cDNA leap product of ~220 bps. *(B) LEAP activity is unaffected by recombinant MOV10:* For LEAP reactions, 1 µg of L1 RNPs were pre-incubated with 0.5, 1, or 2 µg of recombinant FLAG-Tagged MOV10 (FLAG-rMOV10) or FLAG-rMOV10 that was heat inactivated at ~95°C for 10 minutes (FLAG-rMOV10*) in LEAP reaction buffer (minus RACE adapter primer) for 20 min at 37°C. To begin the LEAP reaction, the RACE adapter primer was added to LEAP reactions, which were then incubated fro 37°C for 1 hour. NoRNP = LEAP reaction minus L1 RNPs, Vec = pCEP4 vector, L1 = pJM101/L1.3Δneo, (RT-) = L1 mutant (pJM105/L1.3Δneo) that contains a missense mutation in the L1 RT domain (pJM105/L1.3Δneo), NTC = no template PCR control (H_2O). LEAP experiments were performed twice with similar results.

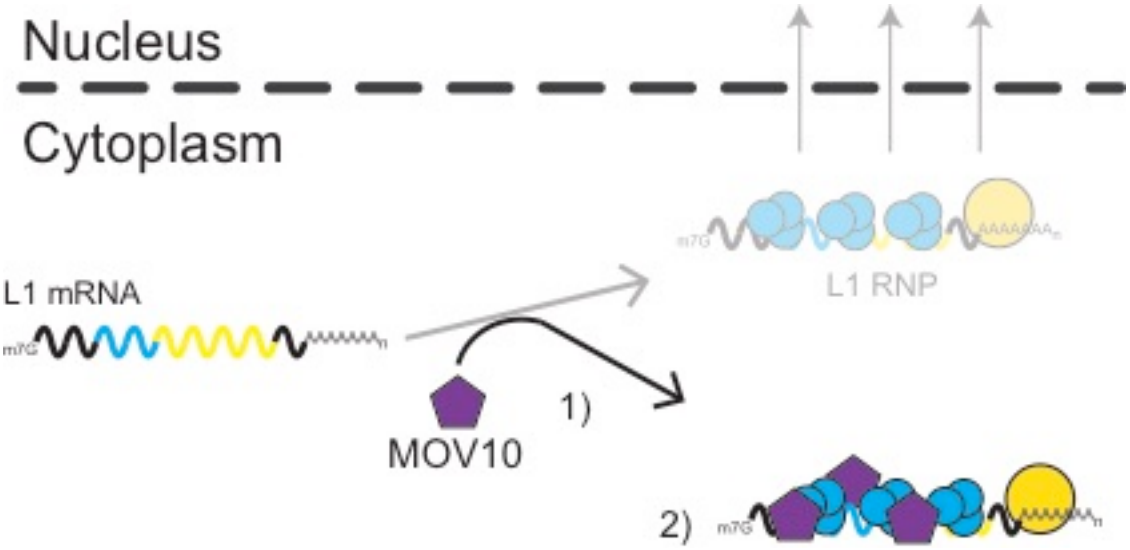

Figure 4.7: Working-model of MOV10-mediated restriction.

1) MOV10 (purple pentagon) binds to L1 RNA in the cytoplasm and 2) sequesters L1 RNA and L1 ORF1p (blue circles) and L1 ORF2p (yellow circles) in the cytoplasm preventing L1 RNPs from accessing genomic DNA in the nucleus.

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Chapter 5

Conclusion

I have identified cellular host factors that associate with L1 ORF1p and showed that the zinc finger antiviral protein ZAP inhibits L1 retrotransposition in cultured HeLa cells. In Chapter 2, I found that mutations in several ORF1p functional domains negatively impact the formation of functional L1 RNPs. Notably, mutations of amino acid sequences that affect ORF1p RNA binding activity generally impaired the ability of ORF1p to localize to L1 RNPs and allowed reverse transcription of L1 RNA to begin within internal L1 RNA sequences. Thus, in agreement with previous studies (Kulpa and Moran 2005, Doucet et al. 2010), the ability of L1 ORF1p to bind to L1 RNA is necessary for the formation of functional L1 RNPs.

The studies in Chapter 2 lead me to hypothesize that other cellular host factors may associate with L1 RNPs to modulate L1 retrotransposition. Indeed, in Chapter 3, I identified cellular host factors that associate with L1 ORF1p. I then used a cultured cell assay to test the effect of over-expression of the ORF1p-associated proteins on L1 retrotransposition. Importantly, I discovered that the antiviral protein ZAP associates with L1 ORF1p and inhibits L1 retrotransposition. Mechanistic analyses demonstrated that ZAP binds to L1 RNA and prevents the accumulation of L1 RNA and proteins, providing insight into how ZAP inhibits L1 retrotransposition. In subsequent chapters, I further investigated the effects of other ORF1p-associated proteins including MOV10 (Chapter 4), PAR-4 (Appendix) and hnRNPL (Appendix) and showed that these proteins also inhibit L1 retrotransposition. In this final chapter I will discuss the significance of some of these findings and suggest possible future directions for this research.

The identification of ORF1p-associated host cell factors

The main tenet of my thesis is that host cell factors associate with L1 RNPs to modulate L1 retrotransposition. To test this hypothesis I used co-immunoprecipitation to purify ORF1p and associated host cell proteins from HeLa cells transfected with engineered human L1 constructs that expressed a version of ORF1p containing a carboxyl terminal FLAG (Hopp et al. 1988) epitope tag. Targeting ORF1p was thought to increase the likelihood of capturing cellular host factors associated with L1 RNPs because: 1) ORF1p is necessary for the formation of L1 RNPs (Kulpa and Moran 2005), and 2) ORF1p is more highly expressed than ORF2p (Doucet et al. 2010). Notably, the composition of a *bona fide* functional L1 RNP still remains unknown and there may exist a constellation of distinct L1 RNP complexes representing different stages in the L1 retrotransposition cycle and/or cellular fates of L1 RNPs (Kulpa and Moran 2005, Taylor et al. 2013).

In the future, it may be informative to study L1 RNPs in different cell types as the composition of L1 RNPs could vary depending on developmental stage or tissues type. For example, a comparison of L1 RNP complexes between cells that permit active L1 retrotransposition (*e.g.*, HeLa cells, hESCs, NPCs) and cell types that do not support L1 retrotransposition (*e.g.*, cell lines derived from primary cells such as fibroblast cell lines) could reveal distinct differences in the composition in L1 RNPs that may reveal differences in the basal L1 RNP complex or identify host factors that either restrict or facilitate L1 retrotransposition.

The ORF1p-FLAG co-immunoprecipitation experiments described in Chapter 3 were conducted on a small scale (*e.g.*, a singleT-175 flask of transfected HeLa cells provided sufficient material for several co-immunoprecipitation experiments). They did not require specialized reagents and all the steps leading up to mass spectroscopy could be easily accomplished in the lab. These methods, which built upon previous methods developed by former lab members (Doucet et al. 2010), have been adapted by other Moran Lab

members to facilitate the identification of cellular proteins that co-purify with L1 ORF2p in a variety of human and rodent cell lines. Other experimental protocols have recently been used to identify L1-interacting proteins in similar cultured human cell lines (Taylor et al. 2013). In contrast to the methods I describe in Chapter 3, this approach utilized inducible expression vectors to express epitope-tagged engineered L1 elements in transfected cells, which required large amounts of starting material (*i.e.*, multi-liter suspension cell cultures) and a specialized cryogenic milling process to prepare cell lysates for immunoprecipitation and mass spectroscopy (Taylor et al. 2013). The use of this approach resulted in the identification of 37 "high-confidence" L1-interacting proteins (Taylor et al. 2013), many of which also were identified as ORF1p-interacting proteins reported in Chapter 3. Thus, the experimental approach described in Chapter 3 provides a relatively simple and straightforward method to identify cellular host factors that associate with the L1-encoded proteins.

Many RNA binding proteins associated with ORF1p

ORF1p-FLAG co-immunoprecipitation experiments resulted in the identification of 39 ORF1p-interacting cellular host factors. Notably, 32/39 ORF1p-FLAG interacting proteins are annotated as RNA binding proteins (RBPs) according to gene ontology (Ashburner et al. 2000) and global analyses of RNA binding proteins in human cell lines (Baltz et al. 2012, Castello et al. 2012). I demonstrated that RNaseA disrupted the association between ORF1p and 13 of the western blot-verified ORF1p-interacting proteins (Fig. 3.1D). Thus, the majority of ORF1p-interacting proteins associate with ORF1p by binding to L1 RNA and/or other RNAs present within the L1 RNP (Mandal et al. 2013).

In addition to the ORF1p-interacting proteins identified in Chapter 3, recent studies also have identified a variety of RNA binding proteins that associate with L1-encoded proteins and/or RNA (Goodier et al. 2007, Doucet et al. 2010, Dai et al. 2012, Goodier et al. 2012, Goodier et al. 2013, Peddigari et al. 2013, Taylor et al. 2013, Zhang et al. 2014). ORF1p is primarily an RNA binding protein that preferentially binds to its encoding L1 RNA transcript (a phenomenon

referred to as *cis* preference) to form an L1 RNP (Martin 1991, Martin and Branciforte 1993, Hohjoh and Singer 1996, Hohjoh and Singer 1997, Kolosha and Martin 1997, Wei et al. 2001). It has been hypothesized that ORF1p coating the RNA may protect it from degradation (Martin 1991, Martin and Bushman 2001) by other cellular factors (*e.g.*, RNA decay factors). Indeed, immunofluorescence microscopy experiments have revealed that ORF1p associates with cytoplasmic RNA binding proteins in the cellular cytoplasm including components of stress granules (SGs) and processing bodies (Goodier et al. 2007, Doucet et al. 2010, Goodier et al. 2013), which are cytoplasmic RNP complexes involved in RNA metabolism and decay (Parker and Sheth 2007, Buchan and Parker 2009, Decker and Parker 2012). Notably, a recent study that examined the biochemical properties of ORF1p hypothesized that ORF1p polymerization prevents its diffusion into the cytoplasm upon translation, which helps to maintain a high local concentration of ORF1p in the vicinity of translating ribosomes and thus favors the association of ORF1p with L1 RNA and not other cytoplasmic RNAs (Callahan et al. 2012). It has also been hypothesized that ORF1p could compete with the translation machinery for the L1 RNA transcript (Kroutter et al. 2009). Following these assumptions one could speculate that ORF1p must compete with other cellular RNA binding proteins for binding L1 RNA. Thus, it is not surprising that L1 RNPs also contain numerous other cellular RNA binding proteins.

What is the significance of the cellular host factors that associate with ORF1p?

To determine if the ORF1p-interacting proteins identified in Chapter 3 were relevant to L1 retrotransposition, I tested whether the overexpression of the ORF1p-interacting proteins affected L1 retrotransposition in cultured human HeLa cells. Importantly, experiments in Chapter 3 showed that the zinc-finger antiviral protein ZAP is an RNA binding protein that restricts human L1 retrotransposition. I also showed that the overexpression of hnRNPL, MOV10, PURA, and PAR-4 inhibited L1 retrotransposition (Figures 3.2C, A1B and A2B). Notably, recent studies have also demonstrated that hnRNPL (Goodier et al.

2013, Peddigari et al. 2013), MOV10 (Arjan-Odedra et al. 2012, Goodier et al. 2012), and PURA (Goodier et al. 2013) inhibit L1 retrotransposition. Thus, certain proteins that interact with L1 RNPs may function to modulate L1 retrotransposition. My thesis data both confirm and extend previous analyses of L1-interacting host factors and will help guide future studies aimed at determining how L1 retrotransposition affects the human genome.

It is noteworthy that many of the ORF1p-interacting proteins that I identified did not significantly affect L1 retrotransposition (Figure 3.2C). This is interesting for several reasons. First, it suggests that simply overexpressing cellular protein in the retrotransposition assay does not adversely affect L1 retrotransposition. These results thus serve as additional negative controls to compare to the effects of ZAP or other cellular factors that restricted L1 retrotransposition. Second, these results suggest that although other RNA binding proteins associate with L1 ORF1p, most of them do not affect L1 activity when overexpressed in cultured cells. Notably, the cultured cell retrotransposition assay is more sensitive to detecting decreases in retrotransposition activity and is thus biased towards detecting restriction factors. Thus, it remains possible that some of the ORF1p-interacting proteins that did not appear to affect L1 retrotransposition could be important for efficient L1 retrotransposition *in vivo*. To determine if any of these ORF1p-interacting host factors are required for L1 retrotransposition, they could be knocked down by siRNA or newer CRISPR (clustered regularly interspaced palindromic repeats) based interference methods (Larson et al. 2013, Mali et al. 2013) to test if depletion of these proteins from cells results in decreased L1 retrotransposition efficiency. Indeed, experiments using siRNA methods have recently been used to show that nucleolin (Peddigari et al. 2013) and poly (A) binding proteins, PABPN1 and PABPC1 (Dai et al. 2012) may be needed for efficient L1 retrotransposition.

ZAP inhibits L1 retrotransposition

Perhaps the most significant finding of my thesis research is that ZAP restricts human L1 retrotransposition. ZAP also inhibited human Alu

retrotransposition as well as the activity of LINE elements from mice and zebrafish. Thus ZAP may function as a host factor that modulates L1 retrotransposition and the activity of other non-LTR retrotransposons. Notably, ZAP also inhibits retroviral activity (Gao et al. 2002). It would be interesting to determine if ZAP also inhibits the activity of other types of endogenous retrotransposons such as endogenous retroviruses, which bare similarity to retroviruses.

Does endogenous ZAP affect L1 activity?

In Chapter 3, I demonstrated that depletion of endogenous ZAP from HeLa cells resulted in an increase in L1 retrotransposition. These data suggest that endogenous levels of ZAP in HeLa cells affects L1 retrotransposition. I also showed that siRNA pools against MOV10 and hnRNPL mimicked previously published results showing that knockdown of these two proteins results in increased L1 activity (Arjan-Odedra et al. 2012, Goodier et al. 2012, Peddigari et al. 2013). These siRNA results are corroborated by genetic assays that show that over-expression of ZAP, MOV10 and hnRNPL inhibit L1 retrotransposition. Finally, fluorescence microscopy data demonstrated that L1 ORF1p co-localizes with endogenous and transfected ZAP in the cytoplasm of HeLa and PA-1 cells. In sum, these data suggest that physiological levels of ZAP in principle could affect L1 retrotransposition.

Future studies could address whether ZAP affects L1 retrotransposition in other cell types or *in vivo*. Indeed, L1 RNA and/or L1-encoded proteins are expressed in a variety of embryonic and adult somatic tissues (Ergun et al. 2004, Garcia-Perez et al. 2007, Faulkner et al. 2009, Belancio et al. 2010, Wissing et al. 2012). In addition, somatic L1 retrotransposition may have an etiological role in certain cancers (Miki et al. 1992, Iskow et al. 2010, Lee et al. 2012, Solyom et al. 2012, Shukla et al. 2013, Helman et al. 2014) and have the potential to contribute to neuronal diversity (Muotri et al. 2005, Coufal et al. 2009, Baillie et al. 2011, Evrony et al. 2012, Evrony et al. 2015, Upton et al. 2015). ZAP RNA is expressed in a variety of tissues, including in the brain and reproductive system

(Gao et al. 2002, Kerns et al. 2008). Notably, APOBEC3B, which has also been demonstrated experimentally to restrict L1 retrotransposition, is expressed in the germline and in human embryonic stem cells (hESCs) (Bogerd et al. 2006, OhAinle et al. 2006, Wissing et al. 2011). Furthermore, previous studies that demonstrated that APOBEC3B could inhibit L1 retrotransposition in HeLa cells (Bogerd et al. 2006) were subsequently verified in hESCs (Wissing et al. 2011). Thus, it is possible that ZAP functions to restrict L1 retrotransposition in tissues or cell types where there is evidence of somatic L1 retrotransposition.

To test whether ZAP, MOV10 or other ORF1p-associated cellular proteins affect L1 retrotransposition in a physiological context, future experiments could test whether the knockdown of ZAP affects L1 retrotransposition in physiologically relevant cell types such as neural progenitor cells (NPCs), human embryonic stem cells (hESCs), and/or induced pluripotent stem cells (iPSCs). Notably, it also may be possible to test if ZAP affects the expression of endogenous L1 RNA and/or proteins in these cell types because they actively express L1 RNA and proteins. To test whether ZAP, MOV10 and/or other host factors restrict L1 activity *in vivo*, tissues and/or cells that express endogenous L1 and/or support somatic retrotransposition (*i.e.*, tumors and brain cells) could be examined to determine whether there is a correlation between increased L1 activity and misexpression of any of these host cell factors.

How does ZAP bind to L1 RNA?

Data indicates that ZAP binding to L1 RNA is critical for L1 restriction; however, the general mechanism by which ZAP recognizes target RNAs is poorly understood. A ZAP consensus sequence/motif has not yet been determined, but evidence so far suggests that ZAP recognizes long RNA stretches (>500 nucleotides) and/or undefined RNA tertiary structure(s) (Guo et al. 2004, Chen et al. 2012). Notably, the ability of ZAP to inhibit non-human LINE elements (Figure 3.3C) suggests that ZAP may not recognize a particular LINE linear consensus RNA sequence, but instead may recognize an unidentified structural feature common to certain LINE RNAs.

In future ZAP studies, several approaches could be used to identify how ZAP binds to L1 RNA. For example, gel shift experiments with a series of L1 deletion mutants (*i.e.*, deletions in the L1 RNA sequence) to identify which L1 RNA sequences were necessary to bind ZAP. Alternatively, PARCLIP experiments also could be used to determine where ZAP binds to L1 RNA and perhaps other LINE and/or viral RNAs. Notably, a recent study using MOV10 PARCLIP experiments has revealed that MOV10 binds to L1 RNA (Gregersen et al. 2014).

How does ZAP inhibit L1 retrotransposition?

Biochemical and immunofluorescence microscopy data from Chapter 3 suggest that ZAP inhibits L1 retrotransposition by binding to and preventing the accumulation of full-length L1 RNA. Indeed, a number of studies have shown that ZAP inhibits retroviral activity primarily by binding to and recruiting other cellular factors to degrade the viral RNA (Guo et al. 2004, Zhu and Gao 2008, Zhu et al. 2011). In addition to affecting L1 RNA, it is also possible that ZAP may affect Alu RNA expression. This hypothesis could be tested by performing Alu Northern blots and/or qRT-PCR in the presence of transfected ZAP. Notably, similar experiments could be used to check the status of the zebrafish LINE RNA and/or mouse L1 RNA to determine if ZAP overexpression also affects RNA from non-human LINE elements.

I also showed that the over-expression of ZAP prevented the accumulation of L1 ORF1p and L1 ORF2p in HeLa cells (see Chapter 3). Notably, a recent study suggests that ZAP inhibits the translation of viral RNA, which may proceed independently and prior to ZAP-mediated viral RNA degradation (Zhu et al. 2012). These data therefore suggest the possibility that ZAP over-expression could in principle also affect L1 mRNA translation. This being the case I cannot definitively state whether L1 RNA degradation and/or the inhibition of L1 translation is primarily responsible for a loss of L1 proteins in the presence of ZAP.

How does ZAP degrade L1 RNA?

Evidence suggests that ZAP binds to viral RNAs and recruits exosome components and/or other cellular factors to degrade the viral RNA (Guo et al. 2004, Zhu and Gao 2008, Zhu et al. 2011). Experiments in Chapter 3 showed that ZAP co-localizes with the stress granule (SG) associated proteins, eIF3 and G3BP (Figures 3.10B and 3.10D), which is in agreement with previously published data showing that ZAP localizes to SGs (Leung et al. 2011). SGs have been suggested to regulate L1 retrotransposition (Goodier et al. 2007) and viral pathogenesis (Reineke and Lloyd 2013). The co-localization of ZAP and L1 ORF1p with SG components is therefore consistent with the hypothesis that ZAP recruits other cellular factors (*e.g.*, RNA degradation factors) to degrade L1RNA. This hypothesis is supported by the facts that SGs contain numerous cellular proteins involved in RNA metabolism, and that SGs exchange components with other cellular RNP complexes (*i.e.*, processing bodies) that may be directly involved with RNA degradation (Parker and Sheth 2007, Buchan and Parker 2009, Decker and Parker 2012). Thus, it is tempting to speculate that ZAP interacts with L1 RNA in the cytoplasm and recruits cellular factors involved in RNA metabolism to destroy L1 RNA and possibly block translation (Figure 3.7).

Notably, human ZAP has been reported to directly interact with two proteins involved in RNA degradation, the exosome component Rrp42 and the RNA helicase p72 (Chen et al. 2008, Zhu et al. 2011). In future studies, it would be interesting to test if knockdown of Rrp42, p72, or another cellular protein involved in RNA degradation reduces ZAP-mediated inhibition of L1 retrotransposition and/or the degradation of full-length L1 RNA. It would also be informative to determine if Rrp42, p72 and or other RNA decay proteins co-localize with ZAP and/or L1 RNA in the cellular cytoplasm. Notably, I tested whether transfected tGFP-tagged ZAP co-localized with endogenous Rrp42 and p72, but preliminary immunofluorescence microscopy experiments produced negative results (data not shown). This result could possibly be due to technical difficulties with reagents (*i.e.*, antibodies) and/or differences in cell lines and endogenous protein expression levels (in our case HeLa cells), and/or that

interactions between ZAP and these proteins are too transient to be detected by direct immunofluorescence. It may be worthwhile to continue to further optimize these experiments using different antibodies and/or cell lines. Alternatively, co-immunoprecipitation experiments could be used to test the interactions between ZAP and Rrp42 and/or other proteins involved in RNA degradation. These experiments would form an excellent starting point for an additional independent study on determining how ZAP inhibits retrotransposition.

RNA degradation vs. translation repression

To test whether ZAP affects L1 translation, ribosome profiling, which quantifies the number of ribosomes bound to a particular mRNA and thus serves as means to monitor mRNA translation (Ingolia et al. 2009), could be used to compare the kinetics of L1 RNA translation to L1 RNA expression in human cell lines that overexpress ZAP. Indeed, ribosome profiling experiments have been used to test whether certain microRNAs affect mRNA translation or mRNA decay in zebrafish embryos (Bazzini et al. 2012). A similar ribosome profiling experiment could be used to address the following question about L1 mRNA translation: are there fewer ribosomes associated with L1 mRNA in the presence of ZAP (Figure 5.1)? In this experiment, a decrease in the ratio of L1 RNA associated ribosomes to total L1 RNA levels in the presence of ZAP would indicate a translation defect (Figure 5.1; right panel). Alternatively, if the ratio of L1 RNA associated ribosomes to total L1 RNA is unaffected in the presence of ZAP, then it is likely that L1 translation is not affected (Figure 5.1; middle panel). Notably, ribosome profiling experiments or any kinetic study of L1 RNA and/or protein expression would necessitate the construction of stable cell lines that expressed inducible L1 and/or ZAP proteins to enable examination of L1 RNA expression over short (hours vs. days) time intervals.

PAR-4: an exception

PAR-4 was one of the few ORF1p-interacting proteins that was identified in Chapter 3 (Appendix and Table 3.1) that is not documented to bind to RNA. Co-immunoprecipitation experiments conducted in HeLa cells with transfected

ORF1p-FLAG demonstrated that RNase treatment was unable to disrupt the interaction between ORF1p-FLAG and PAR-4 (Appendix and Figure A1A) and cultured cell retrotransposition assays demonstrated that over-expression of PAR-4 inhibited both L1 retrotransposition and Alu retrotransposition in HeLa cells (Appendix and Figures A2B and A2C). Thus, the data suggest the possibility that PAR-4 directly interacts with ORF1p and restricts L1 retrotransposition.

How might PAR-4 interact with ORF1p? The carboxyl terminal domain of PAR-4 contains a coiled coil leucine zipper region that binds to other cellular factors, including atypical isoforms of protein kinase C (PKC), Wilms' tumor 1 (WT1) and DNA topoisomerase I (Diaz-Meco et al. 1996, Johnstone et al. 1996, Goswami et al. 2008, Hebbar et al. 2012). The amino-terminal domain of ORF1p is composed of a coiled-coil domain, which mediates polymerization of ORF1p trimers, the basic ORF1p functional unit (Martin et al. 2003, Basame et al. 2006, Khazina and Weichenrieder 2009, Khazina et al. 2011, Callahan et al. 2012). Sequence comparisons between the currently active human specific L1 family (L1PA1) and older L1 families (L1PA5-L1PA2) have revealed that the coiled-coil region of ORF1p may have undergone a brief period of positive selection between L1PA5 to L1PA3B as evidenced by a ratio of non-synonymous to synonymous mutations in the ORF1p coiled-coil region greater than 1 (Boissinot and Furano 2001). Importantly, this signature of positive selection could reflect an interaction between ORF1p and another cellular host factor (Boissinot and Furano 2001). Indeed, coiled-coils mediate direct interactions between a number of cellular proteins (Burkhard et al. 2001).

ORF1p structural data suggests that trimer formation is crucial for L1 retrotransposition as even the subtlest mutations that disrupt the ORF1p trimer have a devastating impact on retrotransposition (Khazina et al. 2011). Indeed, a direct interaction between ORF1p and another coiled-coil protein such as PAR-4 could perturb trimer formation and disrupt L1 RNP biogenesis or L1 RNP function. It would be interesting to further test the interaction between ORF1p and PAR-4, to determine whether these proteins interact directly and if so, how

these interactions influence ORF1p function and L1 retrotransposition. To further test the interaction between ORF1p and PAR-4, co-immunoprecipitation and/or immunofluorescence microscopy experiments using PAR-4 deletion mutants could be used to try to identify which PAR-4 domain is necessary to bind to ORF1p. Notably, I have engineered a series of PAR-4 mutants that could be useful in future PAR-4 experiments (see Appendix).

ZAP, MOV10, hnRNPL, and PAR-4 may function to modulate different steps of the L1 retrotransposition cycle

Several host cell mechanisms may function to modulate L1 retrotransposition such as DNA methylation, small RNA-mediated silencing pathways, splicing, and cellular antiviral factors (Levin and Moran 2011). My thesis results add to this knowledge and suggest that ZAP, MOV10, hnRNPL, and PAR-4 associate with L1 ORF1p to modulate discrete post-transcriptional steps in the L1 retrotransposition cycle (Figure 5.2). The data suggest that the antiviral protein, ZAP restricts L1 retrotransposition most likely by binding to the L1 RNA in the cellular cytoplasm, affecting the stability of full-length L1 RNA and/or expression of the L1-encoded proteins. The RNA helicase and antiviral protein, MOV10 (Burdick et al. 2010, Furtak et al. 2010, Wang et al. 2010, Abudu et al. 2012, Arjan-Odedra et al. 2012, Gregersen et al. 2014) also affected the expression of L1 RNA and/or the L1-encoded proteins. Despite these observations, a restriction defective MOV10 mutant lacking the MOV10 carboxyl terminal domain (MOV10/Δ912-1003) (see Chapter 4) also affected the expression of L1 RNA and/or L1-encoded proteins. These data therefore suggest the possibility that MOV10-mediated inhibition involves an additional mechanism, which I hypothesized to be the sequestration of L1 RNA and/or L1-encoded proteins in the cellular cytoplasm. Notably, a similar mechanism has been proposed to explain the A3G-mediated inhibition of Alu retrotransposition (Chiu et al. 2006) (see Chapter 4: Discussion). In contrast to the antiviral proteins ZAP and MOV10, hnRNPL is a nuclear RNA binding protein that affects RNA splicing of cellular RNAs. My data suggests that hnRNPL affects the normal processing of L1 RNA and prevents the accumulation of full-length L1 RNA. Finally, my data

suggested that PAR-4 binds directly to L1 ORF1p and inhibits retrotransposition. This data further suggests that PAR-4 likely targets a post-translational step in the retrotransposition cycle; however, how PAR-4 interacts with ORF1p and how PAR-4 inhibits L1 retrotransposition requires further investigation.

Clearly, the host cell diverts a diversity of resources to control L1 retrotransposition. These mechanisms are not active in any one cell type at the same time, nor is it the sole function of any one of these mechanisms to control L1 retrotransposition. DNA methylation, for example, is associated with gene regulation and also functions to silence transposable elements. Global DNA methylation patterns are not static as DNA methylation is reset in the germline and once again during embryogenesis, thus providing a window of opportunity for L1 and other transposable elements to be expressed at critical developmental stages. Indeed, a substantial body of evidence shows that defects in methylation result in the derepression of transposable elements with concomitant failures in gametogenesis and embryogenesis (Walsh et al. 1998, Bourc'his and Bestor 2004). The piRNA pathway is confined to the germline and functions to destroy RNA and may also be involved in DNA methylation of transposable elements (Aravin et al. 2007, Aravin et al. 2008, Watanabe et al. 2011). With this being stated, most mammalian piRNAs (~95% of pachytene piRNAs) are not derived from transposable elements (Fu and Wang 2014), implying some additional function for piRNAs besides silencing transposable elements. RNA splicing may participate in the post-transcriptional regulation of L1 RNA (Belancio et al. 2006, Belancio et al. 2008, Belancio et al. 2010). Notably, the degree of L1 splicing is highly variable across different tissues types and thus is likely regulated in a cell type specific manner (Belancio et al. 2010).

Finally, the different host cell factors that restrict L1 retrotransposition are not ubiquitously expressed in all cell types and/or tissues of the body (*e.g.*, ABOBEC3A is only expressed in certain cells of the immune system (Richardson et al. 2014), PIWI proteins are expressed in the germline (Siomi et al. 2011)) and some these host factors (i.e., APOBEC3 proteins (Schumann 2007, Chiu and Greene 2008), TREX1 (Stetson et al. 2008), MOV10 (Arjan-Odedra et al. 2012,

Goodier et al. 2012), SAMHD1 (Zhao et al. 2013), RNase L (Zhang et al. 2014)) also function as host cell antiviral factors. Thus it seems reasonable to conclude that an array of mechanisms is necessary to ensure L1 retrotransposition remains in check in different cell types and during different developmental stages.

Host antiviral factors inhibit L1 retrotransposition

It is interesting to note that a number of cellular host factors that restrict L1 retrotransposition were initially identified as antiviral restriction factors including, APOBEC3 proteins (Schumann 2007, Chiu and Greene 2008), TREX1 (Stetson et al. 2008), MOV10 (Arjan-Odedra et al. 2012, Goodier et al. 2012), SAMHD1 (Zhao et al. 2013), RNase L (Zhang et al. 2014) and now ZAP (Moldovan and Moran 2015). Antiviral restriction factors, which are part of the innate immune system, are proteins that inhibit some aspect of the viral replication cycle typically by recognizing viral components such as viral proteins or viral RNA (Goff 2004, Wolf and Goff 2008). Thus, unlike other innate immune factors such as toll like receptors which "sense" infections and trigger signaling cascades that turn on the transcription of other antiviral genes such as interferons, antiviral proteins directly subvert the viral replication process (Goff 2004, Yan and Chen 2012, Zheng et al. 2012). For example, A3G blocks HIV-1 activity by deaminating retroviral cDNA (Chiu and Greene 2008). Notably, A3A restricts L1 retrotransposition and has recently been shown to deaminate L1 cDNA (Richardson et al. 2014). Active RNase L cleaves viral RNA, which suppresses viral activity and recent evidence suggests RNase L may restrict endogenous retrotransposons principally by cleaving retrotransposon RNA (Zhang et al. 2014). ZAP has also been shown to target the destruction and translation of viral RNAs (Gao et al. 2002, Guo et al. 2007, Zhu et al. 2011, Zhu et al. 2012), which also may apply to L1 and other transposable elements (Moldovan and Moran 2015).

L1 retrotransposition has been active in mammalian genomes for at least 160 million years (Burton et al. 1986, Smit et al. 1995, Yang et al. 2014) and non-LTR retrotransposons like L1 are suggested to be the progenitors of eukaryotic

LTR retrotransposons and vertebrate retroviruses (Xiong and Eickbush 1990, Malik et al. 1999, Malik et al. 2000, Burke et al. 2002, Eickbush and Jamburuthugoda 2008). Interestingly, antiviral factors like the APOBEC3 proteins and SAMHD1 specifically antagonize lentiviruses like HIV-1 (Sheehy et al. 2002, Laguette et al. 2011) and paleovirology estimates that the ancient ancestors of modern primate lentiviruses only date back about 10 million years (Patel et al. 2011, Compton et al. 2013). Based on this information it is conceivable that L1 retrotransposition may have influenced the evolution of cellular host factors that were later co-opted as antiviral restriction factors (Sawyer et al. 2004, Sawyer et al. 2005, Sawyer and Malik 2006, Kerns et al. 2008, Moldovan and Moran 2015). Thus, studying L1 retrotransposition could provide insight into the immune system and viral susceptibility and help identify previously unknown host antiviral factors.

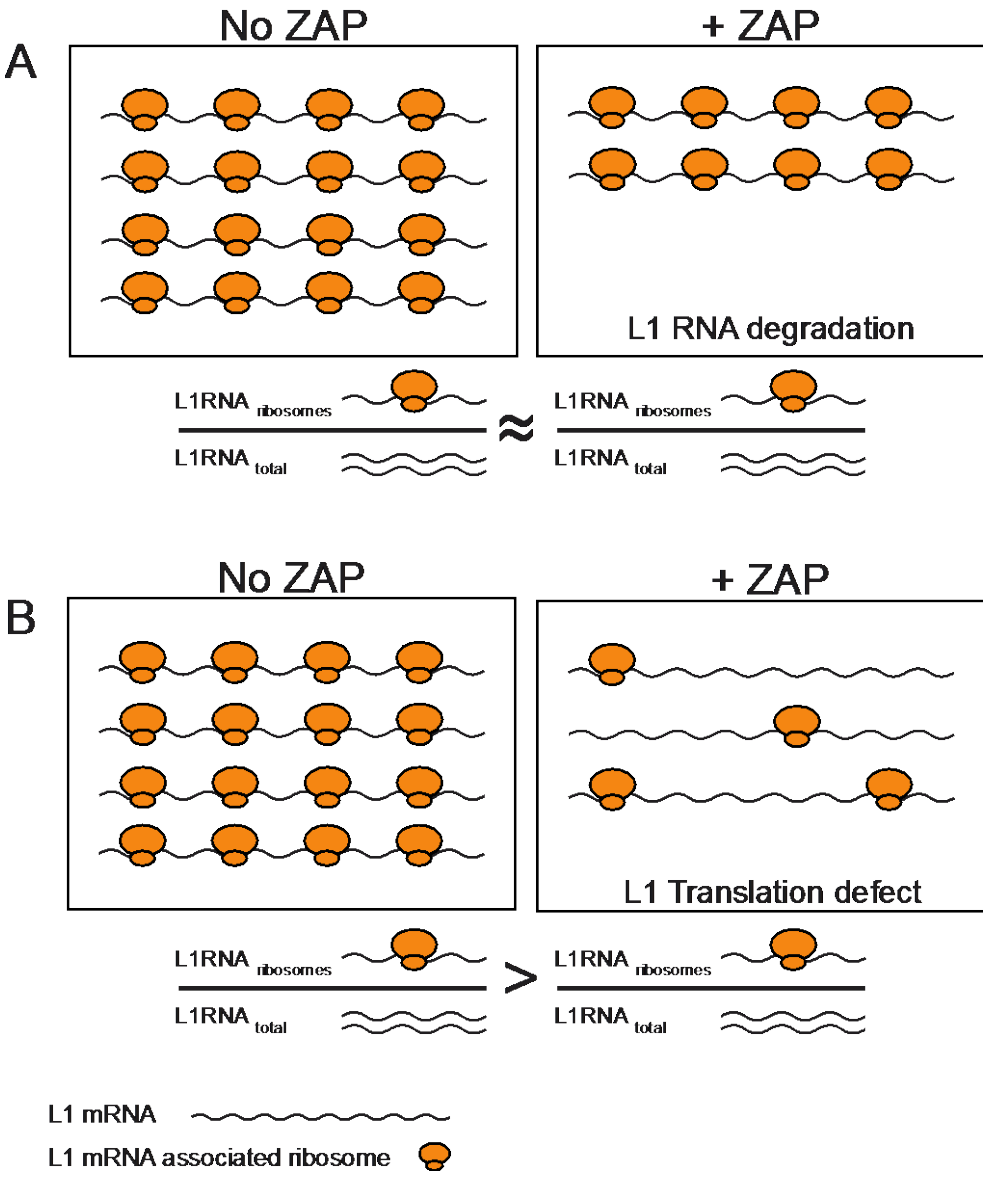

Figure 5.1: Schematic diagram of ZAP ribosome profiling experiment

Possible outcomes of ZAP ribosome profiling experiments: (A) In the presence of ZAP overexpression: no relative change in the ratio of L1 RNA associated with ribosomes to total L1 RNA (compared to no ZAP overexpression) may suggest that L1 RNA is degraded. (B) In the presences of ZAP overexpression: a decrease in the ratio of L1 RNA associated with ribosomes to total L1 RNA (compared to no ZAP overexpression) may suggest a defect in L1 translation.

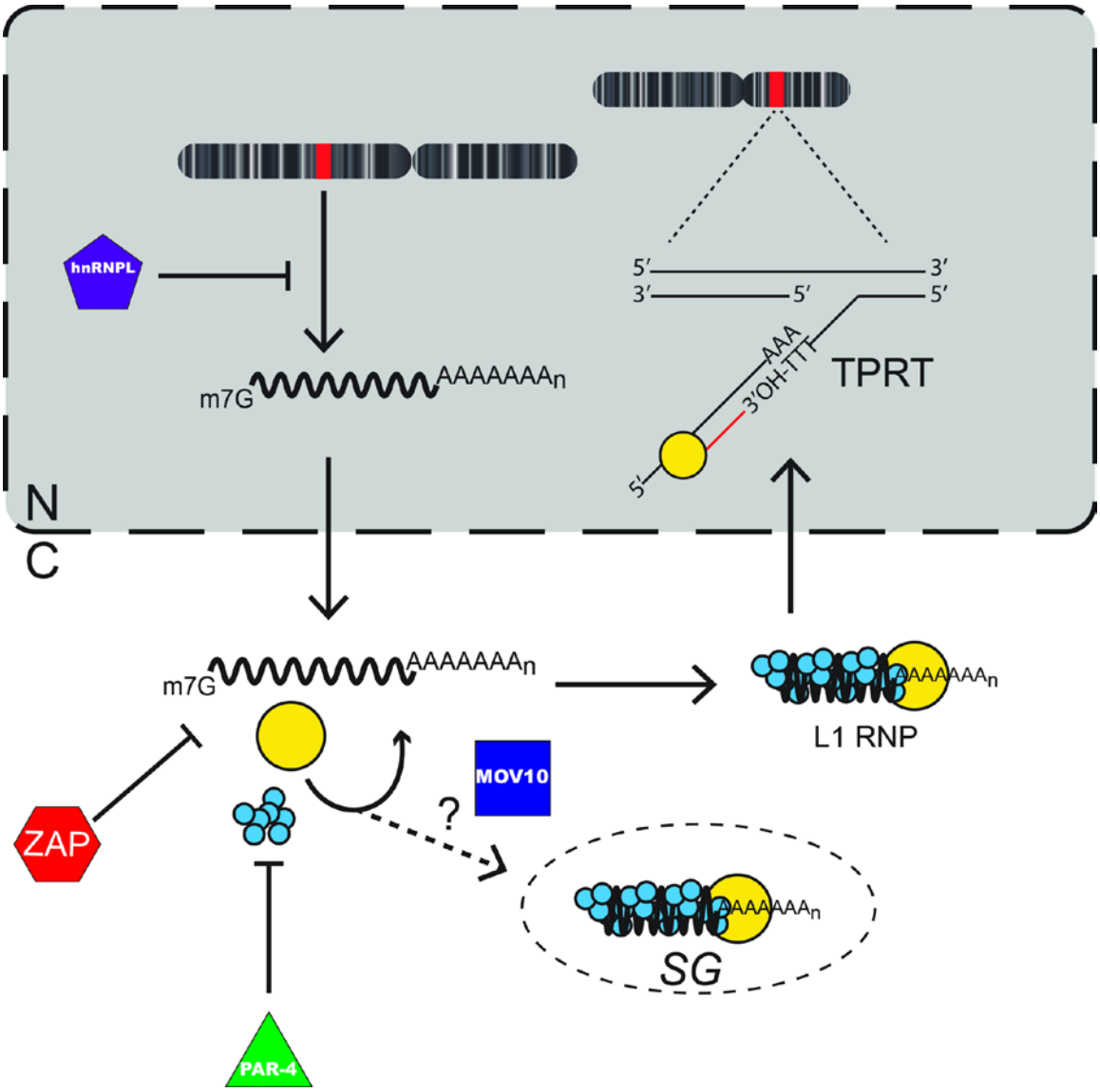

Figure 5.2: ORF1p-associated host factors modulate post-transcriptional steps in the L1 retrotransposition cycle.

Several ORF1p-associated host factors may function to modulate post-transcriptional steps of the L1 retrotransposition cycle. hnRNPL (purple pentagon) inhibits L1 retrotransposition and limits the expression of full-length L1 RNA, perhaps by influencing the way L1 transcripts are processed. ZAP (red hexagon) associates with L1 RNA in the cytoplasm and prevents the accumulation of full-length L1 RNA and the L1-encoded proteins. Although MOV10 (blue square) may affect the expression of L1 RNA and/or proteins, MOV10 may also sequester the L1 proteins and/or RNA into cytoplasmic stress granule (SGs) or SG-like aggregates thereby preventing these critical L1 intermediates from entering the nucleus. Data suggests that PAR-4 (green triangle) inhibits L1 retrotransposition and that PAR-4 may interact directly with ORF1p.

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Appendix 1

PAR-4

Appendix 1 summarizes experiments that test the effect of PAR-4 on L1 retrotransposition. It should be noted that the data in this chapter is preliminary and will require follow-up experiments for verification.

Prostate apoptosis response-4 (PAR-4) was identified as an ORF1p-interacting protein candidate in Chapter 3 (Table 3.1). PAR-4 is a tumor suppressor protein that selectively causes apoptosis in certain cancer cells and has been demonstrated to bind directly to other proteins including atypical isoforms of protein kinase C (PKC), Wilms' tumor I (WTI), and topoisomerase I (TOPI) (Hebbar et al. 2012). The human PAR-4 is 343 amino acids in length and contains several key domains that are 100% conserved across the human, mouse and rat genomes including two nuclear localization sequences (NLS1, NLS2), an alanine rich region, a selective for apoptosis in cancer (SAC) domain located between AA 146-203 that is unique to the PAR-4 protein, and a carboxyl terminal coiled coil/leucine zipper domain (CC/LZ) that mediates protein-protein interactions (Figure A1B) (El-Guendy et al. 2003, Hebbar et al. 2012).

Results and Discussion

PAR-4 interacts with ORF1p

Results from Chapter 3 suggested that the majority of ORF1p-interacting proteins associate with ORF1p by binding to L1 RNA and/or other RNAs present within the L1 RNP (Figures 3.1A-D) (Mandal et al. 2013). Interestingly, ORF1p-FLAG co-immunoprecipitation experiments demonstrated that the interaction

between PAR-4 and ORF1p is not sensitive to RNase A treatment (Figure A1A). Thus, the interaction between PAR-4 and ORF1p is likely not dependent on L1 RNA.

The effect of PAR-4 on L1 retrotransposition

We next tested the effect of PAR-4 overexpression on L1 retrotransposition using the cultured cell retrotransposition assay (see Chapter 3 and Figures 3.2A and 3.2B and Figure A1B). Overexpression of PAR-4 reduced pJJ101/L1.3 retrotransposition to ~50% of control levels (Figure A1B). Notably, PAR-4 overexpression caused substantial toxicity in HeLa cells, reducing the number of blasticidin resistant colonies in pcDNA6/TR control assays to ~40% of control levels; however, this effect has been accounted for through normalization (see Chapter 3 for explanation of the pcDNA6/TR control assays and Figures 3.2A and 3.2B) and thus is independent of the ability of PAR-4 to restrict L1 retrotransposition.

Notably, studies suggest that overexpression of PAR-4 causes apoptosis in certain cancer cell lines (Nalca et al. 1999, Chakraborty et al. 2001) and that PAR-4 pro-apoptotic activity is dependent on the PAR-4 selective for apoptosis in cancer (SAC) domain between AA 146-203 (El-Guendy et al. 2003). Thus, I speculated that the SAC domain was responsible for the elevated toxicity in HeLa cells. I engineered a PAR-4 mutant (PAR-4ΔSAC) that lacks PAR-4 amino acids 146-203 in order to test if the SAC domain was required for L1 restriction and/or PAR-4 toxicity in HeLa cells. Overexpression of PAR-4ΔSAC reduced L1 retrotransposition to similar levels as wild type PAR-4 (~60% of control levels). Critically, PAR-4ΔSAC did not cause noticeable toxicity in pCDNA6/TR control assays (~90% of control levels). It should be noted that western blots will need to be conducted to verify that the PAR-4 mutant proteins are expressed in HeLa cells. Thus, the PAR-4 SAC domain is not required for L1 restriction and is likely responsible for elevated PAR-4 toxicity in HeLa cells.

I next tested if PAR-4 could inhibit Alu retrotransposition. Overexpression of wild-type PAR-4 had a mild inhibitory effect on Alu retrotransposition (~70% of

control levels), whereas overexpression of PAR-4ΔSAC appeared to enhance Alu retrotransposition (~140% of control levels) (Figure A1C). It should be noted that the effect of PAR-4 on Alu retrotransposition was highly variable between experiments ranging from decreasing Alu retrotransposition to as low as ~40% to as high as 90% of control levels. These variations are likely due to the extended G418 selection times required for *mneoI* based assays. PAR-4 overexpression caused a consistent 50% decrease in G418-resistant colonies compared to controls in pcDNA3 control assays (please see Chapter 3 for an explanation of control assays), whereas PAR-4ΔSAC expectedly did not cause a decrease in G418-resistant colony formation (data not shown). Notably, the effect of PAR-4 on G418-resitant colony formation has been accounted for in Alu retrotransposition assays through normalization (see Chapter 3 for explanation of control assays and Figures 3.2A and 3.2B) and thus is independent of the ability of PAR-4 to restrict Alu retrotransposition. Based on the above data it is possible that PAR-4 affects Alu retrotransposition; however, additional experiments will be required to confirm the effects of PAR-4 on Alu retrotransposition.

In sum, these preliminary data suggests that PAR-4 interacts directly with ORF1p and restricts L1 retrotransposition. Importantly, PAR-4 could be the first and only ORF1p-interacting protein to be identified to date. Future experiments will be required to test this interesting hypothesis. To assist with future study of the interaction between ORF1p and PAR-4, several additional PAR-4 mutants have been constructed that contain deletions of various PAR-4 domains. A summary of the effects of these PAR-4 mutants on pJJ101/L1.3 retrotransposition are summarized and presented in Figure A1B.

Methods

Cell Culture

HeLa-JVM cells were grown in high-glucose DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin-streptomycin (Invitrogen), and 0.29 mg/mL L-glutamine (Gibco) (Moran et al. 1996). HeLa-HA (Hulme et al. 2007)

and PA-1 (Zeuthen et al. 1980) cells were grown in MEM (Gibco) with 10% FBS, 100 U/mL penicillin-streptomycin, 0.29 mg/mL L-glutamine, and 0.1 mM nonessential amino acids (Gibco). Cell lines were maintained at 37°C with 7% CO_2 in humidified incubators (Thermo Scientific).

Plasmids

Oligonucleotide sequences and cloning strategies used in this study are available upon request. All human L1 plasmids contain the indicated fragments of L1.3 (accession no. L19088) (Sassaman et al. 1997) DNA cloned into pCEP4 (Invitrogen) unless otherwise indicated. A CMV promoter augments expression of all L1 and cDNA expressing plasmids unless noted otherwise. L1 plasmids also contain an SV40 polyadenylation signal that is located downstream of the native L1 polyadenylation signal. All plasmid DNA was prepared with a Midiprep Plasmid DNA Kit (Qiagen).

pJM101/L1.3: is a pCEP4-based plasmid that expresses a human L1 (L1.3) equipped with an *mneoI* retrotransposition indicator cassette. L1 expression is augmented by a CMV promoter located upstream of the L1 5' UTR and an SV40 polyadenylation signal that is located downstream of the native L1 polyadenylation signal (Dombroski et al. 1993, Freeman et al. 1994, Moran et al. 1996, Sassaman et al. 1997)

pJM101/L1.3FLAG: was derived from pJM101/L1.3 and contains a single FLAG epitope on the carboxyl-terminus of ORF1p. Dr. Huira Kopera (University of Michigan Medical School) constructed the plasmid.

pAlu*neo*^{Tet}: expresses an Alu element cloned from intron 5 of the human *NF1* gene (Wallace et al. 1991) that is marked with the *neo*^{Tet} reporter gene. The reporter (Esnault et al. 2002) was subcloned upstream of the Alu poly adenosine tract (Dewannieux et al. 2003).

pJJ101/L1.3: is a pCEP4 based plasmid that contains an active human L1 (L1.3) equipped with an *mblastI* retrotransposition indicator cassette (Kopera et al. 2011).

pJJ105/L1.3: is similar to pJJ101/L1.3, but contains a D702A missense mutation in the RT active site of L1.3 ORF2 (Kopera et al. 2011).

pJM101/L1.3Δneo: is a pCEP4 based plasmid that contains an active human L1 (L1.3) (Wei et al. 2001).

pcDNA6/TR: expresses the blasticidin resistance gene and was obtained from Invitrogen.

PAR-4: the PAR-4 construct was obtained from OriGene (SC110969).

PAR-4/ΔSAC: was derived by deleting the PAR-4 amino acids 146-203 from the wild-type PAR-4 plasmid (OriGene, SC110969).

PAR-4ΔSAC: was derived by deleting the PAR-4 amino acids 146-203 from the wild-type PAR-4 plasmid (OriGene, SC110969).

PAR-4/ΔNLS: was derived by deleting the PAR-4 NLS2 amino acid sequence wild-type PAR-4 plasmid (OriGene, SC110969).

PAR-4/261X: was derived by substituting the PAR-4 amino acid 261 with a stop codon (OriGene, SC110969).

PAR-4/Δ40-142: was derived by deleting the PAR-4 amino acids 40-142 from the wild-type PAR-4 plasmid (OriGene, SC110969).

Immunoprecipitation and Western Blotting

ORF1p-FLAG immunoprecipitation experiments were detailed in Chapter 3 (Figure 3.1 and Methods). For Western blots, PAR-4 was visualized using anti-PAR-4 antibodies (Origene, TA307434) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Hyperfilm ECL (GE Healthcare).

L1 Retrotransposition Assays

The cultured cell retrotransposition assay was carried out essentially as described (Moran et al. 1996, Wei et al. 2000). For retrotransposition assays with L1 constructs tagged with *mblastI*, HeLa-JVM cells were seeded at ~1-2×10^4 cells/well in a 6-well plate (BD Falcon). Within 24 hours, each well was transfected with 1 μg of plasmid DNA (0.5 μg L1 plasmid + 0.5 μg cDNA plasmid

or pCEP4) using 3 µL of FuGENE 6 transfection reagent (Promega). Four days post-transfection, blasticidin (EMD Millipore) containing medium (10 µg/mL) was added to cells to select for retrotransposition events. Medium was changed every two days. After ~8 days of selection, cells were washed with PBS, fixed, and then stained with crystal violet to visualize colonies. To control for transfection efficiency and off-target effects of cDNA plasmids, in parallel with retrotransposition assays, HeLa-JVM cells were plated in 6-well plates at 500-1,000 cells/well and transfected with 0.5 µg pcDNA6/TR (Invitrogen) plasmid + 0.5 µg cDNA plasmid using 3 µL of FuGENE 6 transfection reagent (Promega). The pcDNA6/TR control assays were treated with blasticidin in the same manner as for retrotransposition assays.

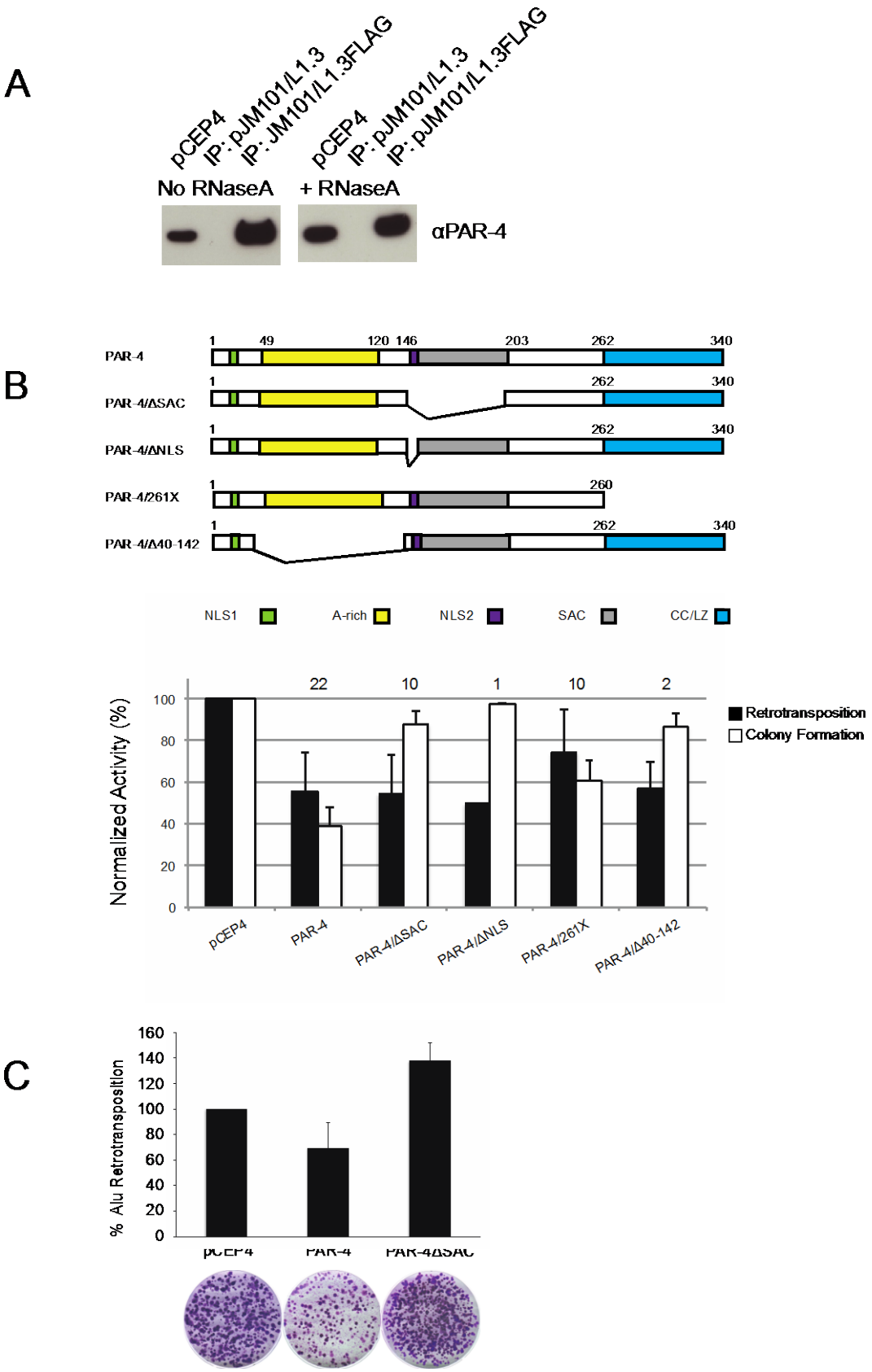
For retrotransposition assays with L1 constructs tagged with *mneoI*, HeLa-JVM cells were transfected as described above. Two days after transfection, cells were treated with medium supplemented with G418 (Gibco) (500 µg/mL) for ~10-12 days. As a control, HeLa cells were plated at ~2×10^4 cells/well in a 6-well plate and transfected with 0.5 µg pcDNA3 (Invitrogen) plasmid + 0.5 µg cDNA plasmid using 3 µL of FuGENE 6 transfection reagent (Promega). The pcDNA3 control assays were treated with G418 in the same manner as for retrotransposition assays.

Alu Retrotransposition Assays

For Alu retrotransposition assays (Dewannieux et al. 2003), ~4×10^5 HeLa-HA cells were plated per well of a 6-well plate (BD Falcon) and transfected with 0.67 µg of pJM101/L1.3Δneo + 0.67 µg of pAlu*neo*^{Tet} + 0.67 µg of cDNA plasmid using 6 µL FuGENE HD (Promega). Three days post-transfection, cells were grown in the presence of G418 (500µg/mL) to select for Alu retrotransposition events. As a control, HeLa-HA cells were plated at ~4×10^5 cells/well in a 6-well plate and transfected with 0.67 µg of pcDNA3 (Invitrogen) + 0.67 µg of pAlu*neo*^{Tet} + 0.67 µg of cDNA plasmid using 6 µL of FuGENE HD (Promega). The pcDNA3 control assays were treated with G418 in the same manner as for Alu retrotransposition assays.

Figure A1: PAR-4 interacts with L1 ORF1p.

(A) PAR-4 interacts with ORF1p-FLAG: Western blot images of the pJM101/L1.3FLAG and pJM101/L1.3 immunoprecipitation (IP) reactions (Figure 3.1A-3.1C). The pCEP4 lanes denote whole cell lysates derived from HeLa cells transfected with an empty pCEP4 vector (~1.0% input). PAR-4 is ~40 kDa in size. Anti-PAR-4 antibodies were used for western blots. Immunoprecipitation reactions were conducted in either the absence (left) or presence (right) of RNaseA (10 µg/mL). *(B) PAR-4 inhibits L1 retrotransposition*: Top panel: Schematics of PAR-4 constructs. Depicted are the relative positions of the nuclear localization signal 1 (NLS1; green box), alanine rich domain (A-rich; yellow box), NLS2 (purple box), SAC domain (grey box), coiled coil/leucine zipper (CC/LZ; blue box). Bottom panel: Results of the retrotransposition assays with PAR-4 and PAR-4 mutants. The X-axis indicates the cDNA co-transfected with pJJ101/L1.3 or pcDNA6/TR. The Y-axis indicates pJJ101/L1.3 retrotransposition activity (black bars), or pcDNA6/TR colony formation activity (white bars). All values have been normalized to the pCEP4 empty vector control (100%). The numbers above the bar graphs indicate the number of independent experiments performed with each cDNA expression construct. Error bars represent standard deviations. *(C) The effect of PAR-4 on Alu retrotransposition:* The X-axis indicates the cDNA co-transfected with pJM101/L1.3Δneo and pAlu*neo*^{Tet}. The Y-axis indicates the retrotransposition efficiency. All values are normalized to the pCEP4 empty vector control (100%). Control assays using a plasmid that expresses the neomycin phosphotransferase gene (pcDNA3) were conducted similarly to pcDNA6/TR control assays as outlined in Figure 3.2B. Representative images of G418-resistant HeLa foci from the Alu retrotransposition assay are shown below the bar graph. The results are the average of two independent experiments. Error bars indicate standard deviations.


A
pCEP4
IP: pJM101/L1.3
IP: JM101/L1.3FLAG
pCEP4
IP: pJM101/L1.3
IP: pJM101/L1.3FLAG
No RNaseA
+ RNaseA
αPAR-4
B
PAR-4
PAR-4/ΔSAC
PAR-4/ΔNLS
PAR-4/261X
PAR-4/Δ40-142
1
49
120
146
203
262
340
260
NLS1
A-rich
NLS2
SAC
CC/LZ
Normalized Activity (%)
22
10
1
10
2
Retrotransposition
Colony Formation
pCEP4
PAR-4
PAR-4/ΔSAC
PAR-4/ΔNLS
PAR-4/261X
PAR-4/Δ40-142
C
% Alu Retrotransposition
pCEP4
PAR-4
PAR-4ΔSAC

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Appendix 2

hnRNPL

Appendix 2 expands the analysis of ORF1p-interacting proteins from Chapter 3 and presents experiments that test the effect of hnRNPL on L1 retrotransposition. It should be noted that the data in this chapter is preliminary and is currently being worked on by, Mr. Peter A. Larson, a graduate student in the Moran Lab.

Heterogeneous ribonucleoprotein L (hnRNPL) is an abundant nuclear RNA binding protein with diverse roles in mRNA processing including RNA splicing (Hui et al. 2003, Guang et al. 2005, Hung et al. 2008). In Chapter 3, hnRNPL was demonstrated to interact with ORF1p by binding to RNA and to inhibit L1 retrotransposition (Figures 3.1A-3.1D and 3.2C). Notably, in a recent study (Peddigari et al. 2013), RNA pull-down experiments revealed hnRNPL associates with LINE-1 RNA from mouse. In the same study, investigators also showed that siRNA knockdown of hnRNPL in cultured mouse cells resulted in an increase in the expression of mouse L1 RNA and proteins suggesting that hnRNPL may function to modulate L1 retrotransposition in mouse (Peddigari et al. 2013). A subsequent study showed that hnRNPL interacts with human ORF1p and that hnRNPL inhibits human L1 retrotransposition suggesting that hnRNPL also may function to modulate L1 retrotransposition in humans (Goodier et al. 2013). Herein, I show that siRNA-mediated depletion of endogenous hnRNPL in HeLa cells led to an ~2-fold increase in human L1 retrotransposition. I also present Northern blot data that suggests that hnRNPL restricts the expression of L1 RNA, thereby providing mechanistic insight on how hnRNPL inhibits L1 retrotransposition.

Results and Discussion

Depletion of endogenous hnRNPL enhances L1 retrotransposition

To test if endogenous hnRNPL restricts L1 retrotransposition, I used small interfering RNA (siRNA) to deplete endogenous hnRNPL from HeLa cells. Breifly, cells were treated with siRNAs and then were transfected with the L1 construct, pLRE3-*mEGFPI* (Ostertag et al. 2000) or the negative control L1 plasmid pJM111-LRE3-*mEGFPI*, which carries two missense mutations that adversely affect ORF1p RNA binding (Moran et al. 1996, Martin et al. 2005, Khazina and Weichenrieder 2009). Treatment of HeLa cells with an siRNA pool against hnRNPL resulted in an ~72% reduction of endogenous hnRNPL protein levels when compared to HeLa cells treated with a non-targeting control siRNA pool (Figure A2A; top panel). hnRNPL siRNA treatment led to an approximately two-fold increase in pLRE3-*mEGFPI* retrotransposition activity when compared to assays conducted in the presence of a control siRNA (Figure A2A; bottom panel). These siRNA data are consistent with hnRNPL siRNA data from a previous study (Peddigari et al. 2013) and suggest that endogenous hnRNPL may affect L1 retrotransposition activity in HeLa cells.

The effect of hnRNPL on L1 RNA expression

To investigate how hnRNPL restricts L1 retrotransposition, I analyzed the effect of hnRNPL expression on the expression of L1 RNA. HeLa cells were co-transfected with pJM101/L1.3Δneo and either hnRNPL or a related heterogeneous ribonucleoprotein (hnRNPQ, also known as SYNCRIP) that does not affect L1 retrotransposition (Figure 3.2C). Polyadenylated RNA from whole cell extracts then was analyzed by northern blot using RNA probes complementary to sequences within the L1.3 5' UTR (5UTR99) and ORF2 (ORF2_5804) (Figure 3.4A). Co-transfection with hnRNPL resulted in a reduction of full-length polyadenylated L1 RNA levels compared to cells co-transfected with either SYNCRIP or an empty pCEP4 control vector (Figure A2B; black arrow in blot). Interestingly, hnRNPL expression prevented the accumulation of smaller L1 RNA species which may have resulted from cryptic splicing and/or premature

polyadenylation (Perepelitsa-Belancio and Deininger 2003, Belancio et al. 2006, Belancio et al. 2008) that were present in the pCEP4 or SYNCRIP lanes (Figure A2B; blue arrows) and resulted in the appearance of a new L1 RNA band at ~2.1 kb (Figure A2B; red arrow). Finally, control experiments revealed that ectopic hnRNPL expression did not affect endogenous actin RNA levels (Figure A2B).

These data suggest that hnRNPL perturbs the expression of L1 RNA. Notably, hnRNPL has been shown to influence the splicing and/or polyadenylation of cellular RNA transcripts (Hui et al. 2003, Hung et al. 2008). Several studies suggest that splicing and/or premature polyadenylation regulates L1 retrotransposition and proteins that regulate splicing have been previously demonstrated to regulate the processing of L1 RNA (Perepelitsa-Belancio and Deininger 2003, Belancio et al. 2006, Belancio et al. 2008, Belancio et al. 2010). Thus, it is tempting to speculate that hnRNPL modulates L1 retrotransposition by influencing the splicing and/or polyadenylation of L1 transcripts. In order to test if hnRNPL affects L1 RNA splicing and/or polyadenylation, future experiments could try to sequence L1 RNA in the presence of ectopic hnRNPL expression using either RT-PCR or by direct sequencing of L1 RNA bands from Northern blots (*i.e.*, isolate and sequence the L1 RNA band corresponding to the red arrow in Figure A2B).

Methods

Cell Culture

HeLa-JVM cells were grown in high-glucose DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin-streptomycin (Invitrogen), and 0.29 mg/mL L-glutamine (Gibco) (Moran et al. 1996). HeLa-HA (Hulme et al. 2007) and PA-1 (Zeuthen et al. 1980) cells were grown in MEM (Gibco) with 10% FBS, 100 U/mL penicillin-streptomycin, 0.29 mg/mL L-glutamine, and 0.1 mM nonessential amino acids (Gibco). Cell lines were maintained at 37°C with 7% CO_2 in humidified incubators (Thermo Scientific).

Plasmids

Oligonucleotide sequences and cloning strategies used in this study are available upon request. All human L1 plasmids contain the indicated fragments of L1.3 (accession no. L19088) (Sassaman et al. 1997) DNA cloned into pCEP4 (Invitrogen) unless otherwise indicated. A CMV promoter augments expression of all L1 and cDNA expressing plasmids unless noted otherwise. L1 plasmids also contain an SV40 polyadenylation signal that is located downstream of the native L1 polyadenylation signal. All plasmid DNA was prepared with a Midiprep Plasmid DNA Kit (Qiagen).

pJM101/L1.3Δneo: is a pCEP4 based plasmid that contains an active human L1 (L1.3) (Wei et al. 2001).

pLRE3-*mEGFPI*: is a pCEP4 based plasmid that contains an active human L1 (LRE3) equipped with an *mEGFPI* retrotransposition indicator cassette (Ostertag et al. 2000, Garcia-Perez et al. 2010). The pCEP4 backbone was modified to contain a puromycin resistance (PURO) gene in place of the hygromycin resistance gene. The CMV promoter also was deleted from the vector; thus, L1 expression is driven only by the native 5′ UTR (Ostertag et al. 2000).

pJM111-LRE3-*mEGFPI*: is identical to pLRE3-*mEGFPI* except that it contains two missense mutations in ORF1 (RR261-262AA), which render the L1 retrotransposition-defective (Moran et al. 1996). Mr. William Giblin (University of Michigan Medical School) constructed the plasmid (Zhang et al. 2014).

hnRNPL: was obtained from Open Biosystems (6174088).

SYNCRIP: was obtained from Open Biosystems (5495201).

pcDNA6/TR: expresses the blasticidin resistance gene and was obtained from Invitrogen.

siRNA knockdown and pLRE3-*mEGFPI* retrotransposition assays

In experiments to study the effect of endogenous proteins on L1 retrotransposition, HeLa cells (~8×10^5 cells) were plated in 60 mm tissue culture dishes (BD Falcon). The next day, the cells were transfected with 50 nM of a

control siRNA pool (D-001810-10, ON-TARGETplus Non-targeting Pool, Thermo Scientific) or siRNA against hnRNPL (ON-TARGETplus Human hnRNPL (56829) siRNA - SMARTpool, Thermo Scientific) or MOV10 (L-014162-00-0005, ON-TARGETplus Human MOV10 (4343) siRNA - SMARTpool, Thermo Scientific) using the DharmaFECT 1 transfection reagent (Thermo Scientific). Twenty-four hours after siRNA treatment, cells were transfected with pLRE3-*mEGFPI* or pJM111-LRE3-*mEGFPI* (5 µg), using 15 µL of FuGENE HD transfection reagent (Roche). After 48 hours, cells were trypsinized and an aliquot of the cells (~2×10^6 cells) was used to monitor endogenous protein levels (72 hours after siRNA treatment) by western blot analysis. Antibodies to hnRNPL (NBP1-67852) were obtained from Novus Biologicals. Blots were analyzed using an Odyssey CLx (LI-COR) with the following secondary antibodies: IRDye 800CW Donkey anti-Rabbit IgG (1:10,000) (LI-COR) and IRDye 680RD Donkey anti-Mouse IgG (1:10,000) (LI-COR). Knockdown efficiencies were calculated using LI-COR Image Studio Software (v3.1.4) and are the average of three independent experiments. Endogenous tubulin was used as the normalization control. The remaining cells were re-plated at ~2×10^5 cells/well of a 6-well plate and cultured in medium supplemented with puromycin (5 µg/ml, Gibco/Life Technologies) to select for cells transfected with pLRE3-*mEGFPI*. After 4 days of puromycin selection, the percentage of GFP positive cells was determined by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

Northern Blots

HeLa-JVM cells were seeded in T-175 flasks (BD Falcon) and transfected with 20 µg of plasmid DNA (10 µg pJM101/L.13Δneo + 10 µg cDNA plasmid) using 60 µL FuGENE HD. Two days after transfection, cell pellets were collected and frozen at -80°C. Frozen cell pellets were then thawed and total RNA was extracted with TRIzol reagent (Ambion), and then poly(A)+ RNA was prepared from total RNA using an Oligotex mRNA kit (Qiagen). Each sample (~1.5 µg of poly(A)+ RNA) was subjected to glyoxal gel electrophoresis and northern blotting using the NorthernMax-Gly Kit (Ambion) according to the manufacturer's protocol. Following electrophoresis, RNA was transferred to BrightStar Nylon

membranes (Invitrogen) and then cross-linked using UV light. For northern blot detection, membranes were prehybridized for ~4 hours at 68°C in NorthernMax Prehybridization/Hybridization Buffer (Ambion), and then incubated with a strand specific RNA probe (final concentration of probe ~3×10^6 cpm ml^{-1}) overnight at 68°C. For band quantification, northern blot films were analyzed using ImageJ software (Schneider et al. 2012).

Strand-specific RNA probes were generated using the MAXIscript T3 system (Invitrogen). The 5UTR99 (Belancio et al. 2006) probe corresponds to bases 7-99 of the L1.3 5' UTR and the ORF2_5804 probe corresponds to nucleotides 5560-5804 of the L1.3 sequence. RNA probe templates for T3 reactions were generated by PCR using pJM101/L1.3Δneo as a PCR template with the following primer pairs:

(5UTR99: 5'-GGAGCCAAGATGGCCGAATAGGAACAGCT-3' and 5'-AATTAACCCTCAAAGGGACCTCAGATGGAAATGCAG-3');

(ORF2_5804: 5'- GACACATGCACACGTATGTTTATT-3' and 5'-AATTAACCCTCACTAAAGGGTGAGTGAGAATATGCGGTGTTT-3').

The T3 promoter sequence (underlined) was added to the reverse primer of each primer pair. The pTRI-β-actin-125-Human Antisense Control Template (Applied Biosystems) was used in T3 reactions as a template to generate the β-actin RNA probe. Each northern blot experiment was independently repeated three times with similar results.

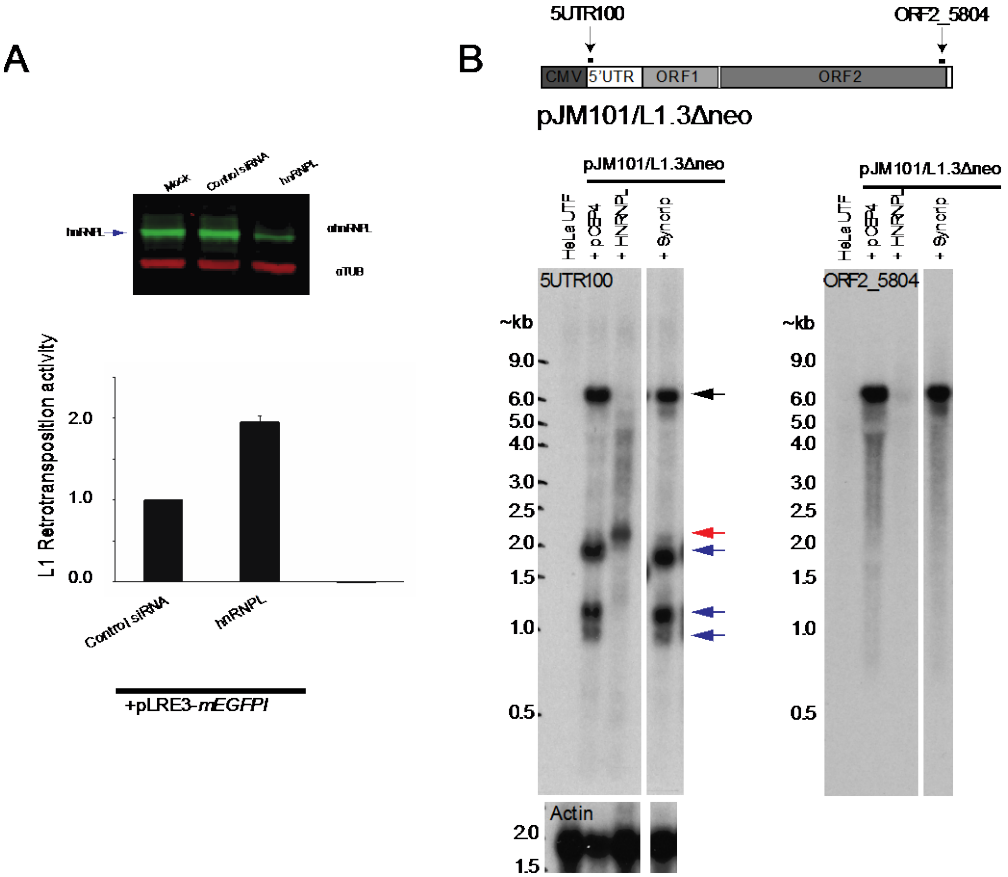

Figure A2: The effect of hnRNPL on L1 Retrotransposition

(A) *The depletion of hnRNPL enhances L1 retrotransposition*: Top panel: Western blots of whole cell lysates derived from mock HeLa cell transfections or HeLa cells transfected with indicated siRNAs. Blue arrow points to the approximate location of hnRNPL (~65 kDa). Bottom panel: The bar graph depicts pLRE-mEGFP1 retrotransposition activity following siRNA treatment. The X-axis indicates the siRNA. The Y-axis indicates the pLRE-mEGFP1 retrotransposition efficiency normalized to the control siRNA (set to 1). siRNA experiments performed once. *(B) Results of northern blots:* Top panel: Schematic of pJM101/L1.3Δneo: Bold black lines indicate the approximate location of probes (5UTR99 and ORF2_5804) used in the northern blot experiments. Bottom panel: HeLa cells were co-transfected with pJM101/L1.3Δneo and either hnRNPL, SYNCRIP, or an empty pCEP4 control vector. Northern blot images depict the effect of hnRNPL overexpression on polyadenylated L1 RNA levels. The constructs transfected into HeLa cells are indicated above each lane. UTF indicates untransfected HeLa cells and serves as a negative control. Probes (5UTR99 and ORF2_5804) are indicated in the top left corner of the respective blots. The black arrow indicates the position of the full-length L1 RNA. The blue and red arrows indicate shorter L1 RNA species. hnRNPL northern blot experiments were repeated three times with similar results. Actin served as a loading control. RNA size standards (~kb) are shown at the left of the blot image.

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