HUMAN LA FUNCTION IN EXPRESSION OF CODING TRANSCRIPTS

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Abstract

The La protein, also referred to as Sjogren's Syndrome antigen B (SSB), is an RNA-binding phosphoprotein first identified as an auto-antigen in patients suffering from Sjogren's syndrome and systemic lupus erythematosus. La proteins are present and indispensable in nearly all eukaryotes and exhibit conserved functions in RNA metabolism. By shuttling between the nucleus and cytoplasm, La interacts with RNA substrates transcribed by both RNA Polymerase III and RNA polymerase II. For human La (hLa), hLa phosphorylated at S366 is typically found in the nucleus and involved in facilitating the processing and maturation of RNA polymerase III transcripts by binding to the UUU-3'OH trailer in a sequence specific manner. In addition to assisting in the processing of nascent RNA polymerase III transcripts, La proteins are associated with promoting cap-independent translation from the internal ribosome entry sites (IRESs) of several cellular and viral coding RNAs. However, the mechanism of La binding to coding RNAs is poorly understood. In this study, we investigated the molecular mechanisms by which La interacts with coding transcripts. In Chapter 2, we show that in addition to a sequence specific UUU-3'OH binding mode, human La (hLa) exhibits both a sequence specific and length dependent poly(A) binding mode mapped to the canonical winged helix face of the eponymous La motif. Moreover, we also demonstrate that cytoplasmic La engages poly(A) RNA in human cells, La entry into polysomes utilizes the poly(A) binding mode, and La promotion of translation from the cyclin D1 IRES occurs in competition with the cytoplasmic poly(A) binding protein (PABP).

During viral infection, tumor progression, and certain forms of cellular stress, the predominantly nuclear hLa protein has been shown to translocate to the cytoplasm where it functions as an IRES *trans*-acting factor (ITAF), controlling cap-independent translation initiation of several cellular IRES-containing mRNAs involved in stress responses, signifying an important

role for hLa during stress. In Chapter 3, we aim to understand the cellular conditions during which La interacts with its cytoplasmic substrates and identify novel RNA substrates of La. We show that cellular stress induced by clotrimazole, an inducer of stress granules and mitochondrial stress, results in translocation of hLa from the nucleus to the cytoplasm. Using polysome analysis and qPCR, we also show that La translocation into cytoplasm is concurrent with increased association of La with actively translating messages, especially messages containing IRESs. Using individual crosslinking immunoprecipitation (iCLIP) analysis, we identify novel hLa associated targets. Taken together, these results suggest that hLa is trafficked into the cytoplasm in response to cellular stress in order to associate with IRES containing messages, and this interaction may occur through contacts made via a novel binding mode with the poly(A) tail.

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List of Abbreviations

32P	Phosphorus-32
3' UTR	3' untranslated region
5' cap	7-methylguanosine cap
5' UTR	5' untranslated region
5' TOP	5' terminal oligopyrimidine
AKT	Protein kinase B (PKB)
BiP	Binding immunoglobulin protein
CCND1	Cyclin D1
cDNA	Complementary DNA
CK2	Casein kinase 2
CLIP	cross-linking immunoprecipitation
Co-IP	Co-immunoprecipitation
CTD	C-terminal domain
D. melanogaster	*Drosophila melanogaster*
DAPI	4',6-diamidino-2-phenylindole
ddH_20	Double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eEF1b	Eukaryotic elongation factor 1b
eIF	Eukaryotic initiation factors
EMCV	Encephalomyocarditis Virus
EMSA	Electrophoretic Mobility Shift Assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
HCV	Hepatitis C virus
HEK293	Human embryonic kidney cells 293
HeLa	Henrietta Lacks Immortal Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
hLa	Human La
hnRNP	Heterogeneous nuclear ribonucleoproteins

HRP	Horseradish peroxidase
iCLIP	Individual-nucleotide resolution cross-linking and immunoprecipitation
IgG	Immunoglobulin G
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factor
kDa	Kilodaltons
KOH	Potassium hydroxide
La	Lupus autoantigen
LAM	La-motif
LARP	La-related protein
MDM2	Murine double minute oncogene
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NRE	Nuclear retention element
NrF2	Nuclear factor erythroid-2 related factor 2
NTD	N-terminal Domain
Opti-MEM	Opti-MEM Reduced Serum Media
ORF	Open reading frame
PABP	Poly(A) binding protein
PAM	PABP interaction motif
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail
pEGFP	GFP vector
PMSF	Phenylmethylsulfonyl Fluoride
PNK	Proteinase K
pre-mRNA	Precursor messenger RNA
pre-tRNA	Precursor transfer RNA
qPCR	Quantitative PCR
RBP	RNA binding protein
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNAse	Ribonuclease
RNA Pol	RNA Polymerase
RNP	Ribonucleoprotein
Rpl9	60S ribosomal protein L9

RRBP1	ribosome-binding protein 1
RRM	RNA-recognition motif
RT-PCR	Reverse transcription polymerase chain reaction
S366	Serine 366
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SBM	Short basic motif
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small (or short) interfering RNA
snRNA	Small nuclear RNA
SSB	Sjogren syndrome antigen B
T. *Brucei*	Trypanosoma *brucei*
T389	Threonine 389
tRNA	transfer RNA
Tris	Tris(hydroxymethyl)aminomethane
U2OS	Human Bone Osteosarcoma Epithelial Cells
uORF	Upstream open reading frame
UV	Ultraviolet
UTR	Untranslated region
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1:

Review of Literature

1.1 Eukaryotic Gene Regulation

1.1.1 Post-transcriptional Gene Regulation

DNA is a repository of genetic information and acts as a blueprint that is passed from generation to generation [1(p30)]. DNA is transcribed into RNA, and RNA is translated into protein. Although it was once widely believed that only DNA and protein were important to the central dogma, while RNA was simply an intermediate accessory, in the past 30 years, extensive research has shed light on the equally important role of RNA in expressing genetic information [1(p37)]. The "RNA World Hypothesis" proposes that RNA was the precursor to all currently existing life, since it has a functional versatility unlike that of DNA or protein [2]. To translate DNA-encoded information into protein and accurately express genes, a plethora of intricate mechanisms have to occur precisely.

Gene expression begins in the nucleus, where DNA binding proteins bind to DNA sequence elements and recruit RNA polymerases (RNA Pol) for RNA synthesis [3]. Precursor messenger RNAs (pre-mRNAs) are single-stranded mRNAs synthesized from a DNA template which undergo various post-transcriptional modifications including 5' capping, 3' polyadenylation, and splicing [4–7]. Once exported through nuclear pores into the cytoplasm, mature RNA transcripts are transported to predestined subcellular regions for translation and storage [8,9] **(Figure 1)**. Translation factors and ribosomes are then recruited to mRNAs to initiate translation and produce protein. Eventually, mRNAs are degraded by exonucleases [10].

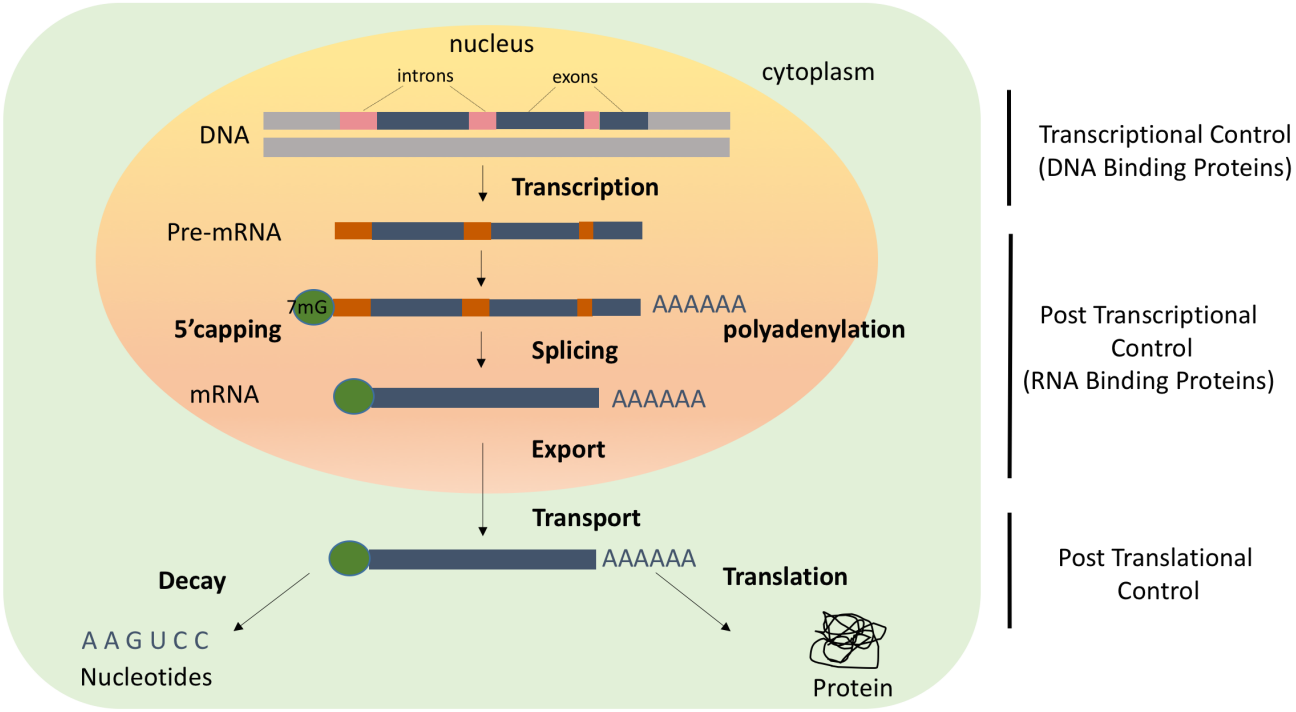


Figure 1: Schematic of the eukaryotic gene expression

During transcription, DNA is converted to make nascent mRNA precursors in the nucleus. mRNAs undergo a series of modifications including splicing, capping, and polyadenylation before becoming mature transcripts. mRNAs are then exported through nuclear pores into the cytoplasm, where they are destined to numerous subcellular regions. The transcripts, along with translation factors and ribosomes, make proteins. Finally, mRNAs undergo exonuclease-mediated degradation through various decay pathways. Figure adapted from [3].

The recent development of new genomic and proteomic tools has led to significant progress in the field of post-transcriptional regulation. Although DNA binding proteins are the key regulators of transcription, many post-transcriptional processes are also regulated by RNA-protein and RNA-RNA interactions. These ribonucleoprotein complexes (RNPs) are essential for the control of various aspects of gene regulation including capping, splicing, A-tailing, export, and transport [6,11]. More than 500 proteins are predicted to function as RNA binding proteins (RBPs) in budding yeast *Saccharomyces cerevisiae*, and it is predicted that RBPs comprise 3 to 11% of

proteomes in bacteria, archaea, and eukaryotes [12–14]. Through proteomic screens and other RNA binding assays, the continuous discovery of novel RBPs further confirms the significance of this protein family in the regulation of gene expression.

1.1.2 RNA Binding Proteins

From the moment it is transcribed, an RNA molecule is never alone, as RBPs bind nascent RNA targets to drive forth both transcription and translation, thus playing a significant role in various aspects of gene expression [7]. For example, in humans, capping enzymes add a 7-methylguanylate (m^7G) cap to nuclear pre-mRNA to prevent degradation [15]. Once exported to the cytoplasm, several eukaryotic initiation factors (eIFs) bind the 5' cap to stabilize the mature mRNA and recruit ribosomes to initiate eukaryotic translation. Similarly, polynucleotide adenylyltransferase (PAP) is an enzyme that adds adenine residues at the 3'OH end of newly synthesized pre-mRNAs in the nucleus [16]. The nuclear Poly(A) Binding Protein (PABP-N1/ PABP II) binds the nascent, developing poly(A) tail to protect against degradation and assists in the export of mRNA from the nucleus to the cytoplasm [17,18]. Once in the cytoplasm, the mature mRNA transcript is bound by PABP-C1, which helps to stabilize the mRNA during translation. Hence, both nuclear and cytosolic PABP are necessary to prevent mRNA degradation at various stages. RBPs also assist in the processing and assembly of non-coding RNAs (ncRNAs) into RNP complexes, mediating both splicing and translation [19]. All of these steps are essential and interdependent to ensure proper gene expression [5]. Several studies using different eukaryotic systems have demonstrated the importance of functional RBPs to regulate post-transcriptional modifications and the process of translation [6,20,21].

The ease and accessibility of new genomic tools allows for greater screening of large data sets to detect RBPs and their targets. Currently, the two most popular and widely used techniques include RNA immunoprecipitation coupled with sequencing (RIP-seq) and cross-linking immunoprecipitation (CLIP). RIP-seq involves the immunoprecipitation of RNP complexes, followed by the analysis of the associated RNAs using next generation sequencing. RIP-seq detects stronger and more stable RNA-RBP interactions. Unlike RIP-seq, CLIP employs ultraviolet (UV) crosslinking of RNA to proteins prior to immunoprecipitation of the associated RNAs **(Figure 2)**. Irradiation of cells at 254 nm creates covalent bonds between nucleotides and photo-reactive amino acids that reside in close proximity. Hence, high efficiency crosslinking is crucial for this approach, and this additional crosslinking step alleviates any potential background signals compared to the RIP-seq technique [22,23]. The crosslinking step also allows for the identification of less stable, transient RNA-RBP interactions. After partial RNAse digestion and reverse transcription to make cDNA, the cDNA is amplified using PCR and then prepared for next-generation sequencing. RNA-protein sites can then be identified by mapping reads and aligning them to the appropriate transcriptome. This method was first used to identify RNA targets of the neuron specific splicing factor NOVA1 and NOVA2 in mouse brain [23,24]. Various modifications of the CLIP protocol have been used successfully to identify the RNA targets of proteins such as Argonaute, heterogeneous nuclear ribonucleoprotein C (hnRNP C), polypyrimidine tract-binding protein (PTB), and the Fragile-X mental retardation protein (FMRP) [25–28].

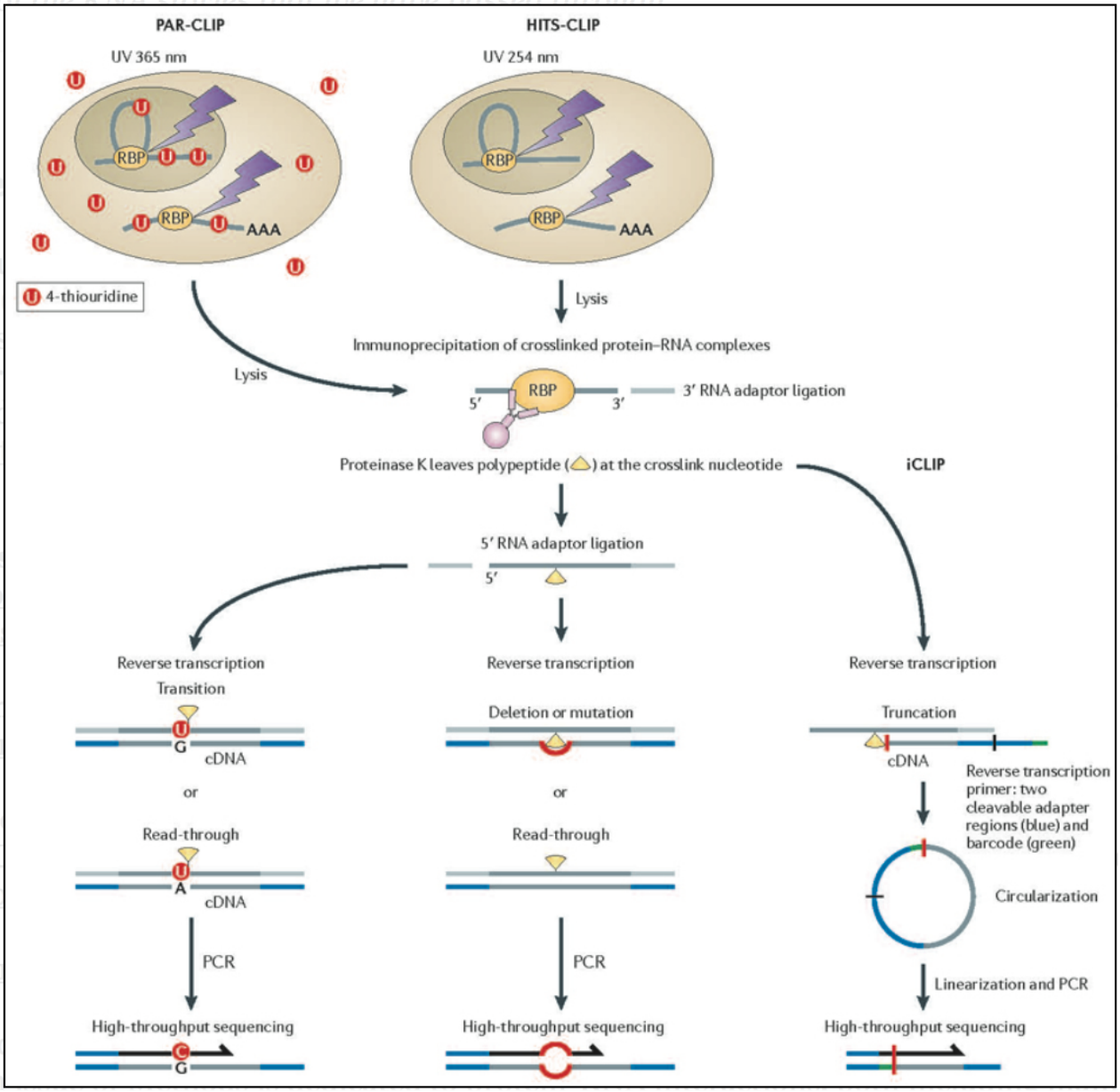


Figure 2: Schematic of CLIP methods

CLIP protocols rely on the use of UV crosslinking RNA with proteins. In PAR-CLIP, cells are cultured in 4-thiouridine containing medium resulting in 4SU-labelled transcripts. In HITS-CLIP and iCLIP, cells are cultured in regular medium and UV crosslinked to RBPs. Partial RNase-digestion of lysates leaves behind portions of RNA directly bound by protein. Radioactively labelled RNA-protein complexes are recovered by immunopurification and size-fractionation. Proteins are degraded via proteinase K treatment to release RNA molecules. RNAs are then converted into a cDNA library and deep sequenced. Figure taken from[29].

1.1.3 Common Features of RNA Binding Proteins

RNA interactions mediated by RBPs can be target specific. Bioinformatics analysis on several RBPs suggests that these proteins contain RNA binding domains that have been highly conserved through eukaryotic evolution [30]. RBPs are often composed of modular structures containing multiple repeats of a few basic domains. Using nuclear magnetic resonance (NMR) with protein-RNA complexes and co-crystal structures of RBPs bound to RNAs, it has been shown that distinct, conserved residues interact with bases and backbones of their RNA substrates [31–33]. These non-covalent intermolecular interactions are generally hydrogen bonding or stacking interactions, mediated by Van der Waals forces between aromatic residues and the purine or pyrimidine rings of RNA [34]. Cooperation between the different modules contribute to the ability of the protein to bind different substrates and to the multifaceted functions of the protein **(Figure 3)**. Modules can also cooperate with enzymatic domains to regulate the activity of catalytic enzymes that associate with RNA.

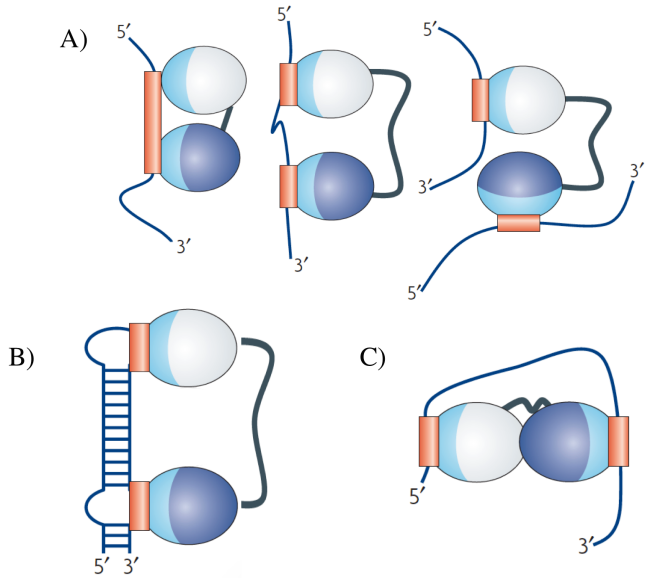


Figure 3: RNA-binding proteins bind RNA using different binding modes

RNA-binding proteins can recognize substrates in a sequence specific, structure specific, or a non-sequence specific manner. **(A)** Multiple domains cooperate with each other to recognize short or long RNA sequences. Longer sequences, or sequences within RNA that are separated by many nucleotides may require more than one domain for recognition. One RBP may also bind more than one RNA at a time. **(B)** RNA binding domains bind RNA in a structure-specific manner as opposed to a sequence-specific manner. **(C)** Domains of an RBP may interact with each other while also interacting with RNA. Figure adapted and modified from [35].

A well-studied example of a RBP consisting of multiple domains that, in concert, facilitate interactions with RNA and other proteins is the cytosolic poly(A) binding protein (PABP-C1). Poly(A) binding protein binds adenosine residues at the 3'OH end of mRNA and assists in mRNA stability and translation. PABP-C1 is comprised of four non-identical RNA recognition motifs (RRMs) followed by a proline-rich region and carboxyl terminal domain, referred to as PABC [36]. While adenine recognition is mediated by conserved residues within the two N-terminal RRMs, the PABC domain is responsible for recognizing a conserved 15-residue sequence, part of the PABP interaction motif (PAM-2) [37]. The PAM-2 motif is found on several proteins that bind to PABP including PABP interacting proteins 1 and 2 (PAIP 1, PAIP 2) and the La-related protein 4 (LARP4) [38,39]. Similarly, the La protein which consists of a La motif and two RRMs, uses a

combination of modules to bind distinct substrates. While the La motif and the N-terminal RRM are involved in binding to RNA polymerase III transcribed substrates of La, the La motif, along with both the N- and C-terminal RRM, are involved in binding a viral substrate of La, the internal ribosome entry site (IRES) of the Hepatitis C Virus [40,41].

In addition to RNA binding domains, the length and flexibility of linker regions inbetween modules allows the RBP to bend, facilitating a greater number of interactions with different substrates. The arrangement and cooperation between these modules are instrumental for the diverse RBP functionality. For instance, in the La protein, the linker that bridges the La motif and N-terminal RRM in its RNA-free form is long and flexible. However, the corresponding linker found within La-related proteins families 4 and 6 (LARP4 and LARP6) appear to be shorter and more rigid [42]. It is hypothesized that the difference in linker flexibility and length may account for the ability of La to function in facilitating the processing of both RNA Pol III and RNA Pol II transcripts, while La-related protein families targeting are restricted to only one of these classes (further discussed in section 1.5).

Greater conservation of RNA binding domains is found in proteins that are orthologous, meaning they are direct evolutionary counterparts, performing similar functions in different organisms. For example, there are more similarities between the RRM1 of yeast PABP and human PABP, than between the RRM1, RRM2, and RRM3 of yeast PABP [43]. There are numerous examples of highly conserved RNA binding domains between different protein families including: the La-motif (LAM), RNA recognition motif (RRM), the SAM (Sterile alpha motif) domain, the K-homology (KH) domain, zinc-fingers (ZnFs), the double stranded RNA-binding domain (dsRBD), the Pumilio domain, and the Piwi/Argonaute/Zwille (PAZ) domain **(Table 1)** [35]. RRM, also known as the RNA binding domain, is comprised of ~80 amino acids and is the most common

motif found in RNA binding proteins. NMR and X-ray crystallography have confirmed that the structure of RRMs consists of a four-stranded anti-parallel β-sheet with two helices packed against it, so that the domain presents a split αβ (βαββαβ) topology [44]. Studies of several RRM-containing RBPs have shown that RNA recognition usually occurs on the surface of the β-sheet, where binding is mediated by three conserved residues: either an arginine or lysine residue, which forms a salt bridge to the phosphodiester backbone, along with two aromatic residues that facilitate stacking interactions with nucleobases [35].

Table 1: Common RNA-binding domains and their properties

Domain	Protein-RNA interactions	Examples
RRM RNA recognition motif	Interactions occur through stacking, electrostatics and hydrogen bonding	La, LARP, PABP
KH K-homology	Interactions occur through hydrophobic interactions between nonaromatic residues and the bases	Nova-1, Nova-2, FMR1
dsRBD double-stranded RNA-binding domain	Shape-specific recognition of the minor–major–minor groove pattern of dsRNA through contacts to the sugar-phosphate backbone	Staufen
PIWI	Highly conserved pocket, including a metal ion that is bound to the exposed C-terminal carboxylate	Argonaute, PIWI
PAZ Piwi/Argonaute/Zwille	Hydrophobic pocket formed by OB-like β-barrel and small αβ motif	Argonaute, Dicer

Table was adapted and modified from [35].

1.2 La Proteins

1.2.1 Overview of La Proteins

First discovered in 1974, La is an RNA-binding phosphoprotein identified as an auto-antigen in patients suffering from Sjogren's syndrome, neonatal lupus, and systemic lupus erythematosus [45,46]. Using La antibodies, levels of circulating La were measured to diagnose patients as having systemic lupus erythematosus and neonatal lupus syndrome [47,48]. Although it is still unclear why La is targeted as an autoantigen, several decades of research have shed light on the multifaceted functions of this protein which has helped to further our understanding of RNA-RBP interactions. Abundantly found at approximately 20 million copies per cell, La proteins were one of the first RBPs to be identified, and thus have pioneered RBP research. La is ubiquitous across eukaryotes and its function in RNA metabolism is highly conserved. While La is an essential gene in all metazoans investigated to date including Trypanosoma, *Arabidopsis thaliana, Drosophila Melanogaster*, and mice, it is dispensable in both budding and fission yeast, making yeast a good model system for genetic studies [49,50].

La shuttles between the nucleus and the cytoplasm interacting with various RNA substrates [51,52]. In humans, hLa (human La) is phosphorylated at serine-366 (S366) and is typically found in the nucleus. La is involved in facilitating the processing of RNA polymerase III transcripts such as U6 snRNA, tRNA precursors, and pre-5S rRNA by protecting the transcript from 3'-5' exonuclease degradation by exonucleases such as Rex1p [53,54]. Transcription termination by RNA Pol III produces a 3′ oligo(U) trailer, which serves as a high affinity binding site for La. Affinity towards this RNA Pol III substrate is dependent on the length of the oligo (U) tract [54,55]. While the interaction of La with substrates that end with the UUU 3'OH trailer have been well characterized,

its interaction with substrates that lack this trailer is still under investigation. Non-phosphorylated hLa is found in the cytoplasm, where it binds and enhances the expression and translation of cellular and viral mRNAs through the use of internal ribosome entry sites (IRES), 5' terminal oligiopyrimidine tracts (5' TOPs), and upstream open-reading frames (uORFs). This interaction has been identified in various cellular IRESs including the binding immunoglobulin protein (BiP) and Cyclin D1 (CCDN1) and in several viral IRESs including Hepatitis C, encephalomyocarditis virus, and poliovirus [41,56–58]. The mechanism by which La binds to the IRES to enhance translation is still under investigation. La binding to non-Pol III transcripts is less well characterized in yeast, although *S. cerevisiae* La (Lhp1p) has been shown to engage mRNAs, in addition to the expected RNA Pol III substrates [59].

1.2.2 Structural overview of La protein

Research on the structure of La has been crucial in unveiling La's complex functions, particularly in understanding its various binding modes. While the N-terminal domain (NTD) is highly conserved amongst eukaryotes, the C-terminal domain (CTD) of La is less conserved and can vary in length [50]. Like many RNA-binding proteins, La is modular. The NTD harbours the La motif (LAM) and a RRM, followed by an α-helix (α3) **(Figure 4).** The C-terminal portion of La harbours a nuclear retention element (NRE) and a nuclear localization signal (NLS). In higher eukaryotes, a second RRM (RRM2) and a short basic motif (SBM) are found in the C-terminal domain. Hence, the size of La can vary from 32 kDa in lower eukaryotes such as yeast, which contains only one RRM, to 50 kDa in humans, which contains two RRMs [50].

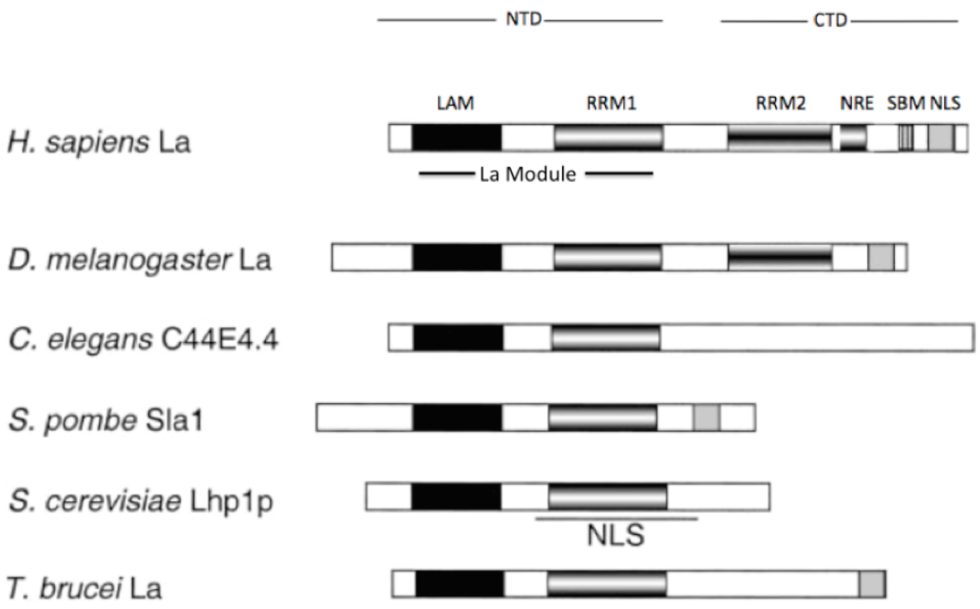


Figure 4: Architecture of La orthologs in eukaryotes

A schematic of the conservation between human La protein, *D. melanogaster*, *C. elegans, S. pombe, S. cerevisiae,* and *T. brucei.* The NTD is highly conserved and contains a La motif (LAM) and a RNA recognition motif (RRM), which are separated by a short linker sequence. The less conserved CTD of hLa harbours a second RRM, a nuclear retention element (NRE), a short basic motif (SBM), and a nuclear localization signal (NLS). The *S. cerevisiae* NLS is hypothesized to be a 113-amino-acid fragment within the RRM. With the exception of the *C. elegans* La protein, the remaining La proteins have demonstrated affinity for nascent RNA polymerase III transcripts. Figure adapted from[49].

The LAM is a highly conserved RNA binding domain that has been the focus of extensive research. This region is also found in several La related protein families and interestingly in a number of proteins that are structurally unrelated to La such as the polyribosome associated proteins Slf1p and Sro9p, both found in *S. cerevisiae* and are predicted to function in translation [60]. Several solved crystal and co-crystal structures of this domain demonstrates that the LAM has an α1α1α2β1α3α4α5β2β3 topology and contains amino acids that make UUU-specific contacts [33,61–63] **(Figure 5a)**. The LAM consists of a winged-helix-turn-helix shape in which many aromatic

residues are conserved [61,64]. Dong *et al*. show that these aromatic residues are conserved in all characterized La proteins and in LAM containing proteins. Through structure based mutagenesis, six residues have been identified as being crucial for binding RNAs with 3' UUU-OH trailers. Of these, four residues are located in the conserved aromatic patch, and two are in close proximity. In addition, mutating an invariant aspartate (D27 in *Trypanosoma brucei* and D33 in hLa) in a hydrophobic pocket reduced La's affinity for a 3'OH, but not for 3'PO_4 groups. Although the ability of La to discriminate between the 3'OH ends of RNA Pol III transcripts and the 3'PO_4 ends of degraded RNA was previously shown by Stefano *et al*., the structural analysis by Dong *et al*. provided the basis for this discrimination[54,61].

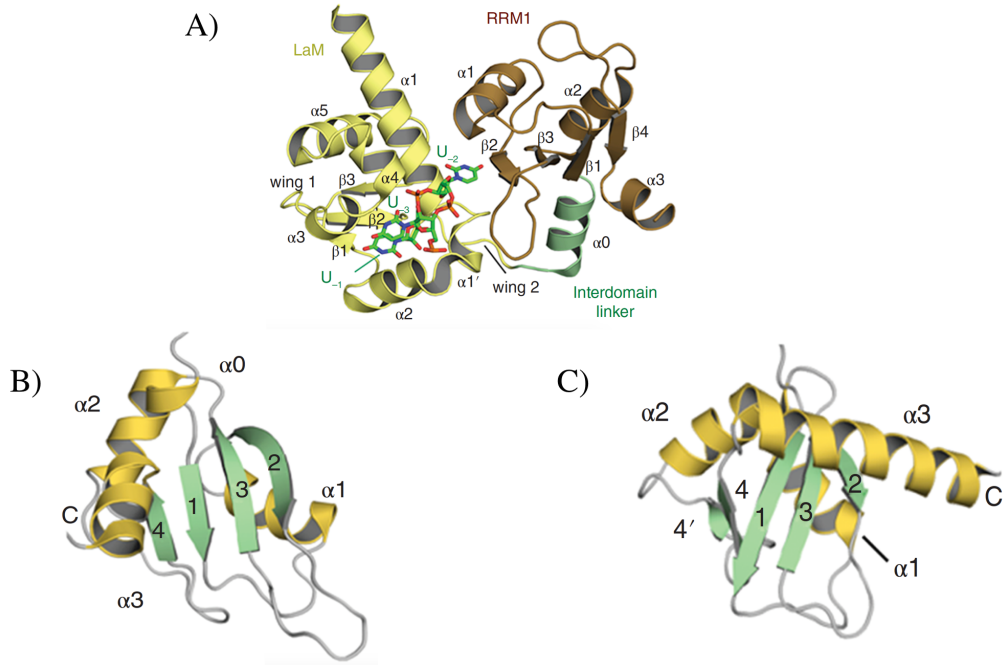


Figure 5: Structure of the LAM, RRM1, and RRM2

(A) The La module, consisting of the LAM and the N-terminal RRM, bind uridylate substrates by forming a binding cleft. The LAM consists of an α1α1α2β1α3α4α5β2β3 topology. High resolution co-crystal structures of the LAM reveal that when bound to the UUU-3'OH of an RNA, a winged helix portion of the LAM does not make any contact with UUU-3'OH and remains unoccupied. A small, flexible linker between the two domains adopts a helical conformation when bound to RNA. This allows both domains to assume proper conformations for RNA recognition. **(B)** The N-terminal RRM has a β1α1β2β3α2β4α3 topology and is hypothesized to bind the body of the substrate. (**C**) The C-terminal RRM has a β1α1β2β3αβ4β4α3 topology and contains a canonical RNA binding β-sheet that is highly obscured by a C-terminal α-helix. Figure adapted from [65].

Solved co-crystal structures demonstrated that the LAM and the RRM bind short oligo (U) ending RNAs through both stacking interactions and hydrogen bond formation [62,63]. While the majority of UUU-3'OH recognition is mediated by the LAM, the N-terminal RRM contributes minimally. The RRM has a β1α1β2β3α2β4α3 topology, with the α3 helix being atypical to the standard RRM fold [62] **(Figure 5b)**. In concert, the LAM and RRM1 form a pocket that binds to

the UUU- 3'OH end of RNA substrates. As the LAM binds to the substrate, the canonical RRM1 RNA binding surface is left relatively available to bind to other substrates. The RRM1 does not seem to play a crucial role in UUU-3'OH substrate recognition. Thus it is hypothesized that the RRM1 binds to substrates in an UUU-independent manner [62]. Mutational analysis indicates that the RRM1 may likely bind UUU-3'OH containing substrates upstream of the 3'OH end [40].

While the function and mechanism of the LAM and RRM1 has been deciphered to some extent, the function of the RRM2 in hLa is still unclear. RRM2 has a β1α1β2β3αβ4β4α3 topology and contains a canonical RNA binding β-sheet that is highly obscured by a C-terminal α-helix [33,41,66] **(Figure 5c)**. Thus, both RRM1 and RRM2 have non-canonical α3 helices C-terminal to the RRM, although they appear to be in different orientations relative to the RRM's RNA binding surface. It has been suggested that RRM2 may be involved in binding mRNA targets to enhance translation. Martino *et al*. hypothesize that the LAM, RRM1, and RRM2 synergistically enhance translation of viral mRNAs such as HCV, yet the mechanism is still unknown [41]. The α3 helix following RRM2 contains the NRE, which is predicted to mediate the structure of the RRM2. Without the NRE, the tertiary structure of the RRM2 is destabilized [67]. The NRE is followed by the SBM, whose function is not clear, but is hypothesized to modulate the access of 5' pre-tRNA leaders to RNase P via engagement of the 5' triphosphate [53]. The NRE and the NLS are involved in the regulation of both nuclear and cytoplasmic activities. La can be phosphorylated at two sites, serine-366 (S366) by Casein Kinase 2 (CK2) and threonine-389 (T389) by AKT-1 [68,69]. Nucleoplasmic La is phosphorylated and associated with RNA pol III transcripts, whereas cytoplasmic La is typically non-phosphorylated and bound to RNA pol II transcripts [70].

1.2.3 Structural overview of La-related proteins (LARPS)

La-related proteins (LARPs) contain the conserved LAM and are involved in gene expression at both the transcriptional and translational level (reviewed in [42,50,65,71]). These proteins have been conserved through eukaryotic evolution, where through the use of modern bioinformatic and structural techniques, five LARP families have been identified in humans: Genuine La, LARP1, LARP4, LARP6, and LARP7 **(Figure 6)**. Like La, the LARP family of proteins all contain a conserved 90-amino acid region called the LAM followed by an RRM, although the RRM structure varies between LARPs. Both La and LARP7 bear the typical canonical RRM1 adjacent to the LAM. However, other LARPs lack the classical canonical RRM and instead have an RRM-like motif [42,50]. The position of the La Module varies between different LARPs. For instance, while the La module is located near the N-terminus in genuine La, the La module is positioned more centrally in the other LARPs.

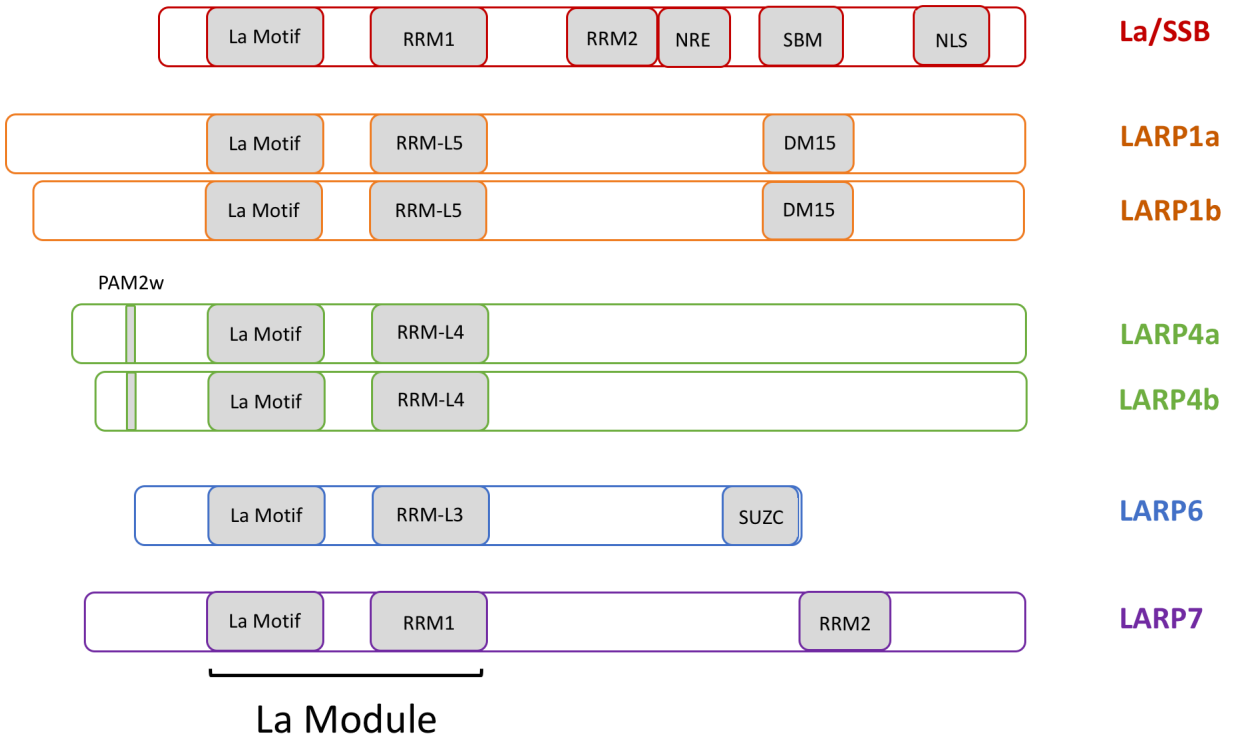


Figure 6: Architecture of the La protein and La-related proteins (LARPs)

Schematic of genuine hLa and hLa-related protein families aligned at the conserved La motif (LAM). hLa consists of two RNA recognition motifs (RRM1 and RRM2), a nuclear retention element (NRE), a short basic motif (SBM), and a nuclear localization signal (NLS). LARP1 consists of a RNA recognition-like motif (RRM-L5) and a DM15-repeat containing region, also known as the LARP1 motif. RNA recognition-like motifs are variations of the canonical RRM found in genuine La and are conserved across LARP families. LARP4 consists of another RNA recognition-like motif (RRM-L4) along with an atypical PAM2 domain (PAM2w). LARP6 contains a RNA recognition-like motif (RRM-L3) and a SUZ-C domain. Lastly, LARP7 consists of two RRMs. Figure adapted from [50].

LARPs vary quite significantly in both their structure and function. LARP1 has two variants: LARP1a and LARP1b. A unique component of LARP1 is a D-repeat containing region (DM15) found at the C-terminus [72]. The DM15 region is comprised of triplicate amino acid repeats and has recently been shown to directly bind the 5' cap of TOP-containing mRNAs [42,73]. LARP4 also has two variants, LARP4a and LARP4b, both of which contain a PAM2w domain at the N-

terminus ahead of the La Module that directly binds to PABP [39]. LARP6 contains a SUZ-C motif in the C-terminal region, a 36 amino acid domain generally found at the C terminus of several RBPs believed to play a role in substrate recognition and protein localization [74,75]. Based on sequence homology, LARP7 is most closely related to genuine La and the only LARP to contain a second RRM. [42]. Although La and LARPs share the LAM, they are all structurally and functionally distinct. Over the past decade, continuous research has shed light on the functionality of the LARP family as these proteins have been found to bind to a wide range of RNA substrates, significantly contribute to cancer progression, and may potentially act as therapeutic targets for a wide range of diseases (further explained in section 1.5).

1.3 The function of La in RNA metabolism

1.3.1 The function of La in non-coding RNA metabolism

Early studies examining La function and its targets were conducted using purified small ribonucleoprotein (RNP) complexes extracted from HeLa cells [54]. Using anti-La sera, it was found that RNPs immunoprecipitated with La mainly consisted of RNAs ending in uridylate residues. A UUU-3'OH trailer is typically found in RNA polymerase III transcribed RNAs such pre-transfer RNAs (tRNAs), pre-5S ribosomal RNA (rRNA), and pre-U6 small nuclear RNA (snRNA). Similarly, La also recognizes and binds certain RNA polymerase II transcribed small RNAs including U1, U2, U3, U4, and U5 snRNAs. These small RNAs have poly(A) tails like other polymerase II transcripts. However, during processing they lose their poly(A) tail and leave behind a UUU-3'OH end [1]. In addition, several studies have implicated La as associating with viral-encoding RNAs including the adenovirus encoded VA-RNA$_1$ and VA-RNA$_2$, the Epstein-Barr

RNAs EBER1 and EBER2 as well as negative-strand viral leader RNAs [76–78]. Using UV cross-linking and RNA foot-printing assays, Mathews *et al.* confirmed that the binding site of hLa bound directly to the U-rich 3' tail of these RNA polymerase III transcripts, with a minimum of three uridines required for recognition [79]. The requirements for the length of terminal uridines varies between organisms, with *S. pombe* requiring four or more terminal uridines and humans requiring a minimum of three terminal uridines [80].

La is amongst one the first proteins to associate with nascent RNA polymerase III transcripts. La was originally hypothesized to play a role in RNA polymerase III transcription. During tRNA processing, the 5' leader of the pre-tRNAs is cleaved by RNAse P, while the 3'OH trailer is cleaved by RNAse Z [19]. Endonucleases cleave the intron of the pre-tRNA and nucleotidyl transferases then add a CCA to the 3' end [53,81]. Transcription is terminated when a stretch of poly-uridylate residues are added to the 3' end by RNA polymerase III. It is believed that La remains bound to these UUU-3'OH ends until processing occurs to prevent exonuclease digest by Rex1p [54,82,83] **(Figure 7)**. During RNA processing and maturation, the terminal uridylates are usually removed resulting in the loss of the La protein binding site. Northern blot analyses on *S. cerevisiae* cell extracts show that without functional La, mutations in the secondary structures of several essential tRNAs and abnormal tRNA processing patterns arise [83]. It is proposed that *Lhp1p*, the yeast La protein ortholog, stabilizes pre-tRNAs in conformations that allow 3′ endonucleolytic cleavage to occur. From this, it has been proposed that La does not have a direct role in transcription, but instead acts as an RNA chaperone to aid misfolded pre-tRNAs to achieve proper folding in order for protection against exonuclease digestion.

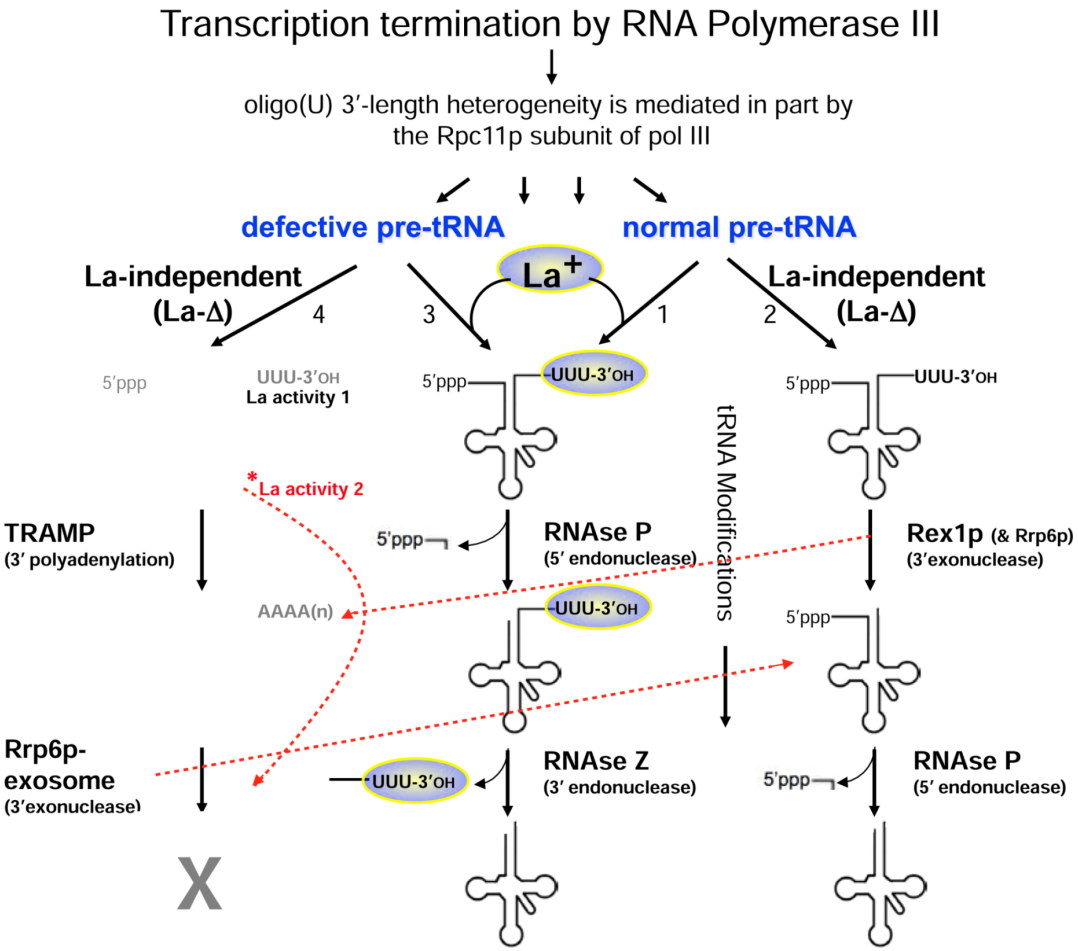


Figure 7: Precursor tRNA processing pathways in the presence and absence of La

Four possible pathways exist which determine the fate of pre-tRNA. pre-tRNA fate determined by the length of the poly-U at the 3' end occurs when the interaction between the pre-tRNA and La is established with a minimum of three terminal U's. The usual pathways of pre-tRNA processing occurs in the presence and absence of La, but processing of a defective pre-tRNA is only made possible in the presence of La. La engages nascent pre-tRNA transcripts and protects them from 3' exonuclease activity, causing the 5' leader to be cleaved first by RNAse P followed by 3' end processing carried out by the 3' endonuclease RNAse Z. In the absence of La, cleavage of the 5' leader and the 3' trailer sequences occurs in the reverse order. A defective pre-tRNA cannot be properly processed in the absence of La, resulting in degradation. Figure adapted from [40].

1.3.1.1 La is an RNA chaperone

One predicted function of La is that of an RNA chaperone [84]. RNA substrates undergo several transition states during the folding process and these states present folding "traps" which prevent RNA from obtaining its native fold [85]. RNA chaperones are proteins that aid in the unwinding and re-folding of RNA substrates into its native fold, which is essential for proper function. These proteins often display strand annealing and displacement activity to resolve these kinetic traps. The chaperone activity of La has been well studied in pre-tRNA substrates. Pre-tRNAs often mis-fold as a result of DNA mutations, hypomodifications, or errors during transcription [86].

La is essential to prevent the degradation of pre-tRNAs by Rrp6, a component of the nuclear exosome that specifically targets defective pre-tRNAs, in a process called nuclear surveillance [86]. Previous research has shown that certain amino acid residues in RRM1 are responsible for RNA chaperone activity. Using a *cis*-splicing assay which relies on the enhanced folding of a self-splicing intron in the presence of an RNA chaperone, Bayfield *et al*. found that mutating basic residues of the β2-β3 loop 3 region of hLa RRM1 resulted in compromised RNA chaperone activity [40]. A few years later, using Fluorescence Resonance Energy Transfer (FRET) based analysis, Naeeni *et al*. further mapped the chaperone activity of La to the RRM1, in the α3 helix as well as the disordered region in the linker before RRM2 [84].

In addition to acting as an RNA chaperone for pre-tRNAs, La is also hypothesized to act as an IRES trans-acting factor (ITAF) for mRNA translation [41,87]. Many ITAFs are also RNA chaperones as these proteins bind to IRES containing cellular and viral mRNAs to induce conformational changes of the IRES and promote cap-independent translation (further explained in section 1.3.2) [87]. A well-studied substrate of La that undergoes conformational changes is the

IRES of the Hepatitis C Virus. Circular dichroism spectroscopy (CD) analysis revealed that Domain IV of the Hepatitis C IRES undergoes minor conformational changes upon hLa binding [41]. More recently, it has been shown that hLa binds near the start codon of the cyclin D1 mRNA (CCND1) and acts as an RNA chaperone to alter the structure of the cellular IRES to facilitate cap-independent translation. However, this chaperone ability can be prevented through T389 phosphorylation by AKT1 [69]. The ability of La to act as an RNA chaperone and the T389 binding site are both critical to promote IRES driven translation of the CCND1 mRNA. The RNA chaperone abilities of La also extend to microRNA (miRNA) processing in mammalian cells [88]. La recognizes the stem loop structure of nascent pre-miRNAs and promotes miRNA biogenesis by protecting them from nuclease-mediated decay. The LAM, RRM1, and RRM2 are all implicated in recognizing and binding the stem loop region of miRNAs.

1.3.2 The function of La in coding RNA metabolism

While the function of La in the nucleus is becoming increasingly well-characterized, the function of La in the cytoplasm is still under investigation. Cytoplasmic substrates of La include upstream open reading frame (uORF) and 5' terminal oligopyrimidine tract (5' TOP)-containing mRNAs, as well as the IRESs of several viral and cellular mRNAs. Experiments by Meerovitch *et al.* in rabbit reticulocyte lysates (RRL) first demonstrated that La has the ability to enhance and correct aberrant translation of polioviral RNA [89]. While La has been implicated in the enhancement of translation of several viral IRESs, many studies aimed at understanding the involvement of La in viral translation have been conducted using the highly structured HCV IRES located in the 5′ untranslated region (5' UTR) of the viral genome [41]. This IRES consists of four domains: 1) Domain I is essential for viral translation, 2) Domain II enhances IRES activity, but is not

necessary for function [90], 3) Domain III has subdomains essential for the recruitment of ribosomes, and 4) Domain IV contains the start (AUG) codon. It was identified that La binds close to the initiator AUG and assists in the assembly of ribosomes in order for IRES-mediated translation to initiate [91].

Martino *et al*. proposed a bipartite model for the interaction of hLa with Domain IV of the HCV IRES and speculate that the LAM, RRM1, and RRM2 are involved [41]. It has been suggested that the LAM and the RRM1 are essential to interact with the single-stranded extension of the IRES element, while the RRM2 is involved in recognizing and binding the stem loop of the IRES. In these experiments, hLa displayed the same affinity for the stem loop when the nucleotide sequence was mutated but the same secondary structure was maintained, suggesting that La may be involved in recognizing the secondary structure of the stem loop instead of specific sequences. This research suggests a non-sequence specific mechanism by which La functions in the cytoplasm, and represents a novel finding where the RRM2 is implicated in binding a RNA substrate.

Additionally, La has also been associated with the translation of human immunodeficiency virus type 1 (HIV1) [92]. Svitkin *et al.* demonstrated that La binds the transactivation response element (TAR) at the 5' end of HIV1 mRNAs to form a stable hairpin structure that promotes translation, thereby alleviating translational repression. In addition to binding viral RNA and promoting viral replication, La has been implicated in binding the 5' terminal oligopyrimidine (5' TOP) tracts of many coding RNAs. These RNAs include ribosomal proteins and histone messenger RNAs [68,93,94]. Using RNA immunoprecipitation chip (RIP-Chip) analysis, Inada *et al.* showed that *Lhp1p* in *S. cerevisiae* engages mRNAs in addition to the expected polymerase III substrates [59].

1.4 La association with cellular stress and cancer

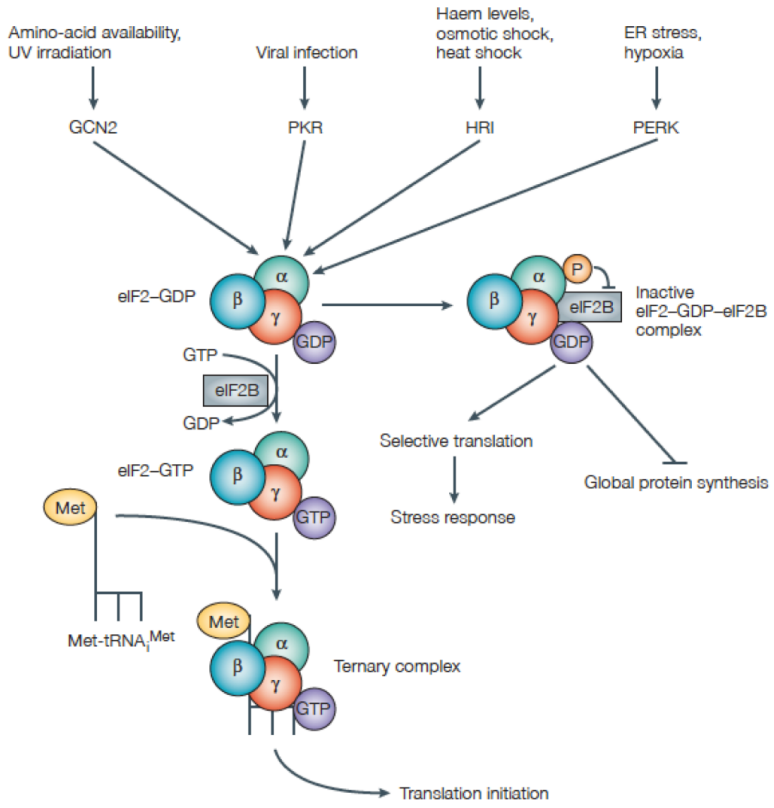
1.4.1 Cellular Stress

The ability of cells to maintain active translation in response to cellular stress is vital for its survival. Cellular stress can be induced by a range of stressors including heat shock, oxidative stress, mitochondrial stress, and serum starvation resulting in a significant decrease in overall translation. Eukaryotic cells have evolved and adopted various mechanisms to adapt and overcome cellular stress or initiate apoptosis. Some of these mechanisms include the repression of cap-dependent translation through the phosphorylation of eIF2α or the inhibition of mTORC1 and through the formation of stress granules to preserve cellular machinery[95,96]. Stress granules are dense aggregations composed of mRNAs and components of the cap-dependent translation machinery, translationally repressed mRNAs, PABP, and other RBPs [97,98]. These membrane-less foci are hypothesized to protect stalled RNP complexes such that they can re-enter into polysomes when cells return to homeostasis[97].

The formation of these granules can be signaled by the phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2) causing inhibition of cap-dependent translation and the subsequent disassembly of polysomes. In unstressed cells, eIF2 serves to mediate binding of the initiator tRNA methionine ($tRNA_i^{met}$) to the 40S ribosomal subunit in a GTP-dependent manner [98]. After initiation has occurred, eIF2 remains bound to GDP in an inactive state until recycled **(Figure 8a)** [99]. In stressed cells, eIF2α is phosphorylated preventing the formation of the eIF2-GTP-$tRNA_i^{met}$ complex, causing an inhibitory effect on translation. Depending on the particular stress, different kinases contribute to this phosphorylation (PERK for ER stress, PKR for viral infection, HRI for heat and osmotic shock, and GCN2 for glucose/ amino acid starvation)[99]. A cohort of RBPs, such as TIA1, TIAR and G3BP, are then recruited to these stalled

translation initiation complex and promote aggregation and formation of stress granules[95,100,101]. Stress granule formation can also be regulated by the mammalian target of rapamycin (mTORC1) complex. mTORC1 is a kinase complex that activates and regulates protein synthesis in response to various cellular and environmental factors including adequate energy resources, nutrient availability, oxygen and proper growth factor abundance [102,103]. In unstressed cells, mTORC1 phosphorylates 4E-BP, allowing eIF4E to bind eIF4G and promote protein synthesis **(Figure 8b)** [104]. However, during nutrient starvation, 4E-BP is dephosphorylated and bound to eIF4E, preventing the formation of the eIF4E/eIF4G complex[103]. This disruption in the formation of the eIF4F complex results in inhibition of translation initiation. Although these mechanisms disrupt the majority of translation during cellular stress, there is increasing evidence that cellular mRNAs containing IRES elements in their 5' UTR can escape recruitment into stress granules and remain actively translated even when cap dependent translation initiation is hindered [87,105,106].

A)



B)

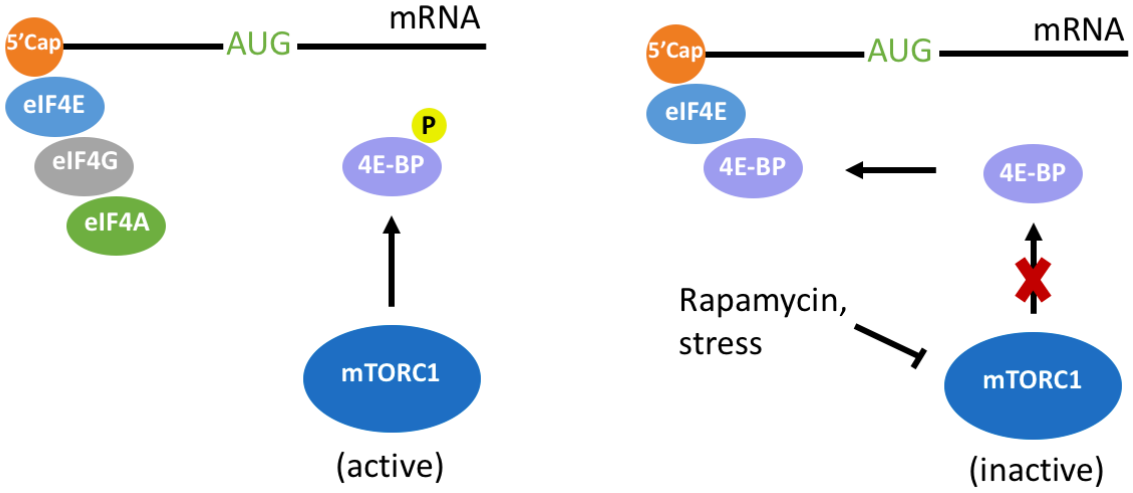


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Figure 8: Mechanisms for translation inhibition through eIF2a phosphorylation and mTORC1 inhibition

(A) Certain stressors stimulate phosphorylation of eukaryotic initiation factor-2α (eIF2α). Depending on the type of stress, different protein kinases are activated: PERK for ER stress, PKR for viral infection, HRI for heme deficiency, and GCN2 for amino acid starvation. EIF2 consists of 3 subunits, eIF2α, eIF2β and eIF2γ. The three subunits form the ternary complex consisting of eIF2-GTP- and $tRNA_i^{met}$. The complex transports the initiator tRNA to the ribosome to form the 43 preinitiation complex. During translation initiation GTP becomes hydrolyzed into GDP, a reaction catalyzed by eIF2β. Before each around of initiation, GDP is exchanged for GTP. During cellular stress, eIF2α is phosphorylated which inhibits the GDP-GTP exchange. This inhibition creates an inactive eIF2-GDP complex, thereby resulting in the inhibition of translation. **(B)** mTORC1 is a kinase complex that regulates protein synthesis. Initiation of cap dependent translation relies on the eIF4F complex, comprised of eIF4E, eIF4G, and eIF4A. The interaction between eIF4E and eIF4G is essential for proper function. In unstressed cells, mTORC1 phosphorylates 4E-BP, allowing eIF4E to bind eIF4G and promote protein synthesis. However, during nutrient starvation, 4E-BP is dephosphorylated and bound to eIF4E, preventing the formation of the eIF4E/eIF4G complex. This disruption in the formation of the eIF4F complex results in inhibition of translation initiation. Figure adapted from [104,107].

1.4.2 La protein involvement in cellular stress

Previous work on viral infection and cellular stress have indicated that La is trafficked to the cytoplasm, with numerous reports citing increased association of La with IRES containing messages under different cellular stresses. Thus far, the IRESs identified as being associated with La under stress include the X-linked inhibitor of apoptosis protein (XIAP), the nuclear factor erythroid-2 related factor 2 (NrF2), the ribosome binding protein 1 (RRBP1), and the binding immunoglobulin protein (BiP) [56,108–110].

One of the earliest reports linking La to an IRES containing mRNA is that of XIAP, a key regulator of programmed cell death (apoptosis) triggered by various apoptotic factors [108]. Under conditions of serum deprivation and radiation-induced apoptosis, when cap-dependent translation is compromised, the XIAP IRES fosters efficient translation. Using gel mobility shifts, UV-crosslinking, and bicistronic reporter assays, Holcik *et al.* demonstrated that La forms a stable RNP complex with the 5′ UTR of the XIAP IRES to stimulate IRES driven translation in HeLa cells.

While previous groups using RRL demonstrated that La binds the 5′ UTR of viral mRNAs to stimulate their translation, Holcik *et al.* were the first to validate that La also binds cellular mRNAs through the use of both *in vitro* and *in vivo* assays and showing that La directly binds the XIAP IRES to modulate XIAP translation.

In a study conducted by Zhang *et al.*, it was shown that nuclear La can migrate into the perinuclear space to engage the NrF2 IRES in response to oxidative stress [109]. The NrF2 protein is a transcription factor that controls gene expression of detoxification genes and is a master regulator of oxidative stress response through its control of expression of antioxidant and detoxification genes. The 5′ UTR of NrF2 contains an IRES element, predicted to be a substrate of La. Immunofluorescence staining of HeLa cells displayed a cytoplasmic distribution of La only 10 minutes following treatment with 100μm hydrogen peroxide (H_2O_2). Tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) along with various *in vitro* and *in vivo* RNA binding assays, confirmed the direct binding of La to the NrF2 IRES in response to H_2O_2 treatment. Thus, it was concluded that oxidative stress results in the nuclear export of La to the cytoplasm and increased association with the NrF2 5′ UTR.

Similarly, *Chan et al.,* reported that oxidative stress results in the increased binding of La to the HCV IRES [111]. Oxidative stress is a prominent feature of patients diagnosed with Hepatitis C and elevated levels of reactive oxygen species (ROS), and reduced antioxidant levels have been detected in both the blood and liver. It was demonstrated that oxidative stress upregulates HCV IRES driven translation resulting in the translocation of La to the cytoplasm. *In vitro* translation assays revealed increased La driven cap-independent translation of the HCV IRES upon oxidative stress in the hepatocellular carcinoma cell line HuH7. The link between how oxidative stress positively affects the ability of La to promote viral replication and the mechanism by which the

HCV IRES induces La cytoplasmic distribution in response to oxidative stress is yet to be established. Further studies examining the interaction of La with other picornaviral and HCV-like IRESs during oxidative stress conditions may reveal more information about the mechanism through which La is recruited to the cytoplasm to promote IRES dependent translation and viral replication.

It has been shown that serum-starvation of both human embryonic kidney (HEK293T) and human hepatocellular carcinoma (Bel7402) cells results in the cytoplasmic localization of La and increased La driven translation of the RRBP1 mRNA [110]. RRBP1 is an ER membrane protein involved in ribosome binding and translocation of nascent proteins, particularly across the rough ER. Increased expression of RRBP1 is observed in some tumour cells implicating RRBP1 in the maintenance of malignancy and adaptation to ER stress. RRBP1 was identified as having an IRES element, which cells rely on to survive during serum starvation, a condition during which they are more susceptible to apoptosis. Serum starvation of Bel7402 cells resulted in increased association of La with both the RRBP1 and the XIAP IRES. Through this study, it was established that La is a positive activator of the RRBP1 IRES *in vivo*, under serum starvation conditions.

Similarly, the cytoplasmic re-localization of La has been observed in cells from the human salivary gland (HSG) of patients with Sjögren's syndrome (SS) [112]. Salivary gland epithelial cells from SS patients secrete fluid rich with a diversity of proteins, a process supported by a hyperactive ER, eventually ending in cellular apoptosis. As a response to the increased ER activity, the unfolded protein response (UPR) is activated to re-establish homeostasis. BiP, a known substrate of La, is also a key player in the UPR and is essential for the folding of misfolded or unfolded proteins within the ER [113]. It is proposed that ER stress-induced apoptosis induces the cytoplasmic redistribution of La to cluster both around and at the surface of the apoptotic bodies in order to

bind the BiP mRNA [112]. La also has increased association with BiP during Human cytomegalovirus (HMCV) infection. Certain viruses, such as the HCMV, inflict stress on cells in order to benefit from already existing translational machinery to promote their own viral replication [114]. By inducing ER stress and modulating the UPR in infected cells, HCMV activates BiP to perform its ER chaperone activities. Consequently, increased activation of the BiP IRES and increased levels of La are observed in HCMV infected cells. Knockdown of La results in both the loss of BiP IRES activity and a reduction of BiP protein levels. It is concluded that infection by HCMV results in increased BiP levels in part due to the ability of La to promote cap-independent translation of the BiP IRES. Although several studies have demonstrated the translocation of nuclear La to the cytoplasm under compromised cellular conditions, the mechanism by which this occurs has yet to be established.

1.4.3 La protein involvement in cancer

The La protein has been linked to cancer related mRNA metabolism, particularly due to its association with the cancer related targets Cyclin D1, Laminin B1, and Murine double minute 2 (MDM2) [56,58,115,116]. Sommer *et al*. observed elevated La protein expression in squamous cancers of the head, neck, and cervix to drive migration and invasion. Elevated levels of La correlated with aberrant levels of CCND1 and it was established that La binds the IRES of the CCND1 mRNA to promote IRES dependent translation, contributing to proliferation [58]. Furthermore, studies show that La binds near the CCND1 start codon and acts as an RNA chaperone to alter the structure of the IRES to facilitate cap-independent translation. However, this chaperone ability is deterred by T389 phosphorylation by AKT1 [69]. It has been concluded that both the ability of La to act as an

RNA chaperone along with the T389 binding site are critical to promote IRES driven translation of the CCND1 mRNA.

La has also been implicated in chronic myelogenous leukemia (CML) through its association with the MDM2 mRNA. CML is triggered by a mutation generating the BCR-ABL1 fusion oncogene, producing abnormal myeloid cells which proliferate in bone marrow and eventually migrate into the bloodstream. The BCR-ABL1 gene is formed when the ABL gene from chromosome 9 joins to the BCR gene on chromosome 22, to form the BCR-ABL fusion gene [117]. Increased expression of La and MDM2 have been detected in BCR-ABL1-expressing myeloid precursor cell lines [116]. Trotta *et al*. demonstrated that the ability of La to recognize and bind to a conserved sequence in the intercistronic region of MDM2 mRNA is necessary for MDM2 protein expression. Moreover, it was shown that the levels of MDM2 and La are proportional, wherein the down-regulation of La leads to a decrease in MDM2, while overexpression of La results in increased MDM2 expression. Since MDM2 is an essential negative regulator of the p53, a guardian of the genome and vital tumour suppressor, this positive correlation suggests that La can indirectly affect the expression levels of p53. It has been hypothesized that the BCR-ABL1 oncogene leads to p53 inactivation, as CML patients often possess inactivating mutations of the p53 gene. Thus, this suggests that the interaction of La with MDM2 can indirectly lead to the progression of cancer through the functional inactivation of p53.

The involvement of La protein in the epithelial to mesenchymal transition (EMT) through its association with the Laminin B1 IRES was first identified by Petz *et al* [115]. EMT results in epithelial cells losing their cell to cell adhesion contacts and becoming malignant, possessing both migratory and invasive properties. This leads to their transition into mesenchymal stem cells, cells susceptible to differentiation and formation of new cell types. The laminins are a family of

glycoproteins found in the extra cellular matrix, and involved in the migratory behavior of malignant cell types. Specifically Laminin B1, promotes cell adhesion, motility, and differentiation and is over-expressed in metastatic cancers such as hepatocellular carcinoma (HCC) [118]. Using bicistronic and RNA affinity assays, it was shown that through its associations with the Laminin B1 IRES, La indirectly promotes translational upregulation during hepatocellular EMT.

1.5 La-related protein function in RNA metabolism

1.5.1 LARP1

While the La protein functions in facilitating the processing of RNA polymerase III and RNA polymerase II transcripts, the LARPs have evolved to target only one of these classes. Although structurally different, there exist many similarities between substrates and functionality of genuine La and the LARPs. The LARP1 family has diverged the most from genuine La. LARP1 was first identified in *Drosophila* and is required for embryogenesis, oogenesis, spermatogenesis, formation of the mitotic spindle poles, successful segregation of mitochondria, and cell cycle progression [119–121]. In humans, LARP1 can be found in both the nucleus and the cytoplasm, but is predominantly cytoplasmic and associated with mRNA translation, stability, and decay [122]. Using co-immunoprecipitation, Aoki *et al*. initially reported that LARP1 promotes translation of 5' TOP containing mRNAs by binding directly to the poly(A) tail to stabilize the structure of the translating message [123]. Later, Tcherkezian *et al*. added to this hypothesis by revealing that LARP1 promotes translation of 5' TOP containing mRNAs via direct interaction with PABP and other cap binding elements, an interaction mediated the DM15 region [124].

While non-phosphorylated LARP1 interacts with both the 5'- and 3' UTR of mRNAs to inhibit their translation, phosphorylated LARP1 dissociates from the 5' UTR and activates translation. In the recent years, great focus has been placed on the involvement of LARP1 with the mammalian target of rapamycin complex 1 (mTORC1) and protein synthesis. mTORC1 is a protein complex that activates and regulates protein synthesis in response to various cellular and environmental factors including adequate energy resources, nutrient availability, oxygen and proper growth factor abundance [102]. In conditions where mTORC1 is inhibited, such as oxidative stress or nutrient starvation, LARP1 is hypothesized to displace the eukaryotic translation initiation factor 4G (eIF4G) from the 5' cap to form a bridge between the 5' TOP sequence and the poly(A) tail **(Figure 9)**. By displacing eIF4G, LARP1 creates a closed loop in which translation is inhibited. However, when protein synthesis and mTORC1 are active, LARP1 forms a translationally active open-loop confirmation to bind PABP to promote translation. A structure based study by Lahr *et al.,* identified the DM15 region of LARP1 as binding directly to the 7-methylguanosine cap of 5' TOP mRNAs [73]. Interactions of LARP1 with the cap and adjacent 5' TOP motif inhibits binding of eIF4E to the cap, thereby preventing the assembly of the eIF4F complex.

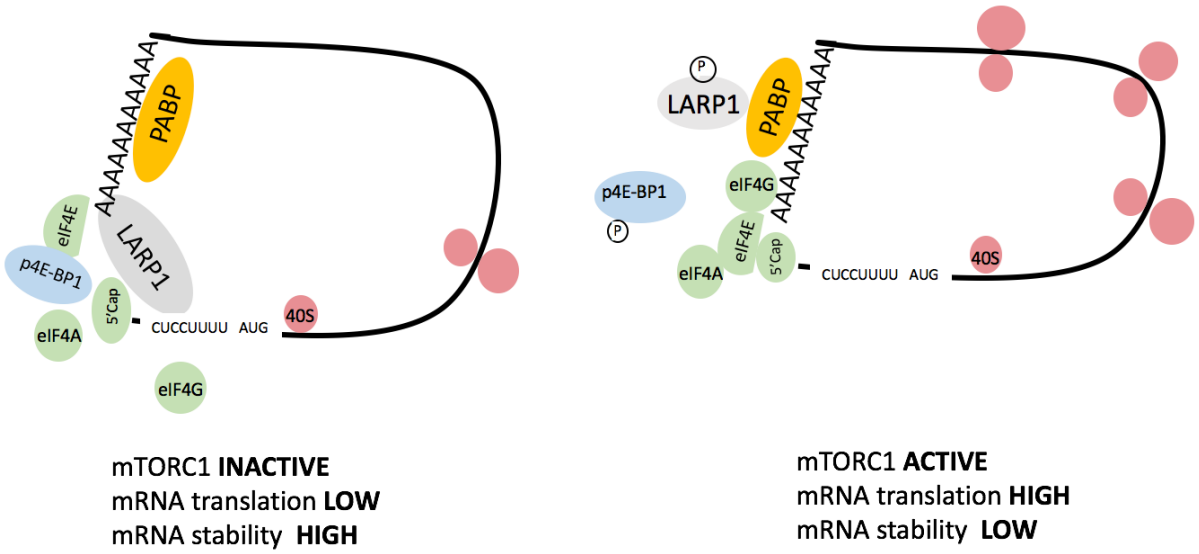

Figure 9: Schematic of hypothesized LARP1 mediated translation of TOP mRNA

LARP1 is hypothesized to both positively and negatively regulate 5' TOPs in response to activation of the mTORC1 pathway. When mTORC1 is inhibited under conditions of cellular stress, LARP1 displaces eIF4G from the 5' cap and binds the 5' TOP sequence, the 5' cap, and the poly(A) tail creating a closed loop formation that is translationally inactive. LARP1 also prevents eIF4E from binding to the cap, thereby blocking the assembly of the eIF4F complex. However, when mTORC1 is activated, 4E-BP1 is phosphorylated releasing eIF4E to join the eIF4F complex. Simultaneously, LARP1 is recruited to bind PABP producing an mRNA in an open-looped formation that is translationally active. Figure adapted from [125].

LARP1 is associated with several types of cancers including cervical, prostate, breast, and hepatocellular [71,126]. Knockdown of LARP1 in cervical cancer cell lines such as HeLa using RNA interference (RNAi) causes cells to undergo apoptosis [127]. In prostate cancer cells, knockdown of LARP1 results in attenuation of tumour migration. It was also shown that the LARP1 mRNA is a direct target of miR-26a and miR-26b, two miRNAs that are significantly downregulated in cancer [128]. Loss of these tumour-suppressive miRNAs in prostate cancer cells enhances invasion through direct regulation of LARP1. In breast cancer, a spliced variant of LARP1 has been identified

suggesting that splicing may occur to alter the conformation of binding domains to bind to different RNA substrates [129].

1.5.2 LARP4

Similar to LARP1, the LARP4 family has been implicated with mRNA translation. LARP4 has two variants, LARP4a and LARP4b, which share only 38% homology [42]. Although LARP4 does not contain a DM15 motif or a second RRM, it does contain a non-canonical PAM2 domain (PAM2w). It is hypothesized that both the LAM and PAM2w directly bind PABP to promote translation [39,130,131]. Both LARP4a and LARP4b are believed to promote mRNA translation via direct interactions with PABP and the ribosome-associated receptor for activated C kinase 1 (RACK1), a 40S ribosome and mRNA associated kinase [39,132]. While mammalian LARP4a displays affinity for poly(A) RNA, suggesting it could bind to mRNA poly(A) tails, LARP4b binds to AU-rich regions in the 3' UTR of mRNAs, implying that these proteins may have distinct functions. It is hypothesized that LARP4b stabilizes the circular conformation of mRNAs by binding both PABP and RACK1 to promote protein synthesis [132]. Recent studies have shown LARP4 to be a vital regulator of cancer cell migration and invasion. Knockdown of both LARP4a and LARP4b has resulted in increased migration of prostate cancer cells and elevated mRNA levels of the tumour suppressor genes p16 and p19, suggesting that LARP4 may play a crucial role in tumour suppression [133].

1.5.3 LARP6

LARP6, originally called Acheron, was first identified in the moth *Manduca sexta* when studying programmed cell death during development of intersegmental muscles [134,135]. Found predominantly in neurons, striated skeletal muscle, and cardiac muscle, LARP6 plays a key role in differentiation, proliferation, and apoptosis of myoblasts-nascent muscle fibers [120]. In addition,

this protein influences cell adhesion, morphology, and cytoskeletal organization. LARP6 shuttles between the nucleus and cytoplasm, and participates in the nuclear export and regulation of type I collagen mRNAs, the most abundant vertebrate structural protein [136]. A unique feature of LARP6 is the conserved SUZ-C motif in the C-terminus believed to participate in substrate recognition [74]. Type I and type III collagen mRNAs contain a unique conserved stem-loop structure in the 5' UTR. LARP6 binds to the 5' stem-loop structure in a sequence specific manner to stabilize mRNA and affect its translation [137,138]. While the ability of LARP6 to bind collagen mRNA was originally attributed to the LAM along with an additional 40 amino acid motif at the N-terminal that is absent from other LARPs, recent studies suggest that the inter-linker domain between the LAM and the RRM within the La module may play a critical role in facilitating this binding [139]. Generally, LARP6 acts as an oncogene by enhancing angiogenesis and tumor growth, and has been found to be highly expressed in breast cancer in the myoepithelial cells of the mammary gland [140].

1.5.4 LARP7

Human LARP7 mediates the transcription and regulation of the 7SK RNA by binding, in a sequence specific manner to the UUU-3'OH end [141,142]. The abundantly expressed non-coding 7SK RNA controls eukaryotic transcription by regulating the activity of the positive transcription elongation factor b (P-TEFb), a cyclin dependent kinase required for RNA polymerase II transcription elongation [143]. By binding to both the UUU-3'OH and the 5' capping protein methylphosphate capping enzyme (MePCE), LARP7 circularizes the 7SK snRNP to create a stable structure protected from exonuclease digestion.

In addition to binding the U-rich sequence at the 3'OH end of the 7SK RNA, LARP7 also recognizes and binds a 3' hairpin loop structure near the U-rich tail. These interactions have been mapped to the LAM, the RRM1, and RRM2 in tandem. The complex of LARP7 bound to the 7SK

RNA suppresses the P-TEFb complex, thus suppressing transcription [144]. Knockdown of LARP7 using RNAi results in transcriptional elongation due to 7SK RNA degradation which results in P-TEFb activation. Deletion of the C-terminal RRM results in gastric cancer tumorigeneses, suggesting that the C-terminal RRM plays a crucial role in its interaction with 7SK RNA and that LARP7 is a potential tumour suppressor [145,146]. Analysis of breast cancer tissue from patients show extremely low levels of LARP7 and increased levels are observed in cancer survivors, further confirming the oncogenic role of this protein [147]. A summary of the La-related protein families, their substrates, and functions are listed in **Table 2**.

Table 2: Summary of LARP families with their functions and substrates. Modified from[71]

	La/SSB	LARP1a	LARP1b	LARP4a	LARP4b	LARP6	LARP7
Role in transcription	Protects Pol III transcripts and maturation of pre-tRNA and non coding RNAs	-	-	-	-	Interacts with transcription factors, vimentin, non muscle myosin	Binds 7SK snRNP and negatively regulates RNA pol II transcription
Role in translation	IRES mediated and 5'TOPs	Regulates the stability and translation of mRNAs	-	Promotes mRNA stability	Stimulates translation and circularizes mRNAs	Promotes translation of collagen	-
Known mRNA targets	IRES and 5'TOP mRNAs	mRNAs containing 5'TOPs and mTOR, 5'cap of mRNA	-	Single stranded poly(A) stretches	-	Type 1 collagen	-
Protein binding partners	-	Raptor, eIF4E, eIF4A, 5'cap	-	RACK1, 40S components	RACK1, 40S components	Filaments, RNA helicase, non muscle myosin	MePCE
Binds PABP?	-	yes		yes	yes	yes	no
Substrate recognition motifs	stem loop in IRES mRNAs and miRNAs, and 3'trailer of Pol III transcripts	5'end of TOPs	-	-	-	5'stem loop in alpha collagen	3'end of 7SK RNA
Associated cancers	head, neck, liver, cervix	cervix, liver, breast, prostate, lung	-	prostate	acute myeloid leukemia	breast	cervix, gastric, breast
Tumor suppressor or oncogenic?	proto-oncogenic	proto-oncogenic	-	tumor suppressor	proto-oncogenic	proto-oncogenic	tumor suppressor

While the genuine La protein assists in both processing of RNA polymerase III and RNA polymerase II transcripts, the various LARP families target only one of these classes. The LARP7 family is most closely related to genuine La both functionally and structurally. Like La, members of the LARP7 family bind RNA polymerase III transcripts using a UUU-3'OH dependent RNA binding mode and assist in transcription. Members of the LARP1, LARP4, and LARP6 families are predominantly involved in various aspects of mRNA stability and translation, and have been reported to interact with PABP. Of these, both human LARP1 and LARP4 have been shown to directly engage poly(A) and demonstrate affinity for adenylates in a length dependent manner. However, the mechanism through which these LARPs engage poly(A) sequences is still unknown. LARP6 binds to the 5' stem-loop structure of collagen mRNAs in a sequence specific manner. The ability of the La/LARP families to bind such a vast range of substrates is attributed to subtle amino acid variations within the La Module along with the diverse domains found in each of these proteins (e.g., DM15 in LARP1 and PAM2w in LARP4). Further structural and biochemical studies will reveal novel targets and functions of the LARP families.

1.6 Summary and Statement of Purpose

La proteins are present in nearly all eukaryotes and have conserved functions in facilitating the processing of RNA polymerase III transcripts. hLa is an RNA-binding phosphoprotein that shuttles between the nucleus and cytoplasm interacting with various RNA substrates [46,52]. Human La phosphorylated at S366 is typically found in the nucleus and involved in the processing of RNA polymerase III transcripts such as U6 snRNA, tRNA precursors, or pre-5S rRNA and serves to protect transcripts from 3'-5' exonuclease degradation by binding the UUU 3'OH trailer in a sequence specific manner [53,54,79]. While the interaction of La with substrates that end with the uridylate trailer has been well characterized, its interaction with substrates that lack this trailer is

still under investigation. Non-phosphorylated hLa, found in the cytoplasm, binds and enhances the expression and translation of cellular and viral mRNAs through the use of the IRES, 5' TOPs, and uORFs. La associated viral IRESs include Hepatitis C, Hepatitis B, encephalomyocarditis viruses, and poliovirus [41,57,87]. La associated cellular IRESs include BiP, Laminin B1, XIAP, and CCND1 [56,58,108,115]. These cytoplasmic substrates are often involved in cell cycle regulation, apoptosis, tumour suppression, and stress responses, signifying a potentially important role for La during cellular stress conditions. Although La proteins are hypothesized to promote cap-independent translation of these coding RNAs, the context and mechanism by which La binds and affects translation of coding RNAs has yet to be established.

The objective of this study is to further understand the mechanisms by which La affects translation and interacts with its cytoplasmic targets, as these structures lack a UUU-3'OH trailer. Like hLa, certain LARP families, specifically hLARP 1 and 4, have also been implicated in mRNA translation and protein expression through their contacts made with the poly(A) tail directly and/or with PABP [39,124,132]. Given the established role of both La and the La-related proteins in the translation of cellular mRNAs, we began by testing whether La also regulates mRNA translation via contacts made through the poly(A) tail. In Chapter 2, we show that in addition to sequence specific UUU-3'OH binding, hLa exhibits a sequence specific and length dependent poly(A) binding mode and that La may regulate IRES mediated translation through contacts made with the poly(A) tail. Using electromobility shift assays (EMSA) and CLIP, we show that hLa exhibits a sequence specific and length dependent poly(A) binding mode. Through mutational analysis, we mapped this poly(A) binding mode to the winged helix face in the LAM previously shown to be vacant during uridylate binding. We also show La entry into polysomes utilizes the poly(A) binding mode and that the ability of La to promote translation using the CCND1 IRES occurs

competitively with PABP. These results provide a mechanism through which hLa may interact with its cytoplasmic substrates to drive cap-independent translation.

The second aim of this study is to understand the cellular context in which La interacts with its cytoplasmic substrates. Several reports have shown that during conditions of viral infection, cellular stress, and tumour progression, the predominantly nuclear hLa protein can translocate to the cytoplasm where it functions as an ITAF, controlling cap-independent translation initiation of several cellular IRES-containing mRNAs. These La associated cytoplasmic substrates are often involved in cell cycle regulation, apoptosis, tumour suppression, and stress responses, signifying an important role for La during cellular stress conditions. However, the mechanism for the shuttling of La during cellular stress seems to be context specific. Thus in Chapter 3, we investigate the conditions that initiate this translocation and identify novel stress associated substrates of hLa. We show that cellular stress induced by the mitochondrial inhibitor clotrimazole, can induce translocation of hLa from the nucleus to the cytoplasm. In addition, using polysome analysis and qPCR, we show that translocation of La to the cytoplasm is concurrent with increased La association with actively translating messages. Using iCLIP, we identified specific messages that La interacts with during stress and monitored changes in La association with these messages in the presence and absence of cellular stress. Taken together, this study reveals a novel binding mode and mechanism of action for hLa and demonstrates that hLa is trafficked into the cytoplasm in response to cellular stress in order to associate with stress-related messages and promote cap independent translation.

Chapter 2:

Human La binds mRNAs through contacts to the poly (A) tail

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Stefano Marrella performed the EMSA data shown in Figures 10, 11, 12, Supplementary Figures 1, 2, and Supplementary Table 1. Rawaa Hussain, Leonid Rozenfeld, and Karine Solomon performed previous versions of EMSAs.

2.1 Summary

In addition to a role in the processing of nascent RNA polymerase III transcripts, La proteins are also associated with promoting cap-independent translation from the internal ribosome entry sites of numerous cellular and viral coding RNAs. La binding to RNA polymerase III transcripts via their common UUU-3'OH motif is well characterized, but the mechanism of La binding to coding RNAs is poorly understood. Using electromobility shift assays and cross-linking immunoprecipitation, we show that in addition to a sequence specific UUU-3'OH binding mode, human La exhibits a sequence specific and length dependent poly(A) binding mode. We demonstrate that this poly(A) binding mode uses the canonical nucleic acid interaction winged helix face of the eponymous La motif, previously shown to be vacant during uridylate binding. We also show that cytoplasmic, but not nuclear La, engages poly(A) RNA in human cells, that La entry into polysomes utilizes the poly(A) binding mode, and that La promotion of translation from the cyclin D1 internal ribosome entry site occurs in competition with cytoplasmic poly(A) binding protein (PABP). Our data are consistent with human La functioning in translation through contacts to the poly(A) tail.

2.2 Introduction

La proteins have been characterized in nearly all eukaryotes examined and have conserved functions in the processing of RNA polymerase III transcripts [42]. First identified as an autoantigen in patients suffering from systemic lupus erythematosus and Sjögren's syndrome [45,148], immunoprecipitations using anti-La antibodies revealed that human La (hLa) associates with precursor forms of RNA polymerase III transcripts [55,76,149] as well as the uridylate-tailed adenoviral VA RNA and the Epstein-Barr virus encoded EBER RNAs [150,151]. The importance of the uridylate tail was subsequently validated by experiments showing that the number of uridylates directly influenced the efficiency of La binding to a pre-tRNA or VA RNA substrate, with high-affinity binding generally requiring at least three terminal uridylates [54,79]. Subsequent structural and biochemical work deciphered the specific mechanism of UUU-3'OH recognition, in which the UUU-3'OH motif is sandwiched between the N-terminal La motif (LAM) and RNA recognition motif (RRM1; together the so-called "La module"). Furthermore it was demonstrated that uridylate specific contacts are mediated largely by conserved amino acids on the La motif [62,63]. Surprisingly, these structures indicated that neither of the expected nucleic acid binding surfaces of the La module (the winged helix interaction surface of the LAM nor the β-sheet of RRM1) contribute to UUU-3'OH recognition [62,152], leaving the function of these canonical interaction surfaces unclear.

In addition to a sequence-specific UUU-3'OH-dependent binding mode, other work using a variety of substrates has demonstrated that the canonical RNA binding surface of RRM1, RRM2 and the disordered CTD also contribute to La RNA binding in a relatively non-specific manner [40,41,153,154]. The canonical RNA binding surface of RRM1 enhances human La binding to the main body of pre-tRNAs, which in combination with the UUU-3'OH dependent binding modes, assists

La in the discrimination of pre-tRNA processing intermediates [40,155]. The RRM1, RRM2 and disordered C-terminal regions of La have also been implicated in RNA binding via modes that lack sequence specificity but can nevertheless rely on the presence of RNA secondary structure [41,153,156]. For example, the La motif, RRM1 and RRM2 all contribute to the binding of a small hairpin with a short, single-stranded 3' tail derived from the Hepatitis C IRES, and while the presence of the hairpin and single stranded extension were both shown to be critical for maximal binding, the actual sequence of these was much less important [41]. Often, these UUU-3'OH independent binding modes have been implicated in La function as an RNA chaperone. RNA chaperone activity in human La has been mapped to the RRM1 as well as the disordered CTD [84,154], and it has been proposed that one of the functions of the UUU-3'OH depending binding mode is to recruit non-specific La-associated RNA chaperone activity to UUU-3'OH containing substrates [157]. It is thus hypothesized that binding of La to RNA targets *in vivo* occurs through the co-operation of a number of RNA binding modes in combination [41,49], and that the specificity determinants for some of these binding modes are still nebulous.

Consistent with the presence of UUU-3'OH independent La-RNA binding modes, La proteins also immunoprecipitate coding RNAs lacking this motif [52,59]. Human La was identified as the first *i*nternal ribosome entry site (IRES) *t*rans-*a*cting *f*actor (ITAF) due to its ability to enhance translation of polioviral RNA [158], and has since been shown to likewise enhance cap-independent translation of other (+) stranded viral RNAs associated with challenges to human health. IRESs are also found in some cellular mRNAs, and it has been hypothesized that these can be more efficiently translated under conditions of cellular stress (reviewed in [159]). Consistent with a role in the translation of RNA polymerase II transcripts, La immunoprecipitates mRNAs in a variety of experimental systems, including yeast, *Xenopus* oocytes, and human tissue culture cells

[52,59,160]. La promotes IRES based translation from several cellular mRNAs, including those encoding BiP, cyclin D, NRF2 and laminin B1, as well as the upstream open reading frame (uORF) containing mRNA for the oncogene MDM2 [56,58,109,115,116]. The effect of La on classical cap-dependent translation is less well understood, with various studies indicating that La can activate or inhibit cap-dependent protein synthesis, suggesting that La influence in this process may be context specific (reviewed in [50]). While the mechanism by which La proteins recognize coding RNAs is still poorly understood, it has been hypothesized to rely on electrostatic, RNA structure dependent contacts mapping largely to the RRM1 and RRM2 domains (in those La proteins that harbour a second RRM) [41,50]. Importantly, a sequence specific RNA binding mode for La targets that lack UUU-3'OH has yet to be characterized.

Recently, the study of human La has substantially expanded into the study of the human La-related proteins (hLARPs), which similar to hLa share a La-motif and can harbour RNA chaperone activity but are hypothesized to bind RNA targets distinct from those of La [42,50,161]. Like La, the hLARPs 1, 4 & 4b have also been implicated in the control of protein translation. Notably, these La-related factors are hypothesized to associate with the poly(A) tail directly and/or through interactions with poly(A) binding protein [123,124,132,162,163]. However, a mechanism by which the LARPs may engage poly(A) sequences is still lacking. Given the established role of La and the La-related proteins in the translation of cellular mRNAs, we decided to test whether La might contact mRNAs, at least in part, through the poly(A) tail. In this work, we demonstrate that human La specifically binds poly(A) RNA *in vitro* with significantly higher affinity than other non-uridylate homopolymers, provided the RNA sequence is extended (i.e twenty nucleotides), as is common in the poly(A) tails of mRNAs. We also show the canonical winged-helix face of the La motif plays a role in poly(A) but not UUU-3'OH binding, as mutagenesis of this region impairs

a) poly(A) binding *in vitro*, b) entry of cytoplasmic La into polysomes c) crosslinking immunoprecipitation (CLIP) of poly(A) tails in human cells and d) translation from a bicistronic cyclin D1 IRES containing reporter construct. Together, our data are consistent with a model in which two specific binding modes (UUU-3'OH and poly(A)) direct La RNA chaperone activity to its two classes of RNA substrate in their respective cellular compartments. The importance of the La motif in poly(A) binding may also have relevance for the mechanism of how the cytoplasmic La-related proteins associated in translation perform related functions.

2.3 Materials and Methods

Electromobility shift assays (EMSAs)

All radiolabelled RNAs were PAGE purified after 5' end labeling. Recombinant His-tagged human La [164] or hLa point mutants (generated by QuikChange; Agilent) were purified from *E. coli* first over a Ni^{++} column (His-Trap, GE-Amersham) then a heparin column (Hi-Trap, GE-Amersham). Proteins were then concentrated and quantitated via Bradford and SDS-PAGE, and A260/A280 ratios were taken to confirm purified proteins were free from contaminating RNAs that might have co-purified from *E. coli*. EMSAs were performed as described [40]. Briefly, 3000 cpm (approximately 0.1 nM) of various RNA substrates (IDT) were incubated with various concentrations of recombinant human La or human La mutants in a 20 μl reaction containing 1X EMSA buffer (20 mM Tris pH 7.6, 100 mM KCl, 1mM EDTA and 5 mM β-mercaptoethanol) and 50 ng HepC (5' rCrGrU rGrCrA rCrCrA rUrGrA rGrCrA rCrGrA rArUrC rCrA 3' [41]) or 10 ng C10 as cold RNA competitor. RNAs were initially slow-cooled from 95°C to room temperature and then incubated with protein at 37°C for 20 minutes. Complexes were resolved on 10% (w/v) polyacrylamide nondenaturing gels at 4°C at 100V. Supershifts were treated as supplementary binding events to the primary binding event, and binding curves were fit using a non-linear specific binding curve fitting program (GraphPad, Prism). K_d values were approximated as the concentration of protein at which half of the RNA substrate was bound.

For competition experiments, radiolabelled RNAs were mixed with indicated amounts of cold RNA in 1X EMSA buffer, after which 2uM recombinant hLa was added and incubated for 20 minutes at 37^{o}C, prior to separation on native PAGE as described above.

Cross-linking Immunoprecipitation (CLIP)

CLIP was performed as described [27] with the following modifications. Briefly, two 15cm dishes of HEK293T cells per CLIP were transfected with pEGFPC1-hLa [70], or indicated GFP-hLa variants or myc-tagged pcDNA-PABPC1 [165] (PolyJet, SignaGen). 24 hours post transfection cells were UV-crosslinked (Stratalinker at 254 nm, 1000 mJ) and cells were lysed in RIPA buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF). Lysates were then treated with 3µl of 1:100 RNAse I (100U/µl AM2294 Invitrogen) or 40 µl of a 1:100 dilution of RNase T1 (1U/µl AM2283 Ambion) and 2 µl DNase I (2U/µl AM2238 Ambion) at 37^{o}C for 3 mins. Antibodies used were anti-cmyc (Abcam ab21060) or anti-GFP (Abcam ab1218). coIPs were performed using Protein G magnetic Dynabeads (Invitrogen). Antibodies were incubated with Dynabeads in 150 µl RIPA buffer for one hour at room temperature, then antibodies/Dynabeads were incubated with lysates for two hours at 4^{o}C. Complexes were washed 2x with RIPA buffer and 3x with proteinase K buffer prior to digestion with 10 µl proteinase K (buffer and enzyme supplied by Invitrogen, #100005393 20mg/mL). Eluted RNA was probed with 32P radiolabeled dT(40), stripped and reprobed for pre-tRNA Met-e (probe sequence AAA TTA TTG TGC CCC G) via Northern.

qPCR of hLa precipitated mRNAs

Crosslinking of RNA protein complexes was performed as described [166]. Briefly, one 15cm plate of HEK293T cells was transfected with pEGFPC1-hLa 1-375, pEGFPC1 hLa 1-375 K86A/T87A/K88A or the pEGFPC1 vector control and crosslinked with 1% formaldehyde at room temperature for 10 minutes. The reaction was stopped with 0.25M glycine pH 7.0 for 5 minutes at room temperature. Cells were washed twice in 1X PBS followed by lysis in 1 mL RIPA

buffer with DNase treatment (4U, 5 minutes at 37°C). Immunoprecipitations were performed using anti-GFP and Protein G beads with three 500 uL washes in RIPA buffer then three 500 uL washes in PNK buffer (50 mM Tris-HCl pH 7.0, 5 mM EDTA, 10 mM DTT, 1% SDS) followed by reversal of crosslink by incubation at 70°C for 45 minutes. RNA was Trizol extracted followed by isopropanol precipitation. Input and eluted RNAs were converted to cDNA using BioRad iScript cDNA synthesis kit and assessed by quantitative real-time PCR using SensiFAST SYBR No-ROX Kit (Bioline) using the following settings: Hot start @ 95°C for 5 minutes; (denaturation for 5 sec @ 95°C, annealing/extension @ 60°C for 10 sec) x 40 cycles. Relative abundance was calculated as enrichment over empty vector normalized to input using the ΔΔCt method. Primers used: BiP Forward: GAAAGAAGGTTACCCATGCAGT, BiP Reverse: CAGGCCATAAGCAATAGCAGC; CCND1 Forward: CTCTCCAGAGTGATCAAGTGTGACCC, CCND1 Reverse: TGTGCAAGCCAGGTCCACC; 5.8S rRNA Forward: TCTTAGCGGTGGATCACTCGGC, 5.8S rRNA Reverse: GCTCAGACAGGCGTAGCCC. Values presented represent mean enrichments over a minimum of three biological replicates.

Polysome analysis

Polysome analysis was performed as described [167]. Briefly, two 15cm dishes of transfected HEK293T cells were lysed 24 hours post transfection in hypotonic lysis buffer [5 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, 1.5 mM KCl, 100 μg/mL cycloheximide, 2 mM DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate] and cell debris was removed by centrifugation. 20 A_{260nm} of cell lysate was loaded over a 7 step 20-50% sucrose gradient prepared according to the method of Luthe [168] in 20 mM HEPES-KOH (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$ followed by centrifugation

at 30,000 rpm for 3 hours in a Beckman SW41 rotor. Gradients were fractionated into 500 µL fractions of which 20 µL was separated by SDS-PAGE and blotted using the relevant antibodies. Anti-Rpl9 antibody was from Abcam (ab182556). For puromycin treatment, cell lysates were treated with 25 µM puromycin for 15 minutes and cyclohexamide was omitted [169].

CCND1 IRES luciferase reporter assay

The CCND1 IRES was cloned as a cDNA from HeLa poly(A) purified RNA into the SalI-BamHI site of the pDL-N dual luciferase construct [170] and confirmed by sequencing. This construct was then used as a template for a PCR reaction using primers that added an SP6 promoter upstream of the renilla 5' UTR and a 20A or 40A sequence after the firefly 3' UTR. The PCR product was used as a template for a SP6 RNA polymerase mediated in vitro transcription of capped mRNA using the SP6 mMESSAGE mMACHINE kit from Ambion as per manufacturer's protocol. HEK 293T cells were plated in 6 well plates 18 hrs prior to transfection in antibiotic free media and transfected with 4ug plasmid DNA (pEGFPC1-hLa [70] and PABPC1-cmyc [165] or vector controls) and Lipofectamine 2000 as per the manufacturer's instructions. 24 hours post transfection, cells were re-plated into 96 well plates and transfected with 150ng of mRNA. 24 hours post transfection lysates were measured using Dual-Luciferase Reporter Assay (Promega). To test for reporter mRNA levels, qPCR of the firefly cistron was performed using the Qiagen one-step RT-PCR kit (Qiagen #210210) and TaqMan based primers, normalized to the U5 snRNA as measured by SYBR qPCR as described above. Primers: U5 snRNA For: TGG TTT CTC TTC AGA TCG CAT AAA, U5 snRNA Rev: CCA AGG CAA GGC TCA AAA AAT; Firefly For: GAC GAT GAC GCC GGT G, Firefly Rev: GAC TGG CGA CGT AAT CCA, Firefly TaqMan Probe: /56-FAM/CC GCC GTT G/ZEN/T TGT TTT GGA GCA C/3IABkFQ/. TaqMan probe was from IDT.

2.4 Results

La binds to poly(A) in a sequence specific and length dependent manner

While La preference for terminal uridylates has previously been established, La binding to extended adenylate sequences has not been tested, despite the established association of La with cellular mRNAs in both yeast and humans cells [56,58,59,115,116]. To test La binding to polyadenylate sequences, we compared hLa binding to various RNA homopolymers using electromobility shift assay **(EMSA; Figure 10, Supplementary Figure 1, Supplementary Table 1)**. To better assess the nature of specificity of RNA binding in our analysis, we included one of two competitors in our EMSA analysis. The first was a small hairpin with a single stranded 3' tail derived from a La target, the Hepatitis C IRES, as La has previously been demonstrated to bind to this RNA in a manner that relies on its secondary structure in the absence of sequence specificity [41]. The alternate competitor was a C10 homopolymer, which is not expected to form secondary structure.

Using the hairpin-containing competitor **(Figure 10)**, we found that hLa bound U10 with substantially higher apparent affinity than C10, G10 or A10, consistent with previous results demonstrating high affinity binding of La to terminal uridylates. However, when the length of the homopolymers was increased to twenty nucleotides, the apparent affinity of hLa for A20 increased substantially. This increase in affinity was specific for adenylates, as C20 bound hLa with low affinity, similar to C10 **(Figure 10A, B, Supplementary Figure 1)**. We noted that G20 tended to dimerize, with the free RNA running as two bands **(Supplementary Figure 1)**. Interestingly, hLa shifted the G20 dimer with reasonably high affinity, while the single stranded G20 band had similarly poor affinity as the G10 homopolymer, reminiscent of previous work indicating binding of hLa to RNAs with secondary structure in the absence of sequence specificity [40,41].

We then compared hLa binding to U10 and A20 in the presence of C10 as a competitor to our previous result using the hairpin-containing competitor **(Figure 10 C, D)**. We found that the apparent affinity for the U10 substrate using C10 as a competitor was nearly identical to that when using the hairpin-containing competitor. However, the apparent affinity for A20 increased when substituting C10 as a competitor, to the extent that the apparent K_d of hLa for A20 was only slightly lower than that of U10. These data suggest that a) hLa binds A20 with high affinity in a manner that is length dependent and that discriminates adenylates from C or (single-stranded) G, and b) structured RNAs compete slightly better for A20 binding than single-stranded RNA, suggesting that some A20 contacts may partially overlap with contacts associated with structure dependent, UUU-3'OH independent binding [41].

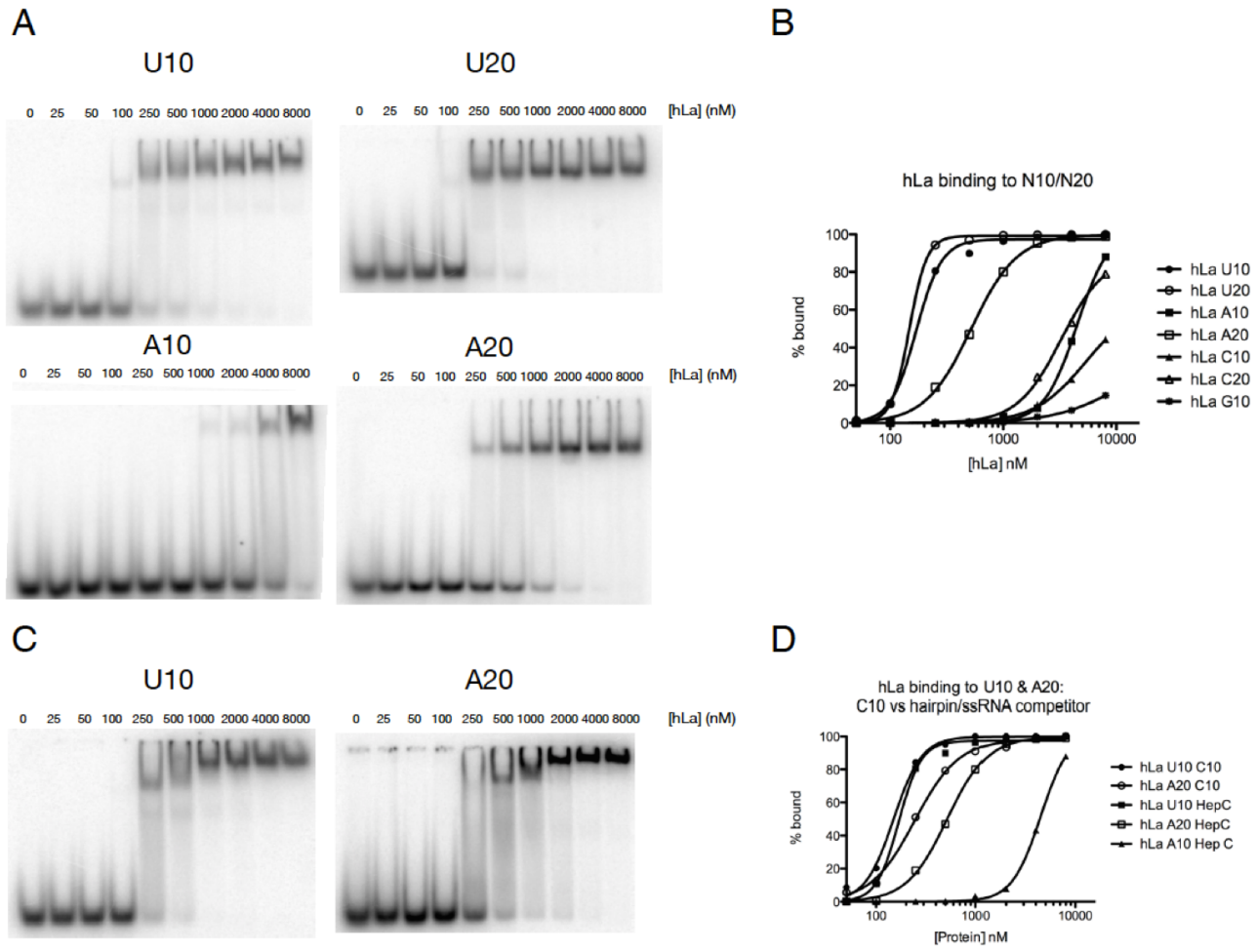

Figure 10: Human La displays length dependent affinity for poly(A)

Human La displays length dependent affinity for poly(A). **(A)** Binding of recombinant human La for U10, U20, A10 and A20 was tested by electromobility shift assay (EMSA) using HepC hairpin RNA as competitor; gels for C10, C20, G10 and G20 provided in Supplementary Figure 1. **(B)** Graphical representation of EMSA results. **(C)** EMSA of human La for U10 and A20 in the presence of C10 as competitor. **(D)** Graphical representation of binding curves comparing La affinity for U10 and A20 with C10 versus HepC hairpin competitors.

In order to better understand the potential overlap between the UUU-3'OH dependent binding mode and those contacts important for A20 binding, we performed direct competition experiments between uridylate and adenylate containing RNAs **(Figure 11)**. We determined an amount of recombinant human La (2 μM) sufficient to achieve >95% binding of radiolabelled A20 (NR lane, Figure 2.2A) or U20 (NR lane, Figure 2.2B) in the absence of competitor, then added this amount of protein to pre-mixed radiolabelled and cold RNAs containing increasing concentrations of cold competing RNA substrates to determine their propensities to compete our radiolabelled RNAs from hLa. In order to demonstrate a successful competition reaction, we performed competitions with the identical cold U20 or A20 RNAs (**Figure 11 A & B,** right hand sides**)**. These experiments revealed that at this concentration, La-RNA binding was occurring in two stages: in the absence of competitor (NR), La protein at 2 μM supershifts U20 and A20 (i.e. greater than one La per RNA, as has been described previously at higher concentrations of La (reviewed in [49]). This supershift would then shift down to a 1:1 La-RNA RNP with increasing like competitor, as the cold RNAs titrated hLa causing a supershift away from the radiolabelled substrates. (**Figure 11 A & B,** right hand sides; 1:1 La-RNA indicated as "1", La-RNA supershifts as "2"). We found that addition of cold competing U10 to radiolabelled A20 titrated La resulting in a supershift away from A20, causing a clear mobility shift to the 1:1 La-A20 species at lower concentration of competitor (**Figure 11A**, left hand side, marked with "*"). However, increasing addition of U10 did not result in a release of A20 from the hLa-A20 RNP even at the highest concentration of U10 competitor tested (32 μM). These data indicate that despite U10 being active in our assay (addition of U10 titrated La that caused a supershift away from A20) it did not result in release of A20 from the A20:hLa RNP, suggesting that the U10 competes poorly for A20 and that their binding modes are at least partially distinct.

We considered that the La RNP in the presence of both A20 and U10 could be a double stranded RNA duplex, and that the La RNP observed with increasing competitor could be the result of La binding to double stranded RNA. To test this, we added increasing concentration of A10 to the hLa:U20 RNP (**Figure 11B**, left panel), as U20:A10 should have a similar propensity to form a duplex as A20:U10. Unlike the A20:U10 competition, however, A10 had no effect on the hLa:U20 RNP, as measured by the persistence of supershift with increasing concentration of A10, consistent with our previous data **(Figure 10)** showing low affinity for A10. In sum, these data are consistent with a relatively poor ability of adenylates to compete with uridylates on hLa and at least partially distinct poly(A) and UUU-3'OH binding modes.

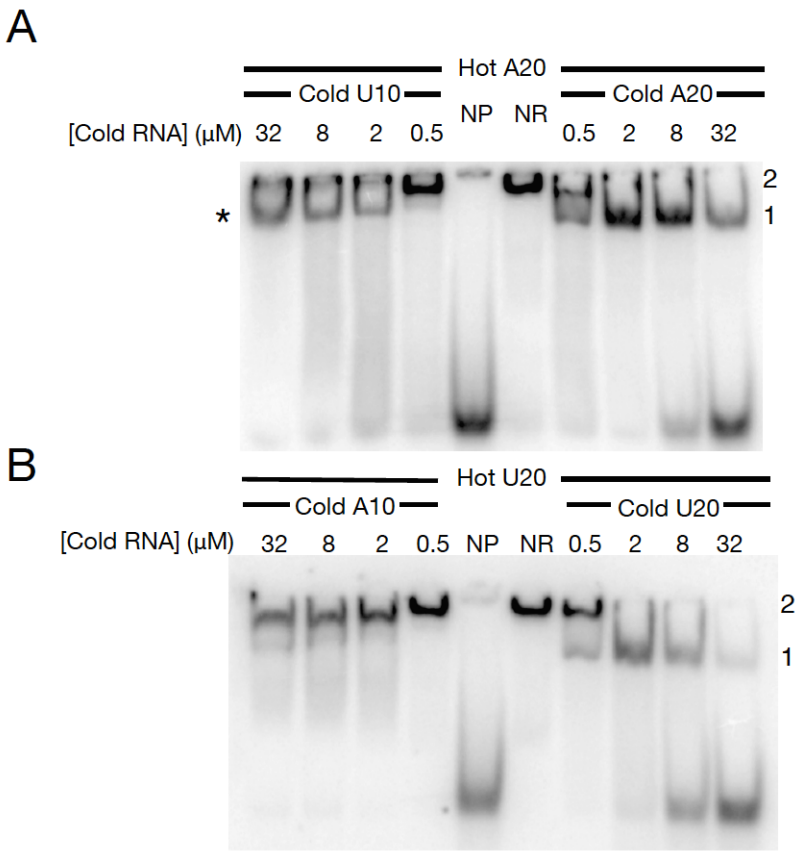

Figure 11: Uridylate containing RNAs compete poorly for poly(A) binding to hLa

Sufficient recombinant human La was added (2 μM) to radiolabelled A20 to achieve >95% binding (NR: **N**o cold **R**NA added) and increasing concentrations of various cold RNAs were added to assess their ability to displace A20 from hLa. **(A)** Comparison of U10 (left-hand series) and A20 (right-hand series) for radiolabelled A20 binding. **(B)** Comparison of A10 (left-hand series) and U20 (right-hand series) for radiolabelled U20 binding. NP: **N**o recombinant hLa **P**rotein added (i.e. free radiolabelled A20 or U20). "1": indicative of 1:1 La-RNA complex; "2": indicative of multimeric La-RNA complex. * (asterisk): New RNP formed between La and A20 with addition of U10.

The winged-helix face of the La motif functions in adenylate binding

High resolution La module/UUU-3'OH co-crystal structures have previously identified the regions of La required for uridylate recognition [62,63], and these surprisingly revealed that the expected nucleic acid binding surface of the LAM (the winged helix face) does not make any contacts to the UUU-3'OH motif **(Figure 12A)**. To further investigate potential differences between the UUU-3'OH and A20 binding modes, we compared U10 versus A20 binding across some La mutants **(Figure 12)**. We tested a mutant we have previously demonstrated as having significantly compromised affinity for terminal uridylates [86] by virtue of mutations to three amino acids critical for UUU-3'OH binding (hLa Q20A/Y23A/D33R) for its differential ability to bind U10 and A20. Consistent with our competition data arguing for at least partially distinct U10 and A20 binding modes, this mutant had nearly negligible U10 binding yet still bound A20. We observed a slight decrease in A20 affinity relative to wild-type, **(Figure 12B, C)**, consistent with either a slight folding defect for this variant, as has been noted previously [40], or the possibility that amino acids critical for UUU-3'OH binding may also play a role in adenylate binding. Nevertheless, the near complete loss of binding of this mutant to U10 relative to the continued binding to A20 are consistent with the uridylate and adenylate RNA binding modes still being substantially distinct.

We considered the possibility that the expected winged helix-fold nucleic acid interaction surface of the La motif might comprise one of the regions important for A20 recognition, as this region has not yet been demonstrated to engage an RNA ligand. We therefore tested several sets of point mutants around the La motif for their differential ability to bind U10 versus A20. The hLa mutant R32A/K34A/K37A, whose mutated amino acids map to neither the winged-helix face nor the UUU-3'OH recognition site, had no defect in binding either substrate, while the hLa

F65A/N66A/E70A and E70A/K74A/K76A mutants, whose mutations map to the canonical winged-helix recognition helix of the La motif, showed slightly impaired binding to both U10 and A20, suggesting they may be misfolded (data not shown). The hLa mutant K86A/T87A/K88A comprises mutations to the first "wing" of the La motif's winged helix fold; while this mutant bound U10 nearly indiscernibly from wild-type, it had a more substantial drop in affinity for the A20 substrate, as well as a more gradual binding curve **(Figure 12E)**, suggesting the possible loss of one of several cooperative binding sites. These data suggest these amino acids contribute to the affinity of La for poly(A) but not UUU-3'OH, even if this mutant still contains other surfaces that contribute to poly(A) binding, consistent with our observed length dependence of the adenylate binding mode.

To further test these ideas, we compared U10 and A20 binding across a series of hLa deletion mutants **(Supplementary Figure 2)**. Consistent with previous work indicating that the LAM and RRM1 form a single binding pocket for UUU-3'OH, deletion of the LAM (hLa dLAM) or RRM1 (hLa dRRM1) resulted in near complete loss of U10 binding. However, each of these mutants were still able to completely shift the A20 substrate, albeit at significantly higher concentrations than the wild-type protein. Deletion of RRM2 and amino acids C-terminal to this (1-235) had no effect on U10 binding, as expected, while A20 binding was slightly enhanced, suggesting that contacts that contribute to A20 binding are contained within the La module and the unstructured region between RRM1 and RRM2. In sum, our results suggest that while the binding of La to UUU-3'OH is an event that relies integrally on the presence of both the LAM and RRM1 forming a single binding site, the adenylate binding mode uses sites on the LAM and RRM1 that function more additively and that cooperate for high-affinity binding.

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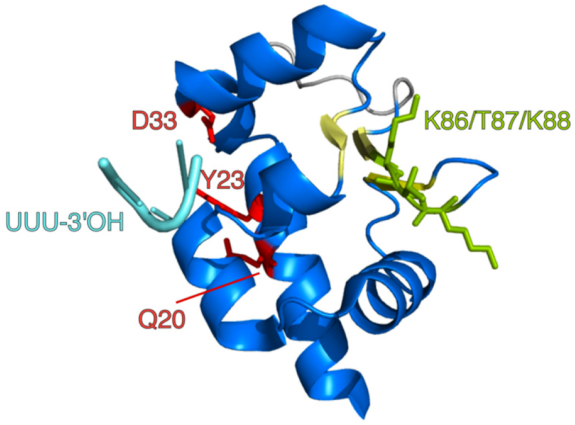
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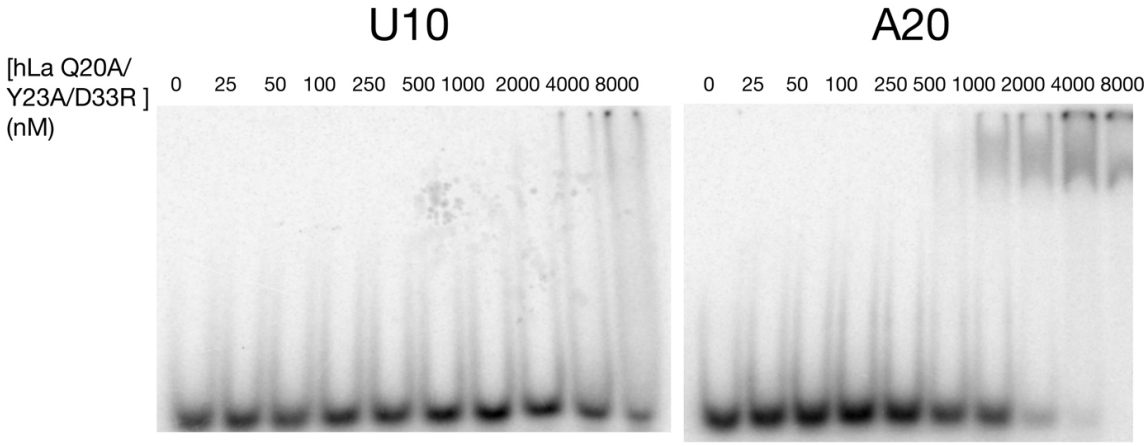
Y23
UUU-3'OH
Q20

B


U10
A20
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Y23A/D33R]
(nM)
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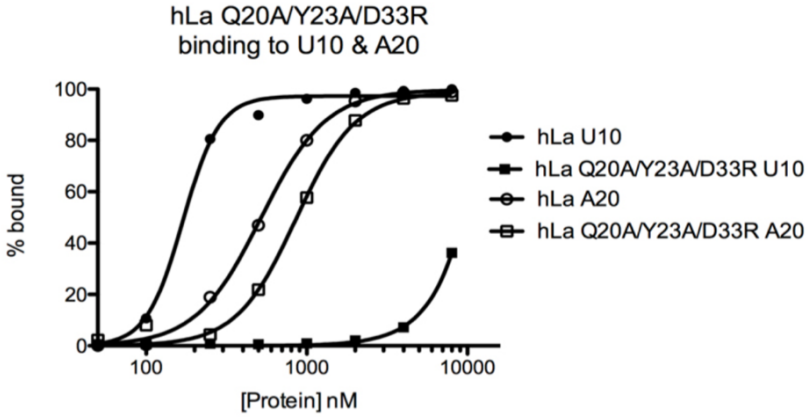
0 25 50 100 250 500 1000 2000 4000 8000

C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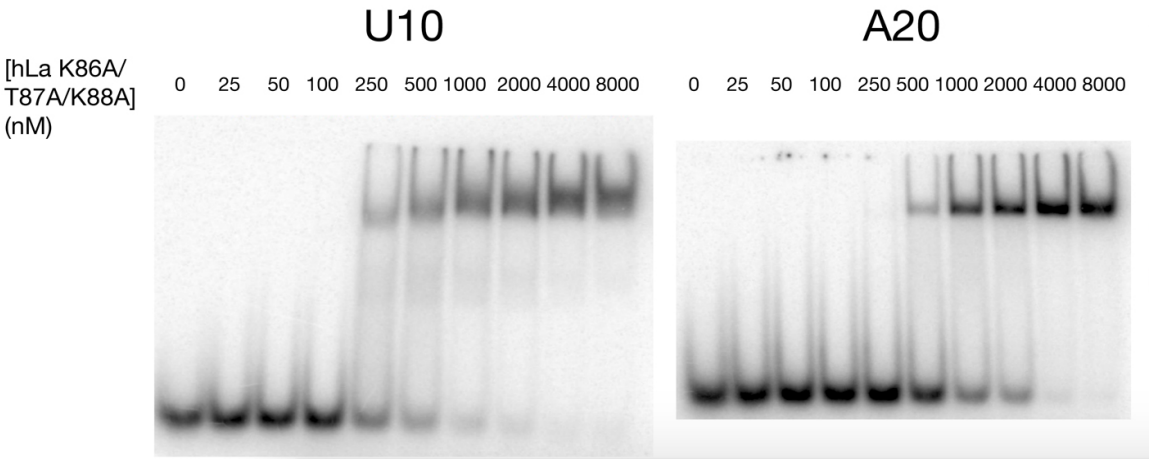
binding to U10 & A20
% bound
100
80
60
40
20
0
100
1000
10000
[Protein] nM
hLa U10
hLa Q20A/Y23A/D33R U10
hLa A20
hLa Q20A/Y23A/D33R A20

D



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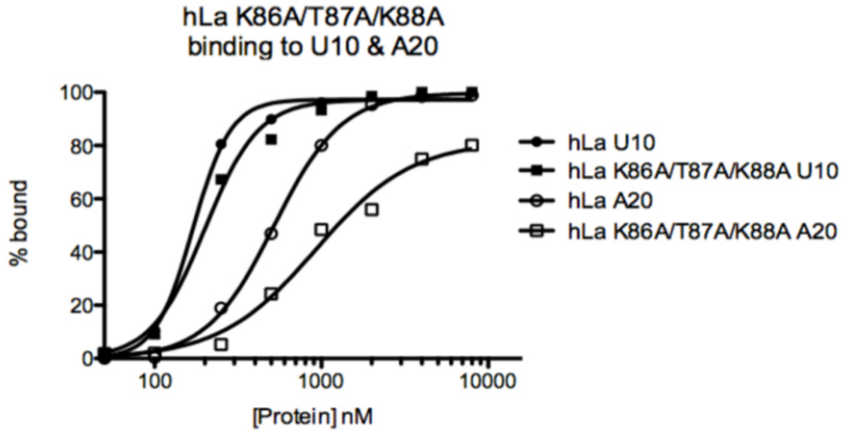

Figure 12: The winged-helix face of the La motif is involved in adenylate binding

(A) Structure of La motif [63] with uridylate binding amino acids labeled in red, UUU-3'OH RNA in cyan, winged helix face of La motif in yellow and mutated amino acids of winged-helix face in green. **(B)** EMSAs of hLa Q20A/Y23A/D33R binding to U10 and A20. **(C)** Graphical representation of hLa and hLa Q20A/Y23A/D33R bound to U10 and A20. **(D)** EMSAs of hLa K86A/T87A/K88A binding to U10 and A20. **(E)** Graphical representation of hLa and hLa K86A/T87A/K88A bound to U10 and A20.

Cytoplasmic La binds directly to poly(A) in human cells

Human La is largely nuclear where it engages RNA polymerase III transcripts, but also shuttles to the cytoplasm where it can accumulate during conditions of cellular stress or viral infection [89,109,171,172]. To test whether hLa can directly engage poly(A) tails in human cells, we performed cross-linking immunoprecipitation (CLIP), which included a limited RNase I digestion step in order to degrade RNA not in direct contact to hLa **(Figure 13A)**. To differentiate hLa RNA binding profiles in the nucleus versus the cytoplasm, we transfected GFP-tagged wild-type and nuclear localization signal deleted (ΔNLS [173,174]; hLa 1-375; localization of constructs in **Supplementary Figure 3**) hLa constructs into HEK293T cells. We also tested our point mutant with compromised A20 binding (K86A/T87A/K88A) in both the full length (nuclear) and 1-375 (cytoplasmic) contexts, as well as a myc-tagged cytoplasmic PABP construct as a poly(A) binding positive control.

We observed that PABP was highly effective in pulling down a range of poly(A) RNA lengths relative to the non-immune sera control, consistent with the heterogeneous length of poly(A) tails observed in the cytoplasm. However, PABP was incapable of pulling down a La RNA polymerase III substrate previously demonstrated to be an unusually stable and a very highly abundant hLa target, pre-tRNA Met-e [175,176], as expected. We found that the cytoplasmic GFP-hLa 1-375 immunoprecipitated poly(A) RNA of similar size range as PABP, and to a substantially greater extent than our nuclear GFP-hLa construct, relative to the poly(A) independent hLa target pre-tRNA Met-e. However, the GFP-hLa 1-375 K86A/T87A/K88A mutant was substantially impaired in poly(A) immunoprecipitation compared to GFP-hLa 1-375, despite still pulling down pre-tRNA Met-e. These data are consistent with hLa binding to poly(A) directly in the cytoplasm and further support the importance of the LAM winged helix in binding poly(A) over UUU-3'OH

containing substrates. To test the nature of this interaction further, we repeated the CLIP using excess RNase T1, which cuts specifically at "G" residues, as this should separate RNA upstream of the poly(A) tail but leave the leave poly(A) tail intact. We found that GFP-hLa 1-375 still immunoprecipitated poly(A) signal similar to PABP **(Figure 13A**, right-hand side**)**, also consistent with hLa 1-375 making contact to the poly(A) tail directly.

To further assess the importance of the winged helix face of the La motif in the binding of previously characterized La mRNA targets, we immunoprecipitated GFP-hLa 1-375 and GFP-hLa 1-375 K86A/T87A/K88A associated transcripts in the context of reversible formaldehyde crosslink and determined their relative abundance using quantitative RT-PCR **(Figure 13B)**. We observed a significant enrichment of GFP-hLa 1-375 with the cyclin D1 and BiP mRNA transcripts relative to the GFP alone control, as expected. However, the ability of GFP-hLa 1-375 to immunoprecipitate these mRNAs was substantially impaired in the context of the K86A/T87A/K88A mutations, and was more similar to the vector control, in agreement with our UV-CLIP data **(Figure 13A)**. As a negative control, we observed no enrichment for the 5.8S rRNA (which lacks a poly(A) tail) relative to the vector control. Thus our immunoprecipitation data suggest that cytoplasmic La binds mRNAs in cells at least in part via the poly(A) tail, and that the winged-helix face of the La motif contributes to this binding, similar to our data *in vitro*.

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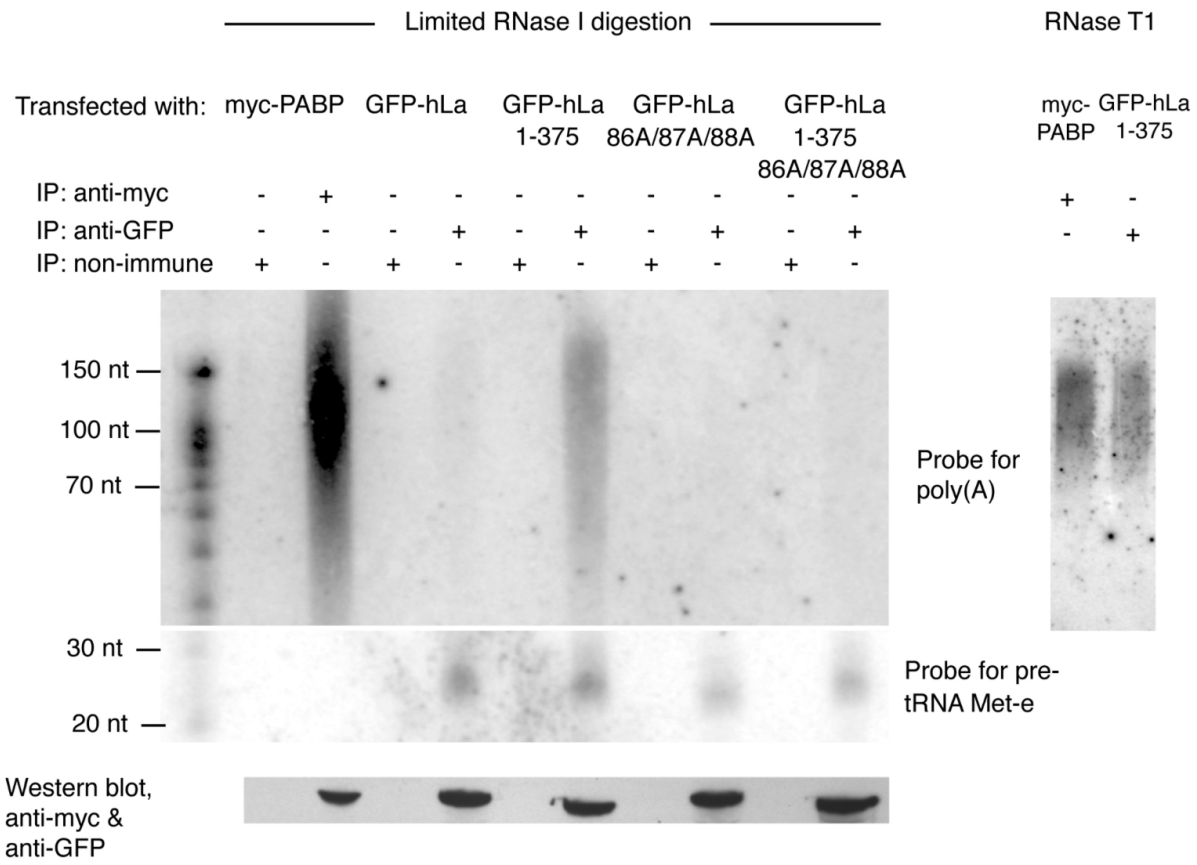

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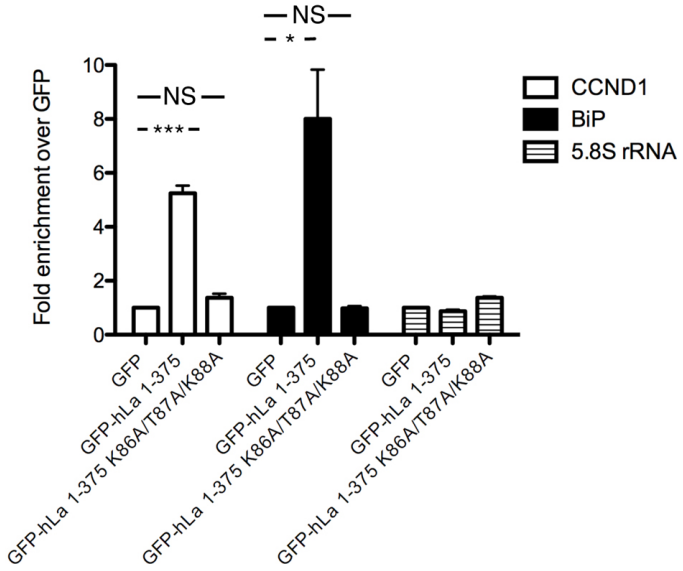

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Figure 13: Human La binds to poly(A) in human cells

(A) Crosslinking immunoprecipitation of transfected myc-PABP and GFP-hLa or GFP-hLa mutants in HEK293T cells. Left: limiting digestion with RNase I; right: Digestion with RNase T1. IP: immunoprecipitation. Presence of poly(A) or pre-tRNA Met-e in immunoprecipitated RNPs was assessed by Northern blot. Bottom: Western blot confirming transfection of myc-PABP or GFP-hLa. (**B)** Co-immunoprecipitation of cyclin D (CCND1) mRNA, BiP mRNA or 5.8S rRNA with hLa 1-375 versus hLa 1-375 GFP-hLa K86A/T87A/K88A relative to GFP vector only control as measured by qPCR. Error bars correspond to standard error of the mean, asterisks highlight statistically significant changes (*= P-value < 0.05; *** = P-value <0.001 two-tailed student t-test).

The poly(A) binding mode contributes to La entry into polysomes

La proteins have been implicated in the translation of several mRNAs and have been previously demonstrated to enter into polysomes [177]. We therefore hypothesized that the adenylate binding mode could play an important role in the capacity of La to enter polysomes via contacts to the poly(A) tail. We transfected GFP-hLa and the cytoplasmic GFP-hLa 1-375 into HEK293T cells and performed polysome fractionation followed by Western blot against GFP **(Figure 14)**. We indeed observed entry of GFP-hLa 1-375, and to a lesser extent GFP-hLa, into polysomal fractions, confirming that hLa (and predominantly cytoplasmic hLa) associates with translating polysomes, consistent with previous work [177]. To test the importance of the hypothesized poly(A) binding mode, we compared these results to the entry of GFP-hLa K86A/T87A/K88A and GFP-hLa 1-375 K86A/T87A/K88A into polysomal fractions. We observed a substantial decrease in the ability of these mutants to enter into polysomes, and instead observed them in abundance exclusively at the top of the polysome gradient. Addition of puromycin to GFP-hLa 1-375 transfected samples resulted in a collapse of polysomes and a concomitant loss of GFP-hLa 1-375 in the higher molecular weight fractions, confirming that GFP-hLa 1-375 is indeed associating with polysomes. These data are consistent with the winged-helix face of the La motif previously identified as a component of an hLa poly(A) binding mode playing an important role in La engagement of translating mRNAs.

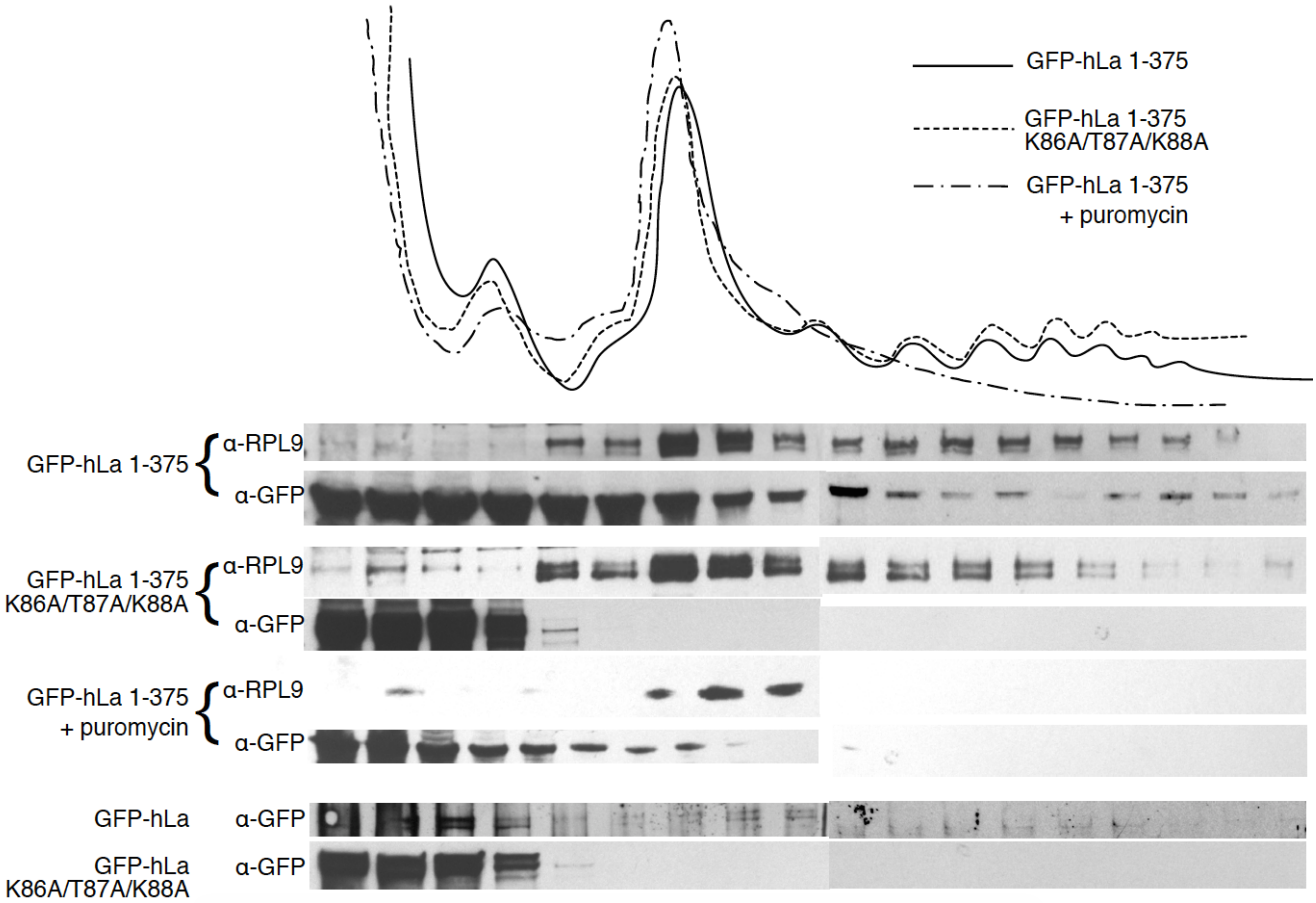

Figure 14: The poly(A) binding mode promotes hLa entry into polysomes

Top: Trace of ribosomes/polysomes fractionated from HEK293T cells transfected with indicated constructs or treated with puromycin. **Bottom**: Western blots versus Rpl9 and GFP-hLa, GFP hLa 1-375 and indicated mutants, as well as a puromycin treated control for GFP hLa 1-375.

La binding to poly(A) in cap-dependent and cap-independent translation

La function has been associated in the enhancement of cap-independent translation, and has been associated with either the enhancement or inhibition of cap-dependent translation depending on the identity of the transcript. To further test the importance of the poly(A) dependent binding mode in translation, we cloned the La-associated IRES from the human cyclin D (CCND1) gene into a dual-luciferase reporter construct, similar to what has been described previously [58], and used this to generate SP6 transcription templates from which we made capped mRNAs for direct transfection into HEK293T cells **(Figure 15A)**. This experimental design allowed us to control the poly(A) tail length of our transfected messages, which we set at twenty or forty adenosines (20A or 40A). These reporter mRNAs were transfected into cells that had been transfected twenty-four hours prior with plasmids encoding GFP-hLa (nuclear), GFP-hLa 1-375 (cytoplasmic) or the poly(A) binding impaired versions of these (GFP-hLa K86A/T87A/K88A or GFP-hLa 1-375 K86A/T87A/K88A), or the GFP vector control, and the effects on cap-dependent and cap-independent translation were assessed. We observed that overexpression of GFP-hLa 1-375 had a substantially greater positive effect on both cap-dependent (renilla) and cap-independent translation (firefly) than GFP-hLa, consistent with cytoplasmic hLa promoting the translation and/or stability of our transfected bicistronic reporter mRNAs **(Figure 15B)**. Notably, GFP-hLa 1-375 K86A/T87A/K88A had a significantly lesser positive effect on translation of both the renilla and firefly cistrons than GFP-hLa 1-375 despite equal levels of GFP-hLa 1-375 K86A/T87A/K88A expression **(Figure 15A)**, consistent with the poly(A) associated binding mode functioning in La enhancement of expression from the reporter. qPCR of the reporter mRNAs eight hours post-transfection revealed that increased expression from the reporter mRNAs was not

due to enhanced mRNA stability in the context of GFP-hLa 1-375 transfected relative to GFP or GFP 1-375 K86A/T87A/K88A transfected cells **(Figure 15C)**.

We then attempted to further design an experiment that might uncouple the potential positive effects of La on translation from those on mRNA stability. While testing for effects on the expression of mRNAs +/- a poly(A) tail in cells is challenging due to the anticipated degradation of messages lacking a poly(A) tail, we hypothesized that if La promotes translation at least in part through engagement of the poly(A) tail, then this might occur in competition with cytoplasmic PABP. We therefore repeated our GFP-hLa and GFP-hLa 1-375 overexpression experiments in the presence and absence of overexpressed myc-PABPC1, bearing in mind that both hLa and PABPC1 have an apparent minimum poly(A) tail binding length: ~ 20A for hLa (this work) and 12-25 nt for PABPC1 [178]. Co-transfection of myc-PABPC1 resulted in further enhanced expression from the renilla reporter relative to hLa 1-375 for the 40A tailed construct but not the 20A tailed construct **(Figure 15D)**, consistent with both PABPC1 and hLa 1-375 promoting cap-dependent translation on this reporter but with the 20A construct possibly being too short for these factors to act additively. Most importantly for this work, co-transfection of myc-PABPC1 had the opposite effect on cap-independent translation, significantly mitigating GFP-hLa 1-375 associated enhancement of firefly expression for the 20A construct **(Figure 15D)**, as well as the firefly/renilla ratio for both the 20A and 40A constructs **(Figure 15E)**. Since lowering the ratio of cap-independent translation to cap-dependent translation in the presence of excess PABP **(Figure 15E)** is not expected to result from altered stability of a bicistronic mRNA, this result is consistent with La-dependent enhancement of expression from the IRES reporter being due, at least partially, to La promoting translation at the IRES through contacts to the poly(A) tail. While the specific nature

by which PABP and hLa functionally interact in this system remains to be determined, these data further corroborate the link between La-associated function in translation and the poly(A) tail.

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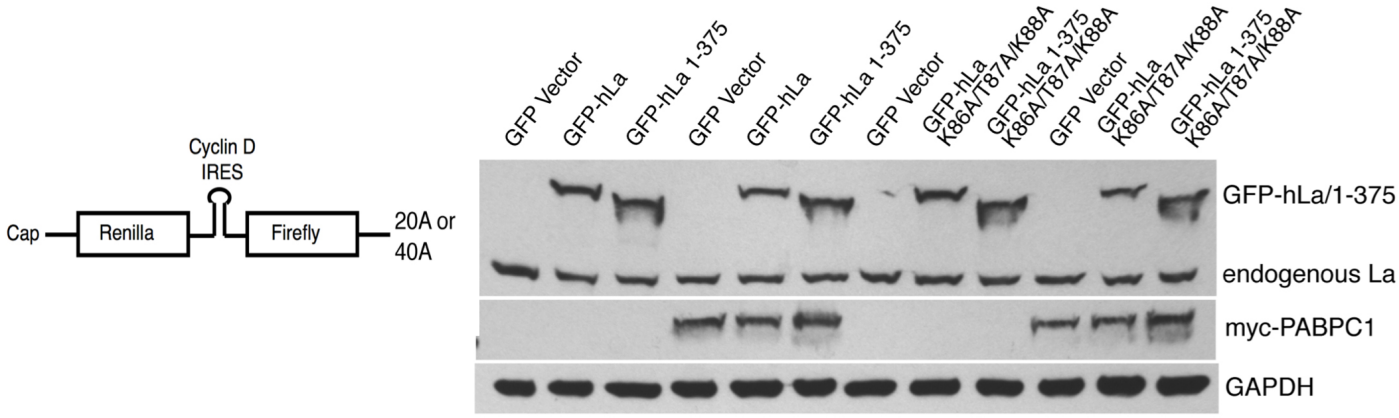
Cyclin D
IRES
Cap
Renilla
Firefly
20A or
40A
GFP Vector
GFP-hLa
GFP-hLa 1-375
GFP Vector
GFP-hLa
GFP-hLa 1-375
GFP Vector
GFP-hLa K86A/T87A/K88A
GFP-hLa 1-375 K86A/T87A/K88A
GFP Vector
GFP-hLa K86A/T87A/K88A
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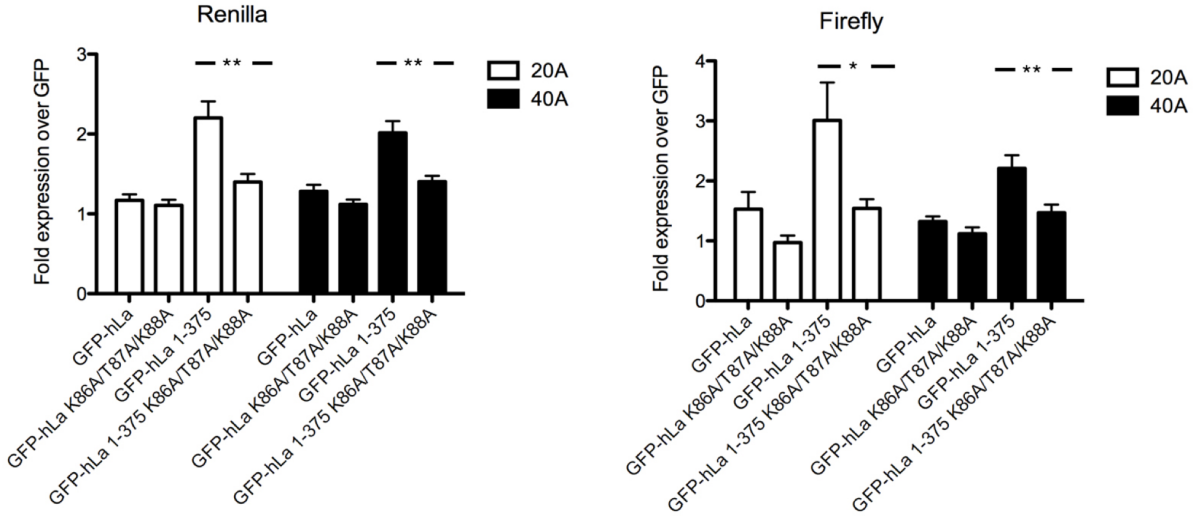
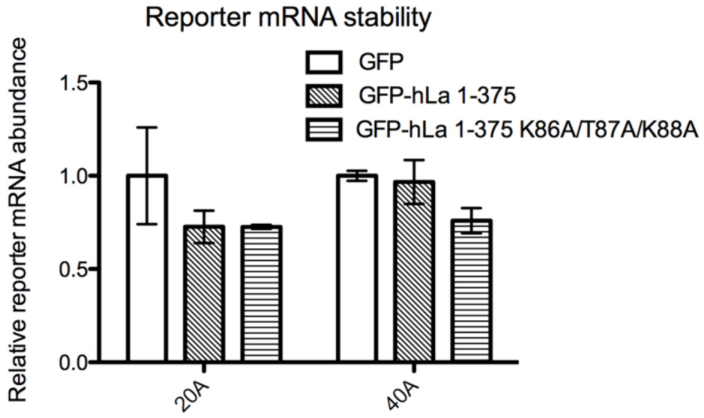
GFP-hLa/1-375
endogenous La
myc-PABPC1
GAPDH

B


Renilla
Fold expression over GFP
20A
40A
**
**
GFP-hLa
GFP-hLa K86A/T87A/K88A
GFP-hLa 1-375
GFP-hLa 1-375 K86A/T87A/K88A
Firefly
*
**

C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Relative reporter mRNA abundance
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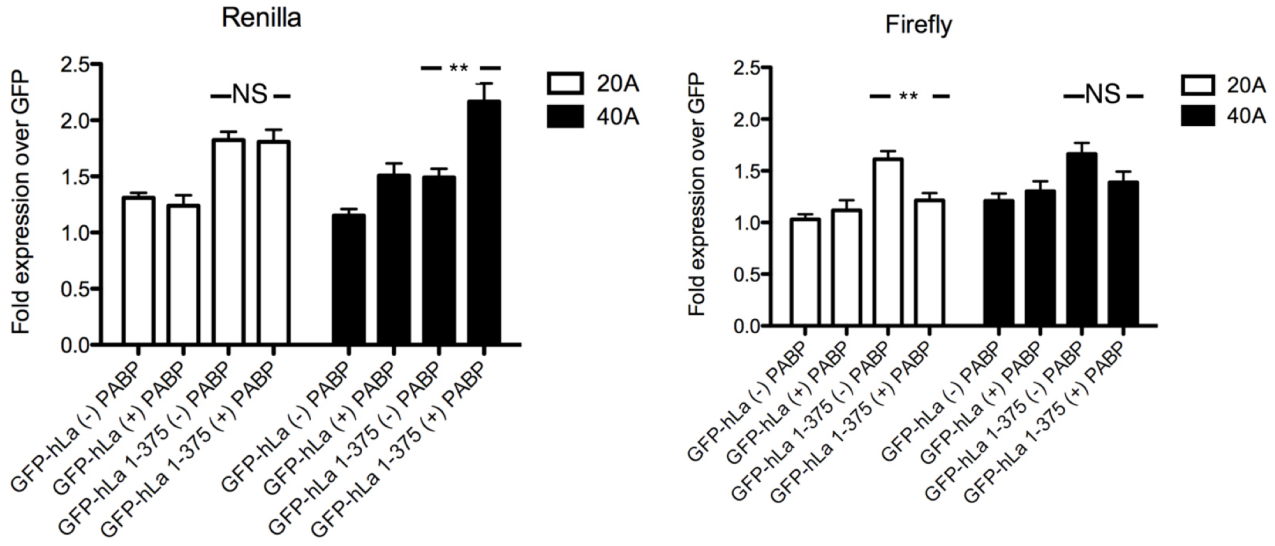
GFP-hLa 1-375
GFP-hLa 1-375 K86A/T87A/K88A
20A
40A

D



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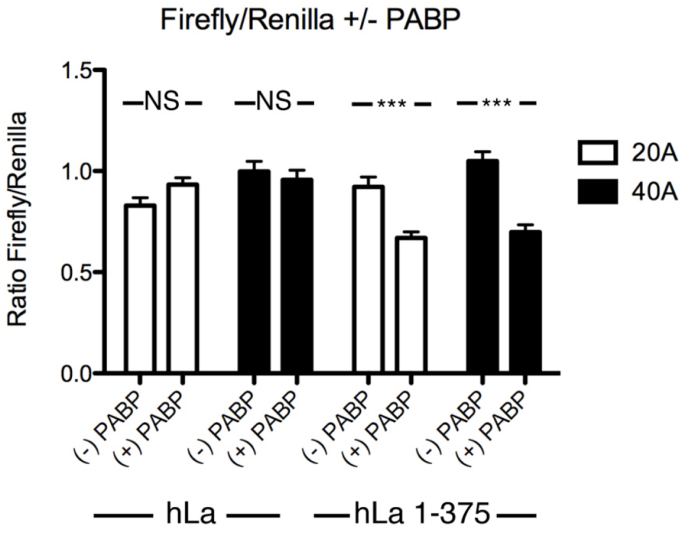

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Figure 15: The human La poly(A) binding mode contributes to La function in translation

(A) Left: Schematic of bicistronic reporter construct used for direct transfection into HEK293T cells. Right: Western blots of transfected myc-PABP, GFP-hLa, GFP-hLa 1-375 or K86A/T87A/K88A mutants of these. GAPDH shown as loading control. **(B)** Relative expression of cap-dependent (left; renilla) and cap-independent (right; firefly) reporter genes in the presence of overexpressed GFP-hLa, GFP-hLa 1-375 or the K86A/T87A/K88A mutants on the 20A or 40A tailed mRNA reporter constructs normalized to level expression in GFP vector control. ** = $P<0.001$. **(C)** Enhanced expression from bicistronic reporter mRNAs upon co-expression of GFP-hLa 1-375 is not due to enhanced reporter mRNA stability. Total RNA was isolated and levels of transfected 20A or 40A bicistronic reporters were assessed by qPCR eight hours post-transfection in cells that had been previously transfected by indicated GFP or GFP-hLa 1-375 constructs. Reporter mRNA levels are provided relative to amounts in the GFP-vector transfected cells after normalization for total RNA abundance via qPCR for the U5 snRNA. **(D)** Effect of overexpression of myc-PABP on the GFP-hLa and GFP-hLa 1-375 associated expression of cap-dependent (left, renilla) and cap-independent (right, firefly), normalized to the expression levels in the context of the GFP vector control +/- overexpression of myc-PABP. ** = $P<0.01$. **(E)** Ratios of renilla/luciferase expression in the context of GFP-hLa or GFP-hLa 1-375 expression +/- the expression of myc-PABP. *** = $P<0.001$. Error bars: SEM.

2.5 Discussion

Human La binds to poly(A) sequences *in vitro* and in human cells

In this work, we propose that human La binds adenylate sequences with high affinity, and that this binding mode represents at least one important way that La contacts coding RNAs in the cytoplasm. La proteins have been associated extensively with the engagement of mRNAs with consequent effects on their translation, but until this work, it was previously hypothesized that La engages such coding transcripts exclusively via non-specific recognition of structured RNA elements. In this work, we demonstrate that human La has higher affinity for adenylate containing sequences compared to poly(C) or single stranded poly(G) in a length dependent manner. We show that point-mutation of the winged-helix face of the La motif results in decreased binding to A20 but not a UUU-3'OH containing RNA, consistent with the adenylate and UUU-3'OH binding modes being at least partially distinct. Using UV-CLIP, we demonstrate that cytoplasmic La contacts poly(A) tails directly in a manner analogous to PABP in tissue culture cells, then demonstrate that the adenylate binding mode contributes to immunoprecipitation of previously identified La-mRNA targets, La entry in to polysomes, and La function in translation using a bicistronic cap-dependent/IRES reporter containing defined adenylate lengths. While the complete mechanism by which La binds to poly(A) tail and its consequent functional overlap with the role of PABP have not yet been fully explored, our data are consistent with La binding to poly(A) acting as an important determinant for La function in mRNA expression in the cytoplasm.

La function in cap-independent and cap-dependent translation

In the nucleus, La proteins are recruited to nascent RNA polymerase III transcripts, such as pre-tRNAs, via sequence specific recognition of UUU-3'OH [54,79]. La then assists these targets

attain their native fold via RNA chaperone activity prior to UUU-3'OH processing and removal [40,86,179]. In the cytoplasm, La was identified as the first cellular factor important for the enhancement of IRES dependent, cap-independent translation, a theme that has since recurred for a number of viral and cellular IRES containing transcripts, leading to hypotheses that La may also function as an RNA chaperone for these [99]. For IRES containing cellular mRNAs that contain a poly(A) tail, our data are consistent with a model in which La recognition of poly(A) may direct La-associated RNA chaperone activity to its mRNA targets in an analogous manner to how UUU-3'OH does the same for nascent RNA polymerase III transcripts. In such a scenario, hLa might remain bound at the poly(A) tail and function as an RNA chaperone by looping the RNA between the poly(A) tail and the 5' UTR/IRES, or hLa could first be recruited to the mRNA via the poly(A) tail and then translocate to these sites. Previous work noting a number of potential RNA binding sites on La support the idea that La may be able to engage the poly(A) tail as well as and other regions of an mRNA simultaneously [40,41,153,154,180], but this has yet to be demonstrated formally.

La function in cap-dependent translation appears to be more complex, as various studies have proposed either an activating or inhibitory role for La at the 5' cap. It has been previously noted that mRNAs whose cap-dependent expression is inhibited by La tend to have short 5' UTRs, such as 5' TOPs, while those that tend to be enhanced often have complex 5' UTRs that may rely on La-dependent RNA remodeling for optimal translation, similar to the role proposed for La at IRESs [68]. One factor whose role in the complex interplay coordinating expression at the cap is becoming increasingly appreciated is the La-related protein LARP1. Similar to La, human LARP1 (hLARP1) has also been hypothesized to bind to poly(A) directly [123], as well as via contacts with PABP [162], and reminiscent of La function on coding transcripts, has been associated with both the enhancement and inhibition of cap-dependent translation [124,125]. Very recently, a structural domain

unique to LARP1 family members and lacking in genuine La proteins, the DM15 region, has been demonstrated to bind the 7-methylguanosine cap in the context of a cytosine at +1, as is commonly observed in 5' TOP sequences [73,181]. These insights on LARP1 function in the expression from the cap have recently been expanded by work showing that LARP1 can be phosphorylated directly by mTORC1 and Akt to switch from an inhibitory to an activating role [182], as well other work demonstrating a role for hLARP1 in controlling the stability of 5' TOP mRNAs on 40S ribosomes independent of translation [183], which is reminiscent of other work linking LARP1 family members and mRNA transcript stability [123,184,185]. Future work will be required to deconvolute the complex interplay of LARP1, PABP and La at the poly(A) tail and the various effects of these on mRNA translation and degradation.

Poly(A) binding in the LAM superfamily

While La proteins have documented functions for both RNA polymerase III and coding transcripts, each of the various LARP families target only one of these classes: the LARP7 family members hLARP7 and p65 engage RNA polymerase III transcripts, also using a UUU-3'OH dependent RNA binding mode, and the LARP1, LARP4 and LARP6 families are associated with mRNA translation (reviewed in [50,65]) . While members from the LARP1, LARP4 and LARP6 families have been documented to interact with PABP [121,130,132,163], several LARPs have also been hypothesized to engage poly(A) directly, with human LARP4 and LARP1 demonstrating affinity for adenylates in a length dependent manner, similar to La [123,163]. Consistent with our findings, one study that screened for factors that could be affinity purified in a poly(A) dependent manner identified not only hLARP1, but also human La [123]. Future structural work will be very helpful in determining the precise contacts between La and poly(A) and whether LARPs that engage poly(A)

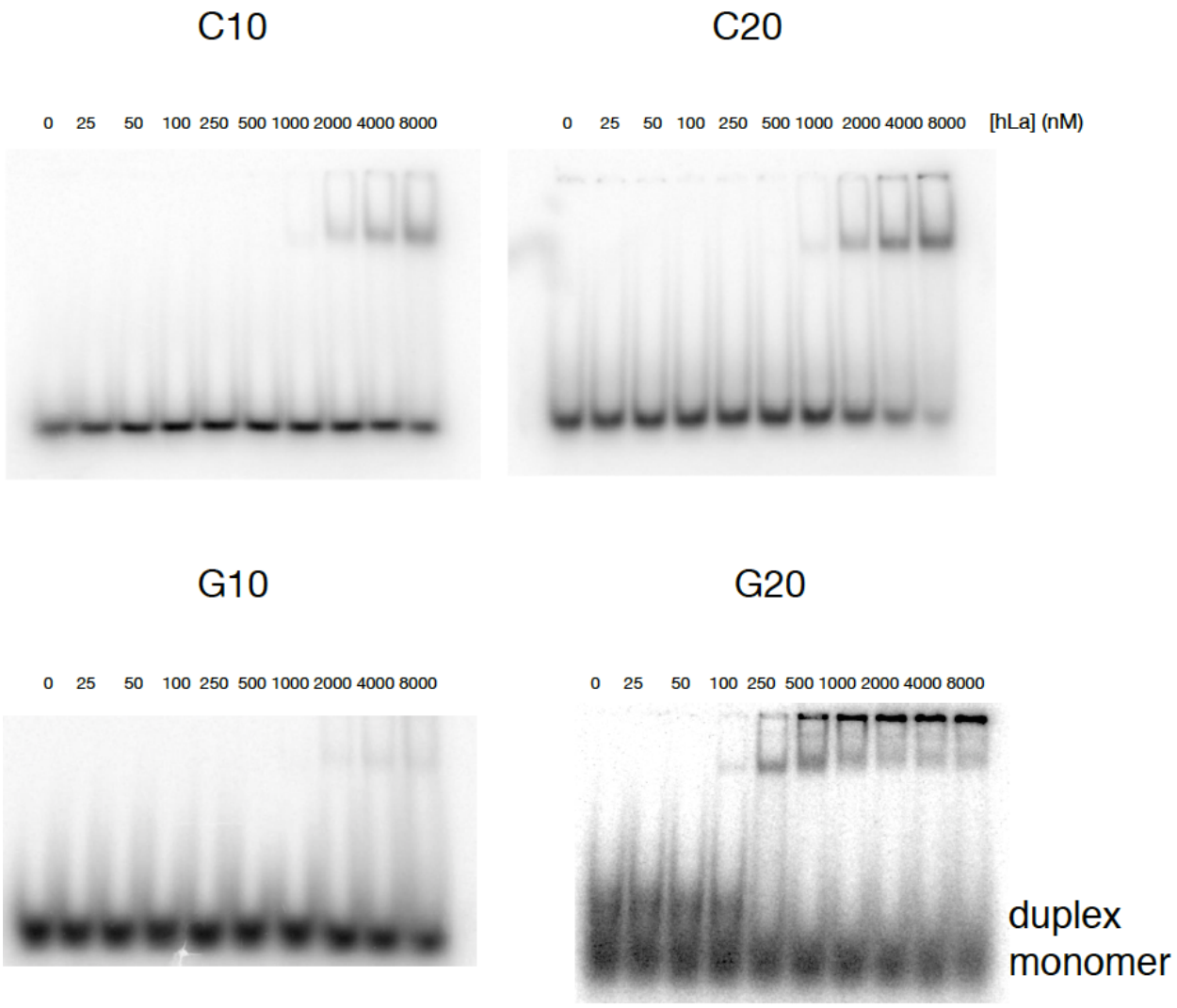
use the same LAM surface as in hLa, given the high conservation of the La motif between the La and La-related proteins.

Our data suggest that the hLa LAM and RRM domains are important for both the UUU-3'OH and poly(A) binding modes, but that the respective RNA binding surfaces on these domains do not entirely overlap. In La proteins, the linker region bridging the LAM and RRM domains is flexible in the RNA-free form, but for those LARPs that have been tested, this appears to not be the case [63]. Notably, the observation that this linker region is much shorter in La modules of the LARP4 family and in hLARP6 [42,139], as well as the observation of LAM-RRM interdomain contacts in the hLARP7 protein [186], has led to the hypothesis that unlike genuine La, the orientation of the linker region in the LARPs is fixed and may constitute an important RNA binding determinant [139]. It will thus be fascinating to investigate whether the flexibility observed between the LAM and RRM of hLa may relate to its capacity to accommodate both the poly(A) and UUU-3'OH binding modes, while the fixation of the orientation between the LAM and RRM in the LARPs is an outcome of their specialization for a single class of target.

2.6 Acknowledgements

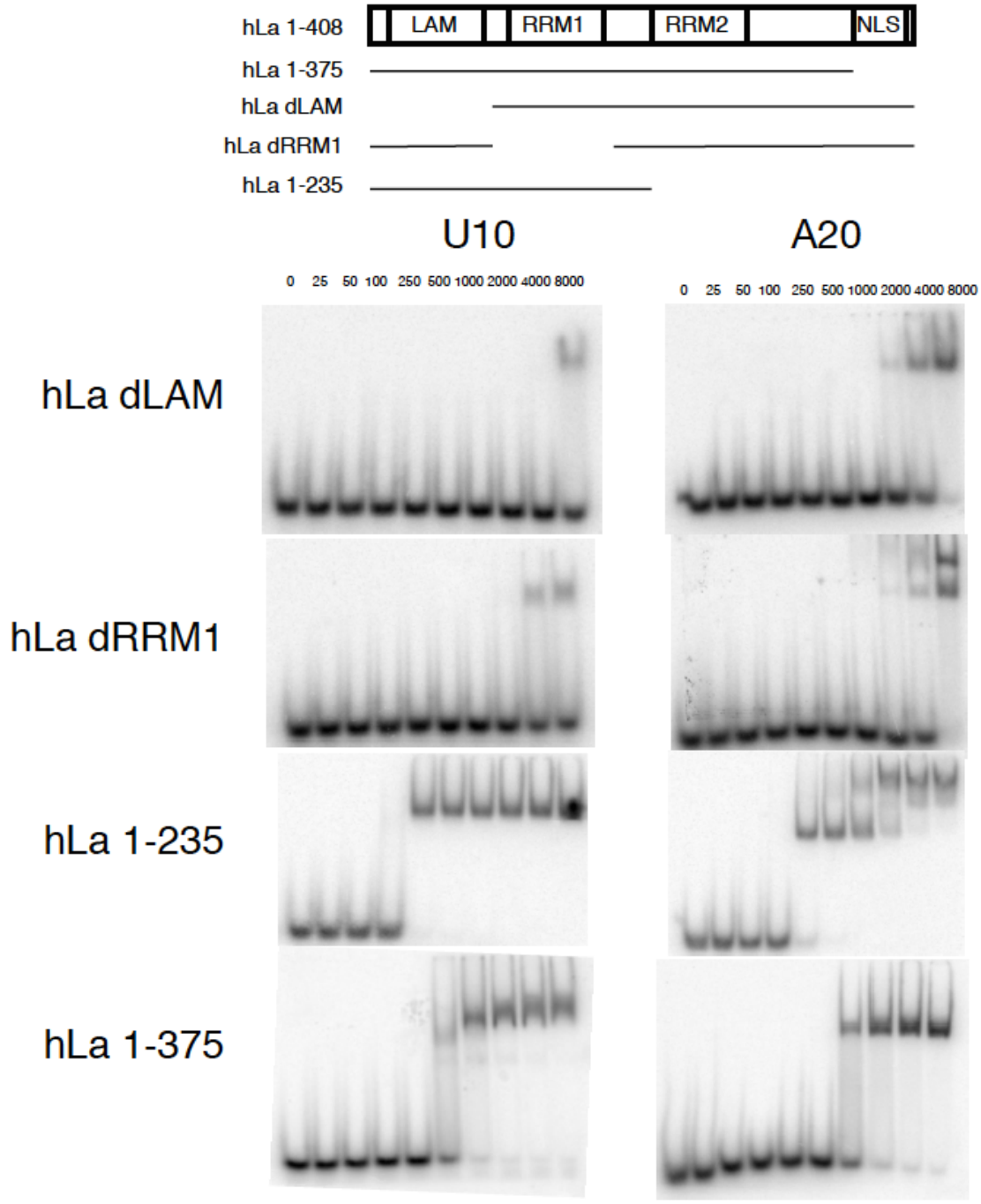
We thank O. Rissland for assistance in the CLIP methodology and A. Ashok & R. Maraia for helpful discussions.

2.7 Supplementary Figures



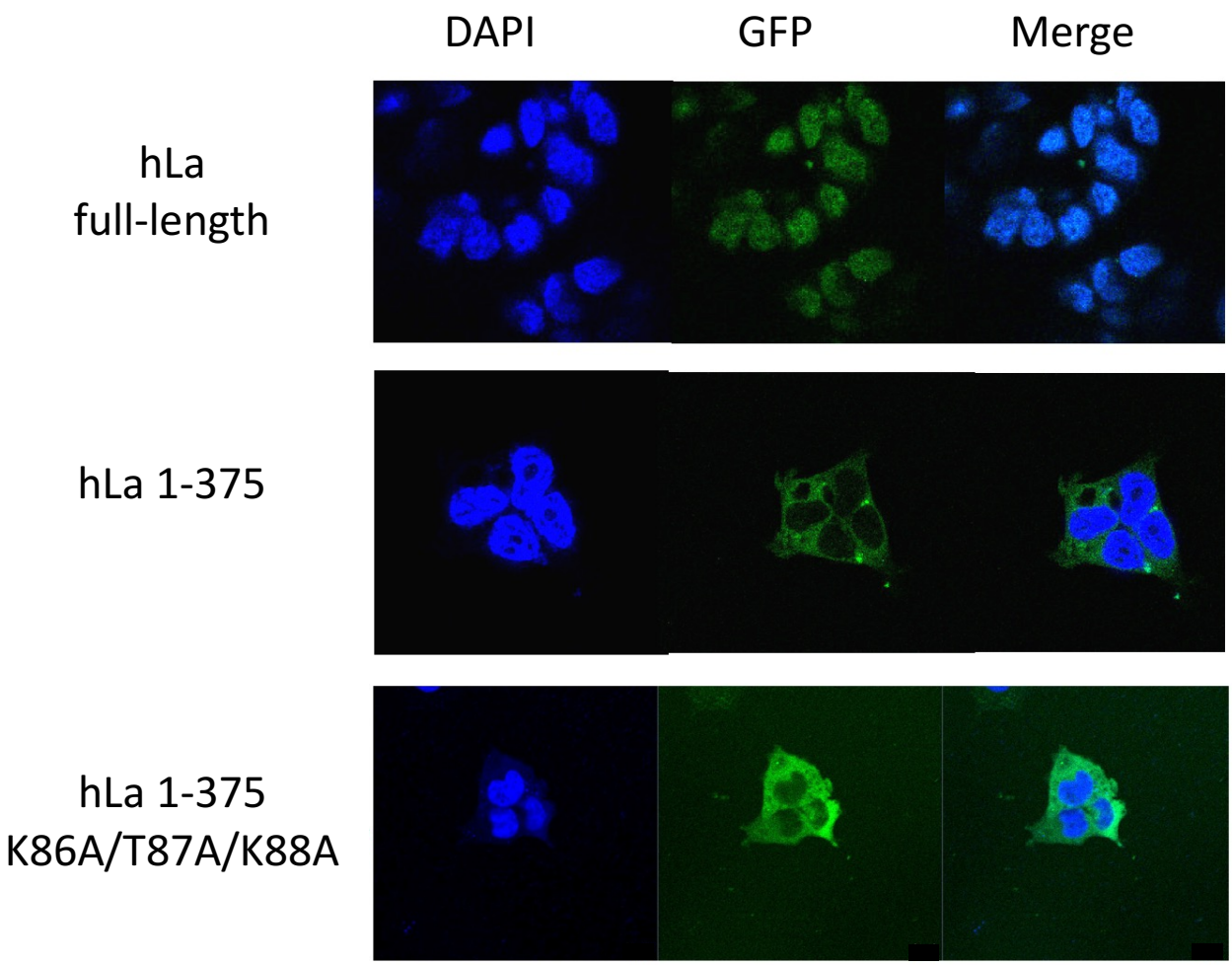
Supplementary Figure 1: Binding of hLa to C and G homopolymers

Binding of wild-type hLa 1-408 to C and G homopolymers in the presence of HepC/ssRNA competitor



Supplementary Figure 2: Binding of hLa deletion variants to U10 and A20

Binding of hLa deletion variants to U10 and A20 in the presence of hepC/ssRNA competitor



Supplementary Figure 3: Localization of GFP-hLa variants

Localization of GFP-hLa and GFP-hLa 1-375 variants in HEK293T cells

	K_d U10	K_{rel} vs. hLa U10	K_d A20	K_{rel} vs hLa A20	K_{rel} A20/K_{rel} U10
hLa	0.17	1	0.4	1	1
hLa K86A/T87A/K88A	0.19	1.1	0.92	2.3	2.1
hLa Q20A/Y23A/D33R	5.6	32.9	1.5	3.75	0.11
hLa 104-408	>10	N.D.	3.9	9.75	N.D.
hLa dRRM1	>10	N.D.	4.4	11	N.D.
hLa 1-235	0.14	0.82	0.17	0.425	0.52
hLa 1-375	0.47	2.7	0.99	2.4	0.9

Supplementary Table 1: Dissociation constants and relative dissociation constants for tested La variants

Dissociation constants (K_ds, in μM) and relative dissociation constants (K_{rel}) for tested La variants. K_d U10 and K_d A20: measured dissociation constants for proteins for U10 and A20 substrate in the presence of HepC/ssRNA competitor, respectively. K_{rel} vs hLa U10 or A20: K_d of indicated mutant for U10 or A20 relative to that for wild-type hLa. K_{rel} A20/ K_{rel} U10: fold change in binding to A20 relative to fold change in binding to U10.

Chapter 3:

Cellular stress induces cytoplasmic localization of La and increased association with stress associated mRNAs

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Manuscript in preparation

Manuscript draft by:

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Author Contributions:

Kyra Kerkhofs performed the immunofluorescence staining shown in Supplementary Figures 4, 5a, and 5b. Steven Hébert and Claudia L. Kleinman performed the bioinformatics analysis for the iCLIP.

3.1 Summary

Although the human La protein is mostly associated with the processing of nascent RNA polymerase III transcripts, La proteins are also hypothesized to promote cap-independent translation of several cellular and viral coding RNAs, but the context and mechanism by which La binds to coding RNAs is yet to be established. During conditions of viral infection, cellular stress, and tumor progression, the predominantly nuclear hLa protein has been shown to translocate to the cytoplasm where it functions as an (IRES) *trans*-acting factor (ITAF), controlling cap-independent translation initiation of several cellular IRES-containing mRNAs including BiP, Laminin B1, NrF2, XIAP and CCND1. These La associated cytoplasmic substrates are often involved in cell cycle regulation, apoptosis, tumorigenesis, and stress responses, signifying an important role for La during cellular stress conditions. Although a mechanism for nuclear-cytoplasmic shuttling of La has been described for polioviral infection, the mechanism for shuttling of La during conditions of cellular stress is yet to be determined. Here, we show that cellular stress induced by clotrimazole, a mitochondrial inhibitor and inducer of stress granules, causes translocation of hLa from the nucleus to the cytoplasm. Using polysome analysis coupled with qPCR, we show that this translocation to the cytoplasm is concurrent with increased association of La with actively translating messages. Using iCLIP, we identify novel substrates of La that contain IRES elements and are translated during cellular stress. Taken together, our data show that hLa is trafficked into the cytoplasm in response to cellular stress to associate with translating messages during suboptimal cellular conditions.

3.2 Introduction

Gene regulation in eukaryotic cells is an intricate process where the expression levels of proteins are regulated by the efficiency of mRNA translation[187]. As the ability of cells to maintain active translation in response to cellular stress is vital for its survival, cells have evolved and adopted various mechanisms to adapt and overcome cellular stress. The well characterized cap-dependent translation mode relies on the recruitment of the small ribosomal subunit to the 5' 7-methyl guanine cap to commence scanning of the transcript until the initiator methionine codon has been identified. This is followed by the recruitment of the large ribosomal subunit to begin translation of the message[188]. In addition to the general recognition of the 5' cap, the ribosome can also initiate translation via an internal ribosome entry site (IRES) in a process termed cap independent translation. Internal ribosome entry sites were originally found in picornaviruses and are believed to have evolved for the purpose of recruiting the ribosome to initiate translation of uncapped viral mRNAs [189,190].

In addition to viruses, IRES elements have been identified in the 5' UTR of a subset of cellular mRNAs including Nrf2[191], myc [192,193], XIAP [108], CCND1 [194], and BiP [195]. Sequence and structural comparison between cellular IRESs indicate very little conservation and a common mechanism by which they function has not yet been determined; however, it is predicted that these cellular IRESs have evolved in order to maintain and promote protein synthesis during suboptimal cellular conditions where cap-dependent translation has been compromised such as mitosis, oxidative stress, apoptosis, mitochondrial stress, heat shock, and osmotic shock [196]. Although very little is known about how mRNAs are translated under cellular stress, there is increasing evidence that cellular mRNAs containing IRES elements in their 5' UTR can escape recruitment into stress granules and are actively translated even when cap dependent translation is hindered [87,105,106].

Stress granules are dense aggregations composed of mRNAs and components of the cap-dependent translation machinery formed in cells undergoing a multitude of external stresses, and are associated with a general shift from cap-dependent to cap-independent translation. These membrane-less foci are hypothesized to protect stalled RNP complexes such that they can reenter into polysomes when cells return to homeostasis [197].

While cap-dependent translation is regulated by several eukaryotic translation initiation factors, IRES elements not only require initiation factors, but also rely on IRES trans-acting factors (ITAFs) [198,199]. While the exact function of these ITAFs is not yet fully understood, it is hypothesized that ITAFs function as RNA chaperones, assisting in inducing conformational change of the IRES to make the start codon accessible for the ribosome [87,92,139]. ITAF requirements can vary for each IRES and these proteins often undergo various modifications including phosphorylation, increased protein expression, and nuclear-cytoplasmic shuttling [200,201]. The human La protein was the first ITAF to be characterized and modification of La, specifically nuclear-cytoplasmic shuttling and changes in phosphorylation, has been observed further signifying a role for this protein in regulating IRES-mediated translation during cellular stress [158].

Since its discovery, La has been linked to the translation from the IRESs of several viral RNAs including poliovirus, Hepatitis C virus, and encephalomyocarditis virus [57,158,176]. Emerging studies have linked the La autoantigen to the IRES of numerous stress associated messages including the extracellular matrix signaling factor laminin B1, the X-linked inhibitor of apoptosis protein (XIAP), the stress associated ER chaperone protein BiP, the cell cycle regulator CCND1, and the master regulator of oxidative stress and transcription factor Nrf2 [56,58,108,109,115]. Additionally, La has been implicated in cancer progression, malignant epithelial to mesenchymal transition (EMT), and BCR/ABL associated leukomogenesis through its association with the IRES

containing cyclin D1 and laminin B1 mRNAs and the upstream open reading frame (uORF) containing MDM2 mRNA [58,115,116].

La proteins are abundantly found in nearly all eukaryotes and function in facilitating the processing of RNA polymerase III transcripts[42]. First identified in patients suffering from systemic lupus erythematosus and Sjögren's syndrome, the predominantly nuclear human La protein is most associated with the processing of nascent precursor transcripts via sequence specific recognition of the UUU-3'OH trailer along with the uridylate-tailed adenoviral VA RNA and the Epstein-Barr virus encoded EBER RNAs[45,54,55,76,79,148–151]. During conditions of viral infection, cellular stress, and tumor progression, hLa has been shown to translocate to the cytoplasm controlling cap-independent translation initiation of several cellular IRES-containing mRNAs, although the mechanism and the context is not yet fully understood [109–111].

In this study, we aimed to further characterize the context and mechanism by which La associates with these stress associated messages and aim to identify novel RNA substrates of La under normal and stress conditions. Given the previous association of La with coding transcripts that contain IRESs and uORFs that are upregulated during cellular stress, we considered whether La may be recruited to the cytoplasm to assist in translation of messages that escape recruitment into stress granules. To observe hLa relocalization in the presence of stress granules, we treated cells with clotrimazole, a well-characterized inducer of stress granules and inhibitor mitochondrial function. Using immunofluorescence, we show that induction of stress using clotrimazole results in a gradual shift in the localization of the La/SSB protein from the nucleus to the cytoplasm; however, La is not recruited into stress granules. Using polysome analysis coupled with qPCR, we show that several previously identified La associated messages that contain IRES elements are dependent on La for translation during cellular stress. Using iCLIP, we have identified novel

substrates of La and mRNAs that are upregulated during clotrimazole induced cellular stress. Interestingly, many of our novel La substrates contain previously established IRES elements in their 5' UTR. Using qPCR analysis, we show that these messages are not only upregulated during cellular stress, but are also dependent on La for optimal translation during suboptimal cellular conditions. Taken together, our data show that hLa is trafficked into the cytoplasm in response to clotrimazole induced cellular stress to associate with messages that continue to translate during compromised cellular conditions and rely on alternate mechanisms to promote translation.

3.3 Materials and Methods

Drug Administration and Immunofluorescence

HEK293T, U2OS, and HeLa cells from ATCC were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Cells were stressed with 20uM clotrimazole in serum free media for the mentioned duration. Unstressed cells were mock treated with DMSO.

Immunofluorescence was performed as described in Kedersha and Anderson [197]. 12-18 hours prior to administration of stress, cells were plated on sterile coverslips at ~70% confluency. Following drug treatment, cells were washed once with PBS and immediately fixed with 4% paraformaldehyde for 20 minutes at room temperature. After washing twice with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Cells were then washed twice with PBS and then blocked with blocking solution (1% BSA + 0.02% sodium azide in PBS) for 1 hour at room temperature. Cells were incubated with primary antibodies overnight at 4^{o}C. Primary antibodies used were human anti-SSB ("GO" 1:500) and rabbit anti-eIF4G (SantaCruz sc11373 1:250). After primary incubation, cells were washed twice with PBS and incubated with the appropriate secondary antibody for 1 hour at room temperature. Secondary antibodies used were goat anti-human AF546 (Lifetech A21089 1:500) and goat anti-rabbit AF488 (Invitrogen A11008 1:500). Cells were washed twice with PBS, mounted using Vectashield (Vector Laboratories H-1200), and viewed under a Zeiss LSM700 Confocal Microscope.

Transfection and Polysome Analysis

All cells were cultured in complete DMEM plus 10% fetal bovine serum. For polysome analysis, cells were grown in 15cm dishes and transfected at ~70% confluency. 1 h before transfection, DMEM was replaced by OPTI-MEM. Plasmid DNA (10ug) and siRNAs (final

concentration of 100 nM) were transfected using Lipofectamine (Invitrogen). Cells were collected 48 hours post transfection.

Polysome analysis was performed as described by Dowling R, 2007 [202]. Stressed cells were subject to 20uM clotrimazole in serum free media for indicated times before being lysed in hypotonic lysis buffer [5 mM Tris -HCl (pH 7.5), 2.5 mM MgCl2, 1.5 mM KCl, 100 μg/mL cycloheximide, 2 mM DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate]. Cell debris was removed by centrifugation. 20 A260nm of cell lysate was loaded over a 20-50% sucrose gradient made in 20 mM HEPES-KOH (pH 7.6), 100 mM KCl, 5 mM MgCl2 followed by centrifugation at 30,000 rpm for 3 hours in a Beckman SW41 rotor. Gradients were fractionated into fifteen 500 μL fractions, TCA precipitated, separated by SDS-PAGE, and blotted using the relevant antibodies [Anti-La SSB (Abcam 75927), Anti-PABP (Abcam 21060), Anti-Rpl9 (Abcam 182556), and anti-GFP (Abcam ab1218)]. For puromycin treatment, cell lysates were treated with 25 μM puromycin for 15 min and cyclohexamide was omitted [169].

Quantitative PCR

QPCR analysis of RNA obtained from polysomes gradients was performed by collecting total and polysomal RNA and performing Trizol extraction as per manufactures protocol. RNAs were converted to cDNA using BioRad iScript cDNA synthesis kit and assessed by quantitative real-time PCR using SensiFAST SYBR No-ROX Kit (Bioline) using the following settings: Hot start @ 95^{o}C for 5 minutes; (denaturation for 5 sec @ 95^{o}C, annealing/extension @ 60^{o}C for 10 sec) x 40 cycles. Relative translation efficiency was calculated by taking the ratio of polysomal RNA to total RNA and normalized using the ΔΔCt method. Primers used are listed in **Supplementary Table 2**. Values presented represent mean enrichments over a minimum of three biological

replicates.

Co-Immunoprecipitation

Crosslinking of RNA/protein complexes was performed as described by Niranjanakumari, S, 2002 [203]. Briefly, one 15cm plate of HEK293T cells were stressed with 20uM clotrimazole for 0, 60, or 120 mins. Cells were washed twice with 1X PBS and crosslinked with 1% formaldehyde for 10 minutes at room temperature. The reaction was stopped with 0.25M glycine pH 7.0 for 5 minutes at room temperature. Cells were washed twice in 1X PBS followed by lysis in 1 mL RIPA buffer with DNase treatment (4U, 5 minutes at 37^{o}C). Protein G beads were incubated with anti-La antibody or anti-GFP antibody for one hour at room temperature. Lysates were added to the beads and incubated for 2 hours at 4^{o}C. Beads were washed twice with 500 uLs RIPA buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF), three times with 500 uLs of PNK buffer (50 mM Tris-HCl pH 7.0, 5 mM EDTA, 10 mM DTT, 1% SDS), followed by reversal of crosslink by incubation at 70^{o}C for 45 minutes. RNA was Trizol extracted followed by isopropanol precipitation. Input and eluted RNAs were converted to cDNA using BioRad iScript cDNA synthesis kit and assessed by quantitative real-time PCR using SensiFAST SYBR No-ROX Kit (Bioline) using settings mentioned above.

Individual-nucleotide resolution crosslinking and immunoprecipitation (iCLIP)

iCLIP analysis was performed as described by Broughton *et al.* 2013 and Huppertz 2014 [29,204]. Briefly, HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells were left untreated or treated with 20uM clotrimazole in serum free media for 1 hour, UV-crosslinked (Stratalinker at 254 nm, 150 mJ/cm^2), and lysed in RIPA buffer. Lysates were then

treated with 10µl of 1:250 RNAse I (100U/µl AM2294 Invitrogen) and 2 µl DNase I (2U/µl AM2238 Ambion) at 37^{o}C for 3 mins. RNA/La complexes were immunopurified with 10 µg anti-SSB monoclonal antibody (abcam 75927) conjugated to 100 µl protein G Dynabeads (Invitrogen). RNAs bound by La were dephosphorylated at their 3′ end followed by on bead ligation of the DNA linker 5′-rAppAGATCGGAAGAGCGGTTCAG/ddC/-3′ (TriLink BioTechnologies). After 5′ end radiolabeling, crosslinked La-RNA complexes were size-separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Regions corresponding to 40–70 kDa on the autoradiogram were extracted from the nitrocellulose membrane and RNAs were released from bound protein via proteinase K treatment. After reverse-transcription of the RNA, the cDNA was size-selected by running a 6% TBE-urea gel followed by gel extraction. Purified cDNAs were circularized using CircLigase (epicentre), linearized using BamH1 (NEB) restriction digestion, and PCR amplified using accuprime (Invitrogen) with P3Solexa /P5Solexa primers. (P5 Solexa: 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC T -3'; P3 Solexa: 5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGA ACCGCTCTTCCGATCT-3'. PCR amplified products were confirmed by running a 6% TBE and gel extracted. iCLIP cDNA libraries were submitted to The Centre for Applied Genomics at The Hospital of Sick Children for high-throughput sequencing on an Illumina GA-IIx (50 nt single end reads).

3.4 Results

Mitochondrial stress induces subcellular relocalization of La/SSB

Previous work on viral infection and cellular stress have suggested that hLa can be recruited to the cytoplasm under certain conditions [56,87,109,110]. The first studies showing migration of La from the nucleus to cytoplasm were done in cells infected with poliovirus where it was shown that cytoplasmic translocation of La is mediated by the cleavage of the peptide bond between Gln358 and Gly359 by 3C62, a poliovirus-specific protease [89,173]. In apoptotic cells, hLa is rapidly dephosphorylated by a PP2A-like phosphatase at S366, resulting in the cleavage of the nuclear localization signal (NLS) and subsequent redistribution of the protein from the nucleus to the cytoplasm [205,206]. Both these examples illustrate that cytoplasmic distribution of hLa can occur via multiple mechanisms and may be context specific. To observe hLa relocalization in the presence of stress granules, we treated cells with clotrimazole, a well-characterized inducer of stress granules that inhibits mitochondrial function. Using immunofluorescence, we monitored the subcellular distribution of hLa in response to the formation of stress granules. Consistent with literature, we observed formation of stress granules by clotrimazole at a minimum concentration of 20uM for 30 mins **(Supplementary Figure 4)**. Using this concentration, we performed a time course from 0-120 minutes in 30 minute intervals to observe changes in localization of hLa using HeLa cells. As expected, before induction of stress, La was observed to be predominately nuclear; however, 30 minutes post administration of the drug, we observed the formation of stress granules and a gradual shift of nuclear La into the cytoplasm **(Figure 16)**. By 60 minutes post induction of stress, a greater percentage of La was seen dispersed in the cytoplasm. Approximately 200 cells from each time point were counted and characterized as being nuclear, cytoplasmic, or both. While we visualized migration of La from the nucleus to the cytoplasm, we did not see La co-localize

with stress granules along with our positive stress granule marker, eIF4G. To assess whether this phenomenon was cell type specific, we attempted this experiment in HEK293T cells and U2OS cells and observed similar migration patterns **(Supplementary Figure 5)**. From these findings, we hypothesized that although La is not recruited into stress granules, the formation of stress granules may signal for La to shift to the cytoplasm to interact with cellular mRNAs that become up-regulated during cellular stress. In agreement with our data, recent findings have shown that nuclear La migrates into the perinuclear space to engage the Nrf2 IRES in response to oxidative stress [109] and serum-starvation of cells results in cytoplasmic localization of La and increased La driven translation of the RRBP1 IRES [110].

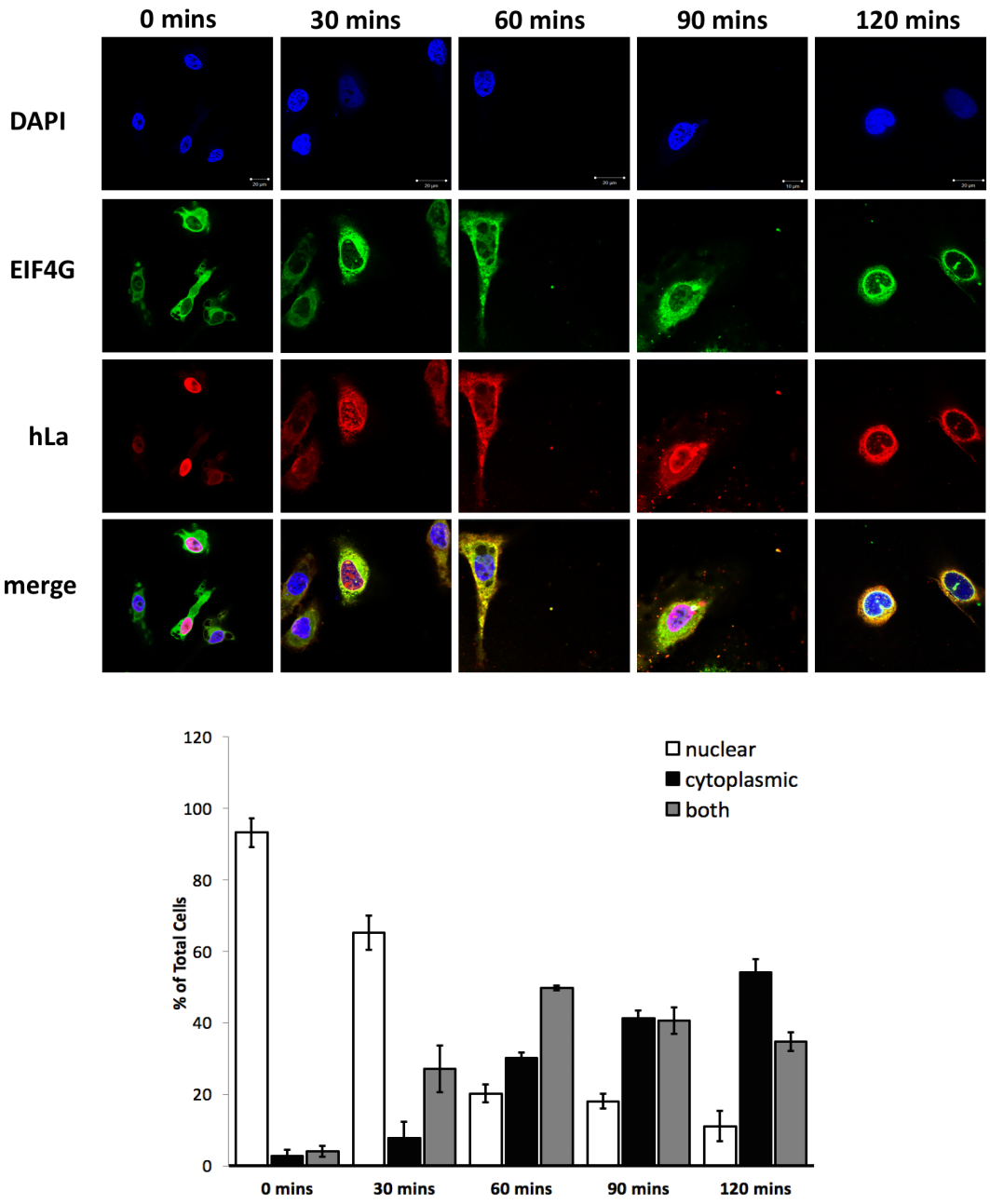

Figure 16: Cellular stress induces cytoplasmic localization of La

HeLa cells were treated with 20uM clotrimazole to induce stress granules. Cells at the 0 time point were mock treated with DMSO. A time course was performed from 0-120 minutes in 30 minute intervals to visualize the movement of La (red) into stress granules. EIF4G was used as a stress granule marker (green). Nuclei were visualized by DAPI (blue). Approximately 200 cells from each time point were counted and characterized as being nuclear, cytoplasmic, or both (n=3).

Cellular stress increases La's association with polysomes

During cellular stress, the majority of mRNA translation is suppressed and stress granules are formed as storage sites for non-stress related messages and stalled translation complexes. Although PABP is not required for the formation of stress granules, it is hypothesized that PABP is recruited to stabilize the stored mRNAs and aid in re-activation of global translation upon the disassociation of the stress granule [207]. Given the previous association of La with coding RNAs to promote cap-independent translation and our recent finding of La directly binding to the poly (A) tail of mRNAs [208], we postulated that La may shuttle to the cytoplasm during cellular stress to assist in cap-independent translation of stress related mRNAs in a situation where PABP may have been recruited away from actively translating messages into the stress granules. We hypothesized that stress induced by clotrimazole would result in increased association of La with polysomes. HEK293T cells were treated with 20uM clotrimazole for 30 and 90 minutes, polysome fractionated, and individual fractions from the sucrose gradients were subject to immunoblotting analysis to localize La and PABP **(Figure 17)**. Treatment of cells with clotrimazole resulted in an accumulation of the 80S subunit and the collapse of polysomes, as expected. Interestingly, despite the decrease in the presence polysomes in our stressed condition, we observed an increase in association of La with polysomal fractions confirming that La (and presumably cytoplasmic La) associates with the remaining actively translating polysomes. Additionally, we observed PABP in both monosome and polysome containing fractions in non-stressed control cells; however, a decrease of PABP in polysomal fractions was observed in stressed cells as has been previously demonstrated[209]. We confirmed these results in the context of a GFP-La transfected plasmid **(Supplementary Figure 6)**. While we observed GFP-La to associate with polysomes 60 minutes post stress, we observed GFP empty vector to be present only in the first few fractions along with

free RNA. To confirm that the sedimentation of our polysomes are dependent on active translation and the observed higher molecular weight complex is indeed caused by polysomes, we treated cells with puromycin, a destabilizer of polysomes that promotes premature termination. As expected, treatment of cells with puromycin resulted in disruption of polysomes and the absence of both La and Rpl9 in polysomal fractions.

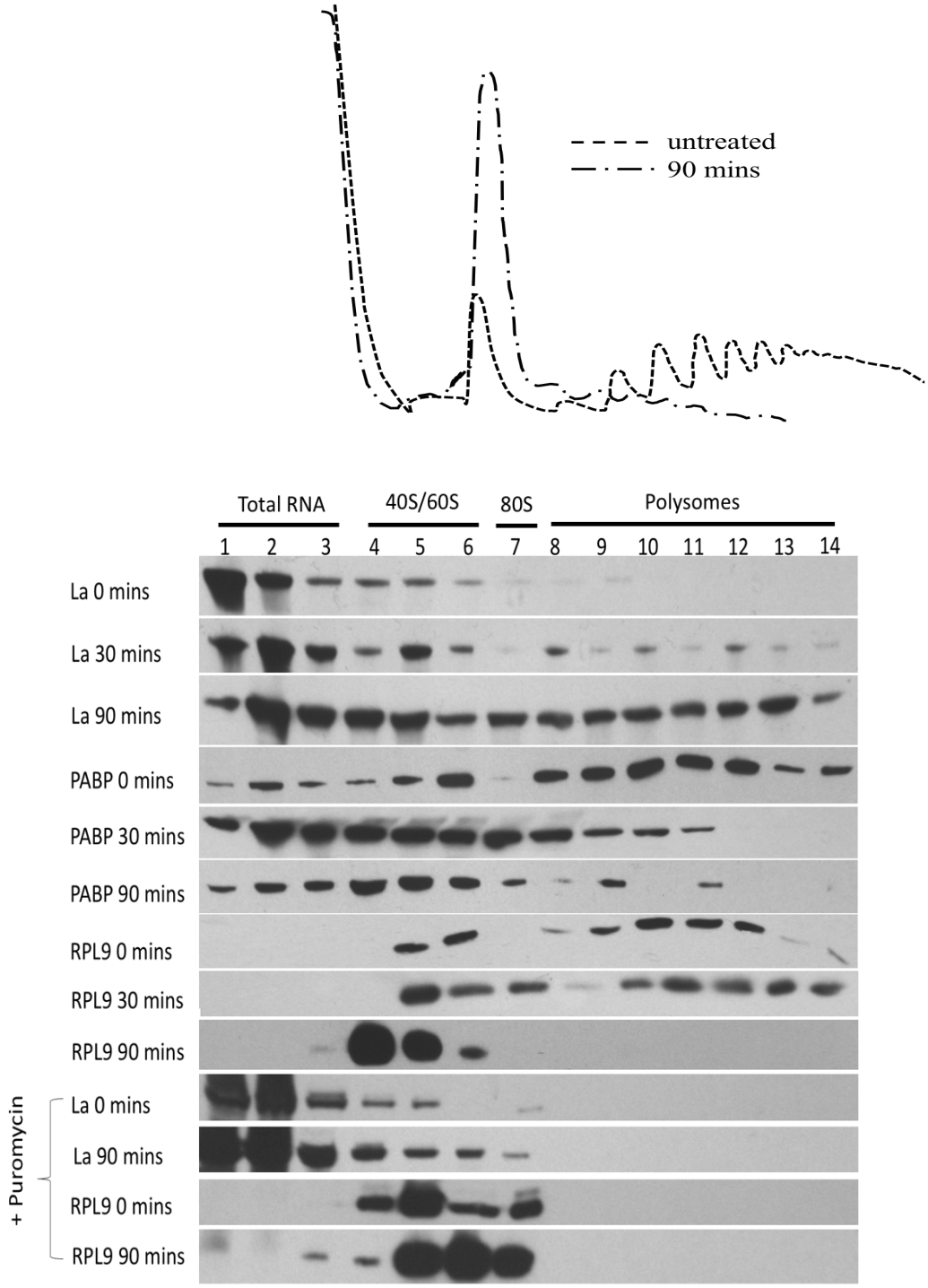

Figure 17: Cellular stress increases La's association with polysomes

Top: Trace of ribosomes/polysomes fractionated from HEK293T cells untreated and stressed with 20uM clotrimazole for 90 minutes. Bottom: Presence of proteins in each fraction was analyzed by western blotting using antibodies against La, PABP, and Rpl9. Presence of polysomes in higher weight fractions was confirmed by treating cells with puromycin.

La affects translational regulation in response to cellular stress

The ability of cells to adapt and respond to cellular cues and stresses is vital for its survival. During cellular stress, a subset of messages (often encoding for proteins that are essential for that specific stress) are translationally upregulated. In an attempt to identify La dependent messages that are upregulated during clotrimazole induced cellular stress, we used polysome profiling as previously described coupled with qPCR. By measuring the percentage of a particular mRNA within polysomal fractions, we were able to assess its translational efficiency. We calculated relative translatability of mRNAs by normalizing abundance in polysomal fractions to abundance in total RNA (poly/total) under different cellular conditions. We first tested known mRNA targets of La including those that contain IRESs and uORFs (BiP, CCND1, LamininB1, and MDM2), 5' TOPs (RPL37 and eEF1b), and a non La related target (GAPDH). We first evaluated changes in translation efficiency of these genes in response to cellular stress **(Figure 18b)**. While we observed a general increase in translation of the BiP, Laminin B1, and MDM2 mRNAs, we saw decrease in overall translation of eEF1b and RPL37 as a result of cellular stress. The observed decrease in translation of the two tested 5' TOP messages is likely reflective of its translational repression as has been observed in human cells previously [68]. We also observed no overall change in translation of GAPDH in response to cellular stress as has been previously demonstrated[210]. In addition, although previous reports have identified La to promote translation from the IRES of the CCDN1 mRNA during tumorigenesis in cervical cancer cells[58], we did not observe any change in translation of this message and suspect this particular mRNA may not be actively translating in cells undergoing mitochondrial stress.

To monitor the dependency of La on these messages to maintain active translation during cellular stress, we performed knockdown of La using a previously validated siRNA[58]. As complete

La knockout is lethal to eukaryotic cells resulting in apoptosis and cell death, we used samples where we achieved ~50% knockdown of La. Experimental trials showing desired levels of La knockdown were used for polysome fractionation and subsequent qPCR **(Figure 18a)**. As expected, knockdown of La resulted in a decrease in translation of BiP and Laminin B1. However, no significant change in translational efficiency was observed for MDM2 and eEF1b. Knockdown of La resulted in an increase in translatability of RPL37, further validating the proposed function of La in inhibiting translation of 5' TOPs [68].

To confirm if the observed changes in translation of our tested messages are a result of direct association with La, we performed a time course and immunoprecipitated hLa in untreated and clotrimazole treated cells along with its associated transcripts in the context of a reversible formaldehyde crosslink and determined relative abundance using qPCR **(Figure 18c)**. As a negative control, we immunoprecipitated using an anti-GFP antibody. With the exception of GAPDH, we observed a general overall increase in association of La with our tested substrates in clotrimazole treated cells, albeit to varying extents. Maximum immunoprecipitation of our substrates was observed after 120 mins of drug exposure. Thus, our immunoprecipitation data suggest that the observed translational changes we see in our polysome analysis are a direct result of La's association with these particular messages.

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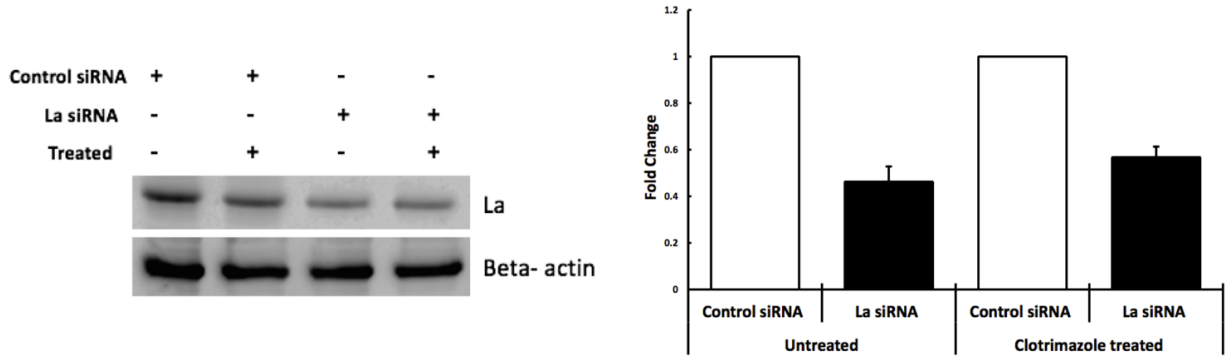

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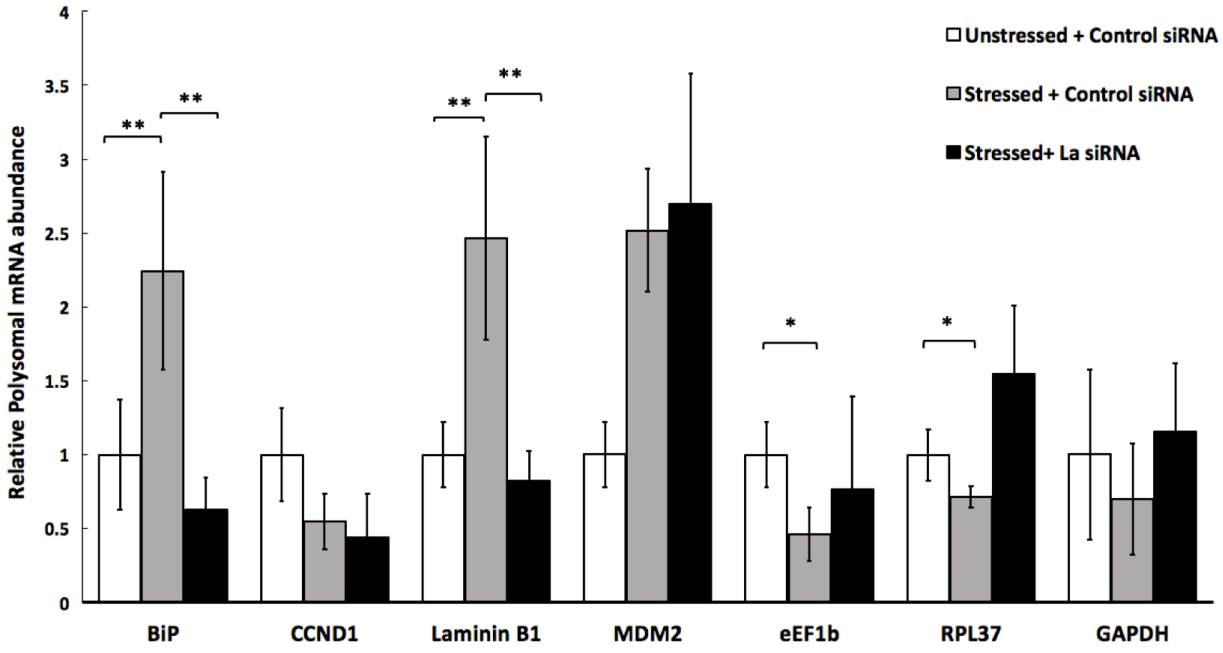

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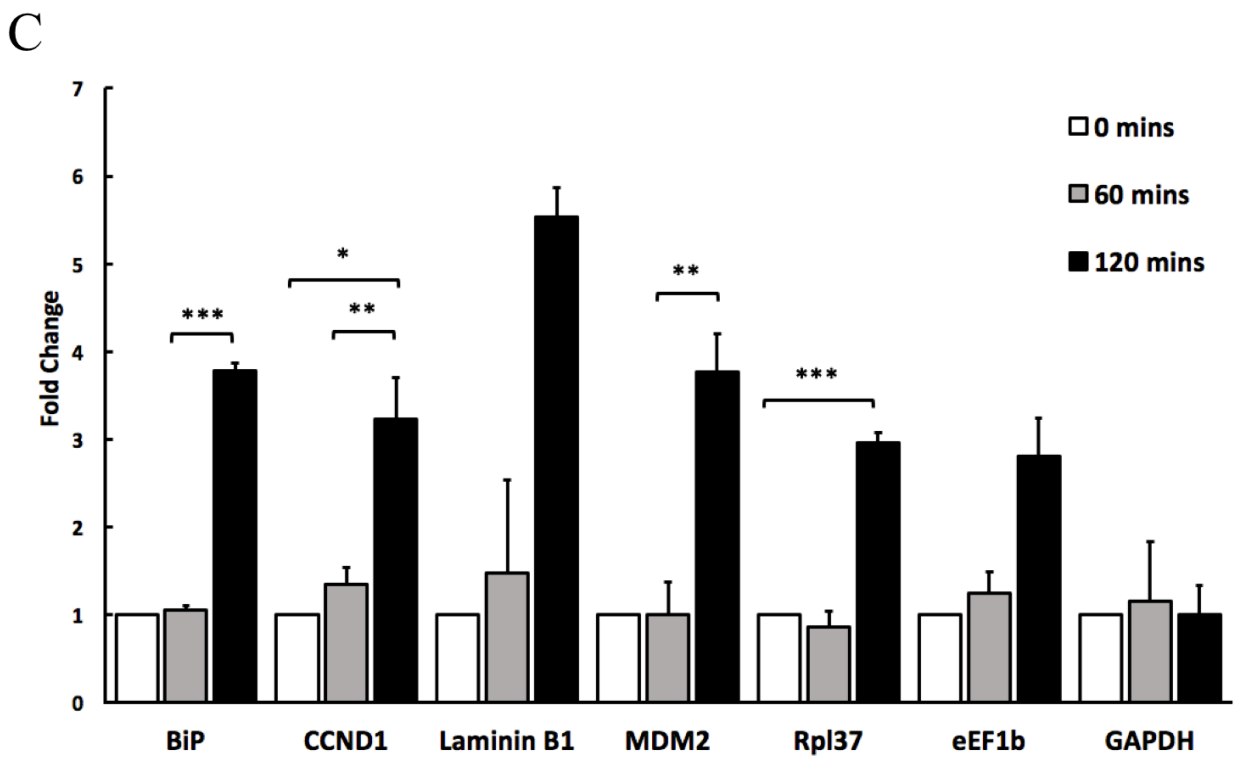

Figure 18: La binds mRNAs to regulate cap-independent translation

(A) HEK293T cells were transfected with a control siRNA or a siRNA targeting La. 24 hours post transfection, cells were treated with 20uM clotrimazole for 60 minutes to induce mitochondrial stress. Cells were then harvested and subject to western blot analysis before preparation for polysome analysis (left panel). Western blots were imaged using the MicroChemi Bioimaging System and quantified using ImageJ. Three biological replicates were performed and the relative knockdown was calculated (right panel). Error bars indicate standard deviation. (**B)** HEK293T cells from (A) were subject to polysome fractionation and subsequent qPCR analysis. Translational efficiency of known La mRNA targets that contain IRESs, uORFs, and 5' TOPs were tested along with a non La related target (GAPDH) by calculating ratio of poly/total RNA. Unstressed cells were set to "1". Error bars indicate standard deviation of three biological replicates and asterisks highlight statistically significant changes (*= $P<0.05$, **=$P<0.01$ two-tailed student t-test). (**C)** Time course of La co-immunoprecipitated with its associated transcripts during cellular stress measured by qPCR. Efficiency of pulldown is measured relative to co-immunoprecipitation with an anti-GFP antibody. Error bars correspond to standard deviation of a minimum of three biological replicates, asterisks highlight statistically significant changes (**=$P<0.01$, *** $P<.001$ two-tailed student t-test).

Identification of new La associated stress targets

To date, IRES containing messages upregulated during cellular stress and interact with La include Laminin B1, XIAP, BiP, CCND1 and Nrf2, and RRBP1 [56,58,108–110,115]. In an attempt to identify novel messages that associate with La during compromised cellular conditions, we performed individual-nucleotide resolution cross-linking and immunoprecipitation (iCLIP). Stressed and unstressed HEK293T cells were UV cross linked to covalently bind protein-RNA molecules and immunoprecipitated using an anti-La antibody. Protein-RNA complexes were subject to partial RNAse I digestion. RNA bound by La was isolated using an anti-La antibody, reverse transcribed, and PCR amplified for next generation sequencing.

Using iCLIP, we not only identified messages bound by La, but we also identified approximate site(s) of the interaction. Interestingly, several newly identified substrates were mRNAs that have previously been demonstrated to contain cellular IRESs such as Bag1, HIF-1 alpha, and TP53 [211–213]. Messages containing previously identified IRES elements, along with messages upregulated in our stress induced condition (particularly those that showed an interaction occuring near the 5' UTR where a potential IRES would be located) were selected for further examination. Presented in **Table 3** is a list of selected genes, along with gene ID and gene description. While IRES elements have been identified within the mRNAs of BAG1, HIF1-alpha, TP53, Apaf1, and RBM3 in human cells, the YAP1 IRES has only been identified in *Saccharomyces cerevisiae* to date [211–217]. To assess the ability of these messages to translate during cellular stress and to measure the dependency of La for efficient translation, we repeated the polysome/qPCR experiment as previously mentioned. As expected, several of our tested IRES containing messages showed increased translation efficiency upon cellular stress **(Figure 19a)**. Although the HSPA1A and BCL2L2 mRNAs do not contain a known IRES, these particular

mRNAs displayed a similar trend to the other tested IRESs. Unlike typical 5' TOPs, which begin with a cytidine followed by a stretch of up to 15 pyrimidine residues, the HSP90AB1 gene, encoding for a member the heat shock protein family, contains a "TOP-like" motif consisting of a shorter stretch of pyrimidine residues [214]. We show that unlike our previously tested 5' TOPs (eEF1b and RPL37), HSP90AB1 is upregulated by cellular stress, but its ability to effectively translate is not deterred by knockdown of La. Lastly, we tested a long non-protein coding RNA, XIST, and measured the effects of cellular stress on the translation of this message. The XIST RNA functions to silence one of the pair of X chromosomes in females in order to provide equal expression of genes between males and females [218]. We show that the translation efficiency of this specific RNA is not affected by the induction of cellular stress nor by the silencing of La. Immunoprecipitation of La with its associated messages once again revealed that the observed translational changes we see in our polysome analysis are a direct result of La's association with these particular messages with more association occurring at either 60 or 120 mins post administration of stress **(Figure 19b)**. Taken together, our data reveal that La associates with several previously unidentified IRES containing messages and that this interaction occurs as a response to cellular stress.

Table 3: Selected target genes from iCLIP

GENE NAME	ENSEMBL GENE ID	GENE TYPE	TRANSLATIONAL ELEMENT	GENE DESCRIPTION
BAG1	ENSG00000107262	Coding RNA	IRES[211]	BCL2 associated athanogene 1
HIF1A	ENSG00000100644	Coding RNA	IRES[212]	hypoxia inducible factor 1 alpha subunit
TP53	ENSG00000141510	Coding RNA	IRES[213]	tumor protein p53
APAF1	ENSG00000120868	Coding RNA	IRES[217]	apoptotic peptidase activating factor 1
YAP1	ENSG00000137693	Coding RNA	IRES[216]	Yes associated protein 1
HSPA8	ENSG00000109971	Coding RNA	IRES[219]	Heat shock protein 70 alpha family member 8
HSP90AB1	ENSG00000096384	Coding RNA	5' TOP-like [214]	Heat shock protein 90 alpha family class B member 1
BCL2L2	ENSG00000129473	Coding RNA	-	BCL2 like 2
HSPA1A	ENSG00000204389	Coding RNA	-	Heat shock protein 70 alpha family member 1A
XIST	ENSG00000229807	Long non coding RNA	-	X inactive specific transcript

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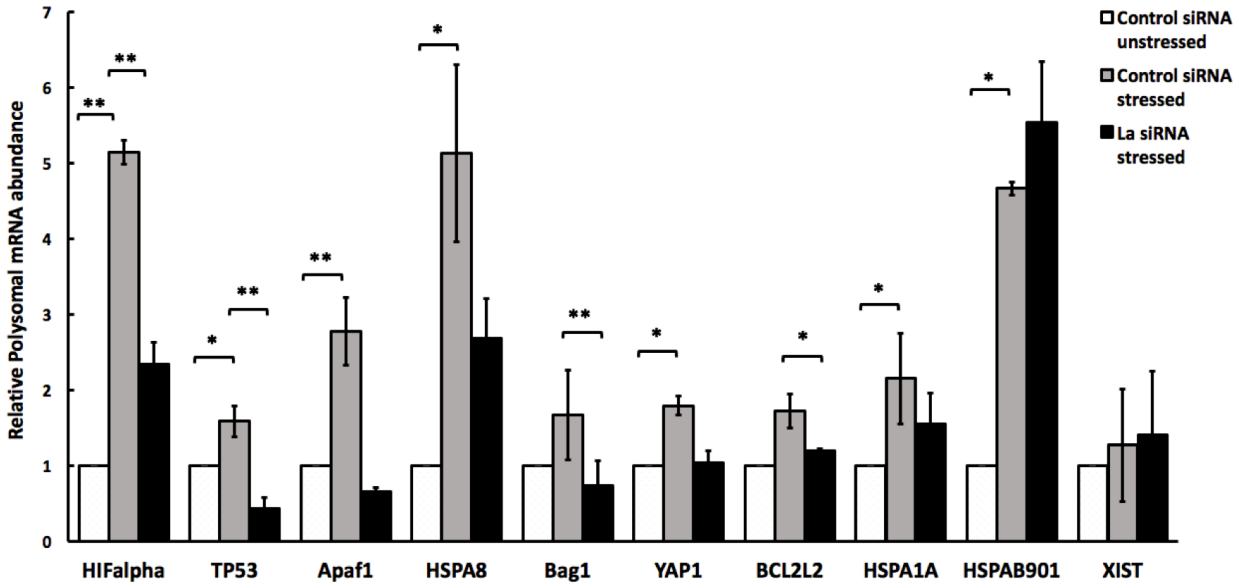

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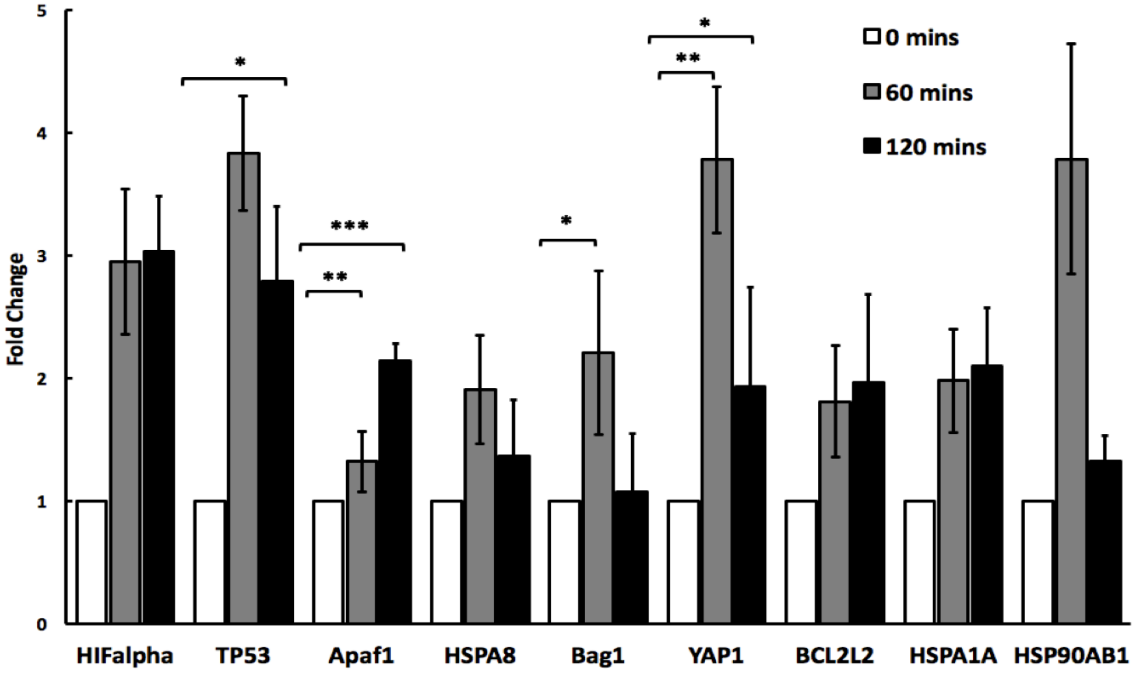

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Figure 19: La's association with mRNAs during cellular stress

(A) HEK293T cells were subject to polysome fractionation and subsequent qPCR analysis as previously described. Translational efficiency of mRNA targets identified from iCLIP analysis was measured by calculating ratio of poly/total RNA. Unstressed cells were set to "1". Error bars indicate standard deviation of three biological replicates and asterisks highlight statistically significant changes (*= P<.005, **=P<.01 two-tailed student t-test). **(B)** Time course of La co-immunoprecipitated with associated transcripts during cellular stress measured by qPCR. Error bars correspond to standard deviation of a minimum of three biological replicates, asterisks highlight statistically significant changes (*= P<0.05, **=P<0.01, *** P<.001 two-tailed student t-test).

3.5 Discussion

Cytoplasmic redistribution of La during cellular stress occurs to promote translation of stress associated messages

Several IRES trans-acting factors are upregulated during suboptimal cellular conditions such as viral infection, cellular stress, and tumor progression. These proteins often undergo various modifications to engage their RNA target including phosphorylation, increased protein expression, and nuclear-cytoplasmic shuttling [200,201]. In this study, we aim to decipher the role of hLa during cellular stress, specifically to further understand the conditions during which this protein is exported to the cytoplasm, the mechanism by which this occurs, and novel RNA substrates bound by La. We report here that the La protein undergoes subcellular relocalization upon exposure to clotrimazole, an inducer of mitochondrial stress and stress granules. Previous reports have shown similar shuttling of nucleoplasmic La to the cytoplasm under conditions of oxidative stress, serum starvation, tumor progression, and viral infection suggesting that this redistribution of La is not specific to mitochondrial stress or to the formation of stress granules [56,58,108–110,115]. Using a combination of polysome analysis and qPCR we show increased association of La with stress associated messages in response to cellular stress, specifically those that contain IRESs and uORFs. Using iCLIP, we then identify several new La associated messages and demonstrate a La dependency for these messages to effectively translate during cellular stress.

Human La function in cap-independent translation

The human La protein is most associated with the processing of nascent RNA polymerase III transcripts, but also plays a role in promoting cap-independent translation for several cellular and viral coding RNAs. In the nucleus, La is recruited to nascent RNA polymerase III transcripts,

such as pre-tRNAs, via sequence specific recognition of UUU-3'OH [54,149]. Although the mechanism by which La interacts with its nuclear substrates is well established, the mechanism by which La interacts with its cytoplasmic substrates appears to be more complex and context specific. Cytoplasmic La is often associated with messages that contain atypical translation initiation contexts such as 5' TOPs and IRESs [68]. Given that a significant 20% of mRNAs in cells contain 5' TOPs and that these mRNAs often encode ribosomal proteins and other components of translational machinery, it can be postulated that hLa's interaction with these messages serves to either directly or indirectly regulate the translation of essential proteins [220]. The role of La in the translation of 5' TOPs has been controversial with some studies suggesting that La enhances translation in *Xenopus* oocytes[177,221], while others suggest that La represses 5' TOP translation in human cells[68,222]. Our data presented in figure 18b, are consistent in showing that La displays an inhibitory effect on 5' TOP translation during cellular stress for some messages. La was the first ITAF discovered bound to poliovirus and since then identified as a key factor for the enhancement of IRES dependent translation by functioning as an RNA chaperone [99,158]. Human La can induce translation of IRES containing viral mRNAs including Hepatitis B Virus [156], Hepatitis C Virus [176], Coxsackievirus B3 [223], and encephalomyocarditis [57], along with cellular mRNAs including BiP [56], XIAP [198], Laminin B1 [115], CCND1 [58], Nrf2 [109], RRBP1[110], and the upstream open reading frame containing mRNA for the oncogene MDM2 [116].

Mechanism of La export to the cytoplasm

One aspect of La shutting yet to be identified is an explicit signal or cue which causes La to undergo subcellular redistribution. In our experiments, we specifically used clotrimazole to induce stress granule forming stress and monitored the migration of La in the presence of stress

granules. While we observed La migrate into the cytoplasm, we did not visualize La to co-localize with the granules. Cytoplasmic redistribution of La has also been reported during glucose starvation, a stress granule forming stress, and oxidative stress caused by hydrogen peroxide, a non-stress granule forming stress [109,110]. Monitoring the migration of La in response to a variety stresses that cause granule formation and those that do not, would help determine whether the cytoplasmic distribution is specific to certain types of stress or if it occurs as a general response to global homeostatic disruption. One question not addressed in this study is the possibility of La returning to its nuclear state once cells return to homeostasis. Cell fractionation of La performed in various studies indicate that protein expression levels remain the same during cellular stress and normal conditions and it is only the nuclear-cytoplasmic distribution that is altered, suggesting the possibility of La returning to the nucleus when homeostasis has been attained [109,115].

Also yet to be identified is a common mechanism for La's recruitment into the cytoplasm. Cytoplasmic translocation of hLa in apoptotic cells occurs through dephosphorylation at S366 resulting in the cleavage of the nuclear localization signal [205,206]. By contrast, murine La phosphorylated at Thr301 by AKT, shuttles to the cytoplasm and regulates mRNA translation in mouse glial progenitors [224]. In human cells undergoing epithelial-mesenchymal transition, it is hypothesized that AKT induced cytoplasmic shuttling of La promotes La binding to the Laminin B1 IRES, but the exact mechanism is unknown [115]. Surprisingly, in HeLa cells and rat cardiomyocytes, oxidative stress induced by H_2O_2 results in no apparent modifications of La suggesting the possibility of a carrier protein being involved in mediating export during this specific scenario [109]. Taken together, these data suggest that nuclear-cytoplasmic redistribution of La can occur via different mechanisms and appear to be context specific. Performing co-immunoprecipitation of La in the presence and absence of cellular stress coupled with mass

spectrometry will allow for the detection of modifications made to hLa and identify possible carrier proteins that mediate nuclear export. There also exists the possibility of La being co-exported with newly synthesized RNA transcripts to the cytoplasm. Inhibiting transcription using actinomycin D and/or alpha-amanitin and monitoring the localization of La using immunofluorescence will allow to assess if La export is mediated by the ability of the protein to bind nascent transcribed RNAs that are destined for the cytoplasm.

While our data do not provide a complete mechanism by which hLa translocates to the cytoplasm, our data are consistent with La being exported into the cytoplasm during cellular stress to assist in translation of upregulated messages. We have demonstrated using iCLIP and qPCR that our tested IRES containing messages are upregulated during cellular stress and display La dependency for translation during suboptimal cellular conditions. What remains to be tested is the specific nature by which La interacts with these messages. Previous research has indicated that the La motif, along with the C-terminal and N-terminal RNA recognition motifs are responsible for IRES dependent translation of the HCV IRES[41]. Hence, it can be speculated that La may promote cellular mRNA translation through the same binding modes. Through the use of bicistronic reporter constructs and hLa-GFP mutants, it will be possible to determine the specific domains of the protein that are involved in binding to IRES elements.

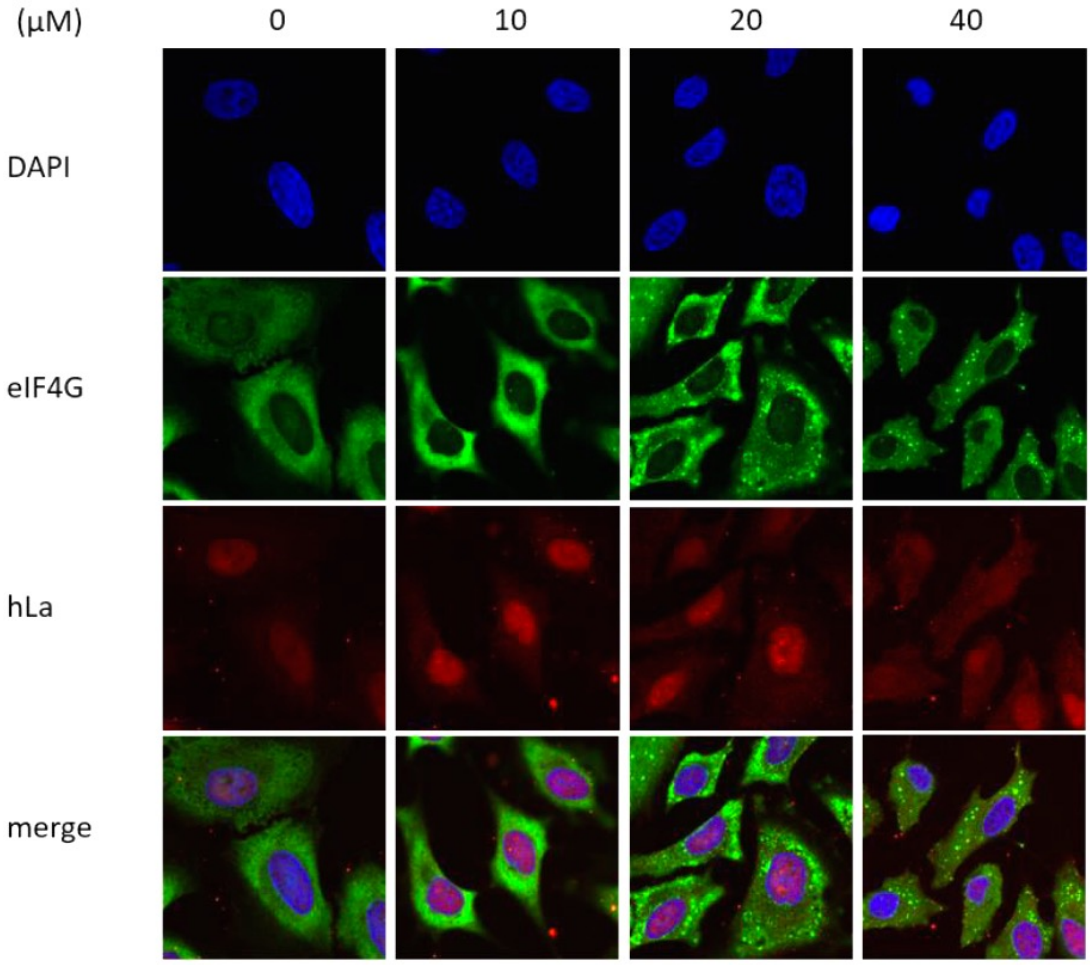
Cellular stress, specifically oxidative stress and mitochondrial stress haven been associated in the pathogenesis of neurodegenerative diseases, including Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS)[225]. In addition, oxidative stress has been implicated in viral replication, and tumor progression. The ability of certain messages to adapt and overcome cellular stress while others remain translationally repressed can have dramatic implications on gene expression. Therefore, understanding the molecular mechanisms by which cells adapt to cellular

stress and promote translation during compromised conditions is imperative for the discovery of novel therapeutic targets for neurodegenerative diseases, viruses, and cancer.

3.6 Acknowledgements

We thank Amy Pasquinelli and Marc Fabian for assistance in the iCLIP methodology and Claudia L. Kleinman and Steven Hebert for the bioinformatics analysis.

3.7 Supplementary Figures



Supplementary Figure 4: Stress granules induced using clotrimazole

Stress granules were induced at a minimum concentration of 20uM clotrimazole in HeLa cells for 30 minutes.

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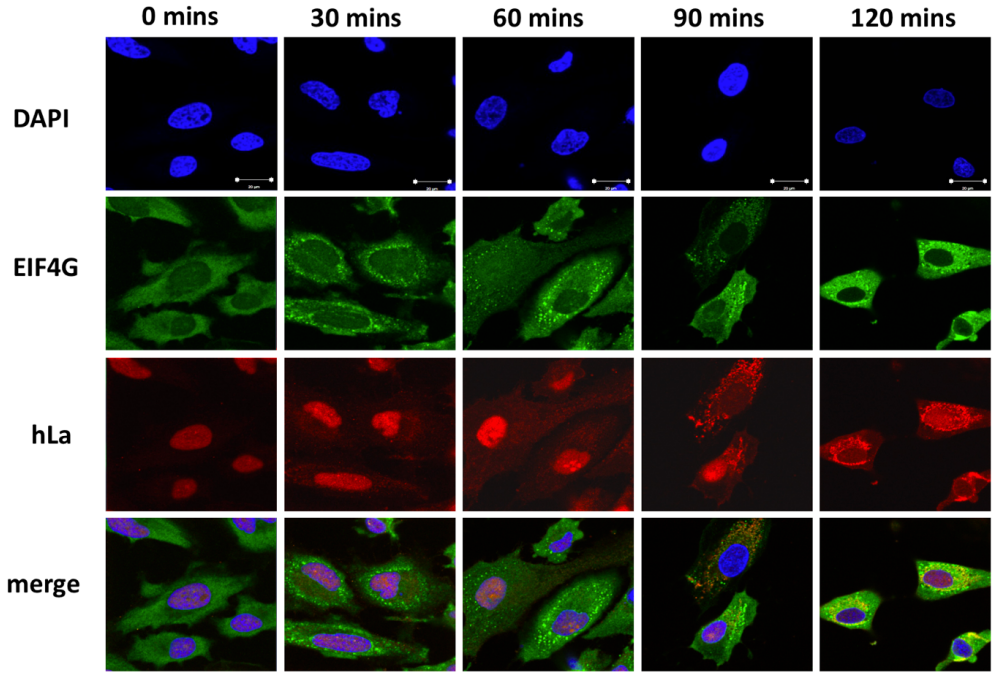

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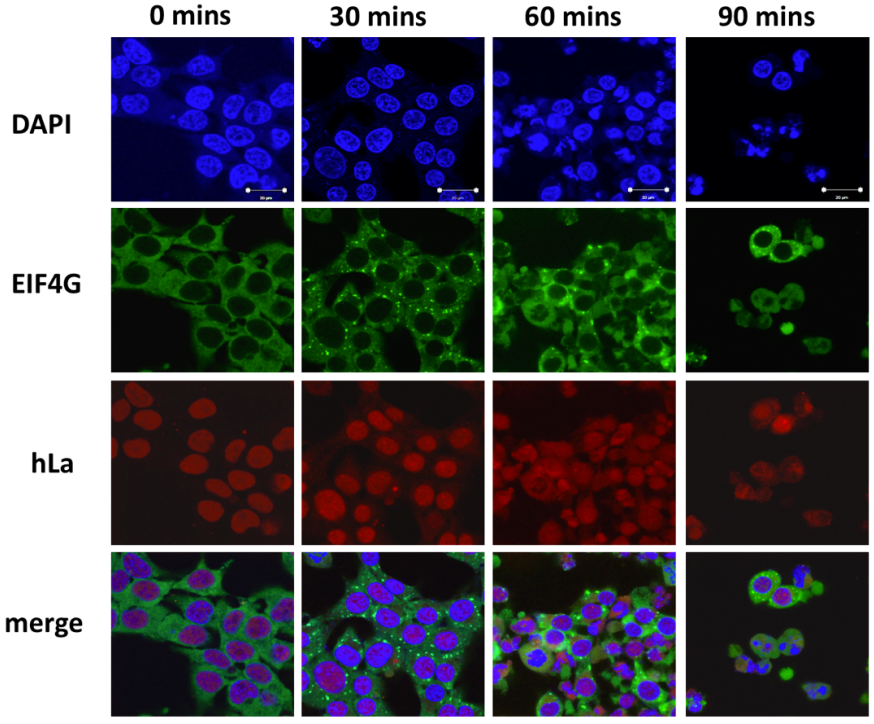

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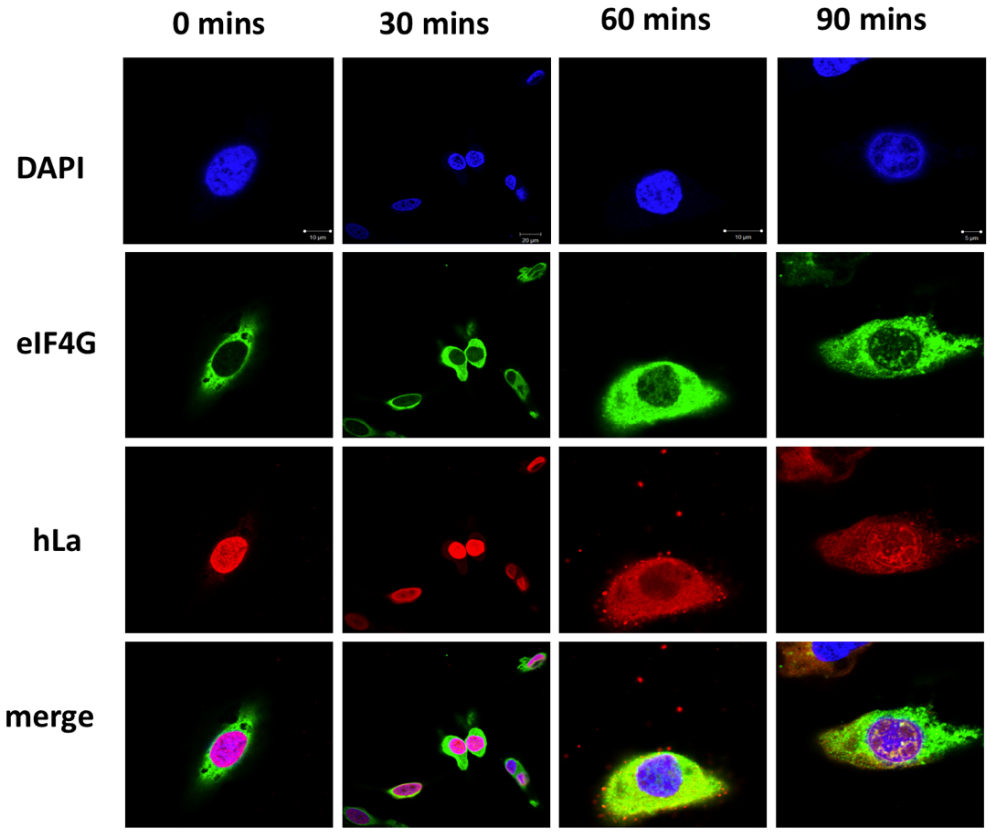
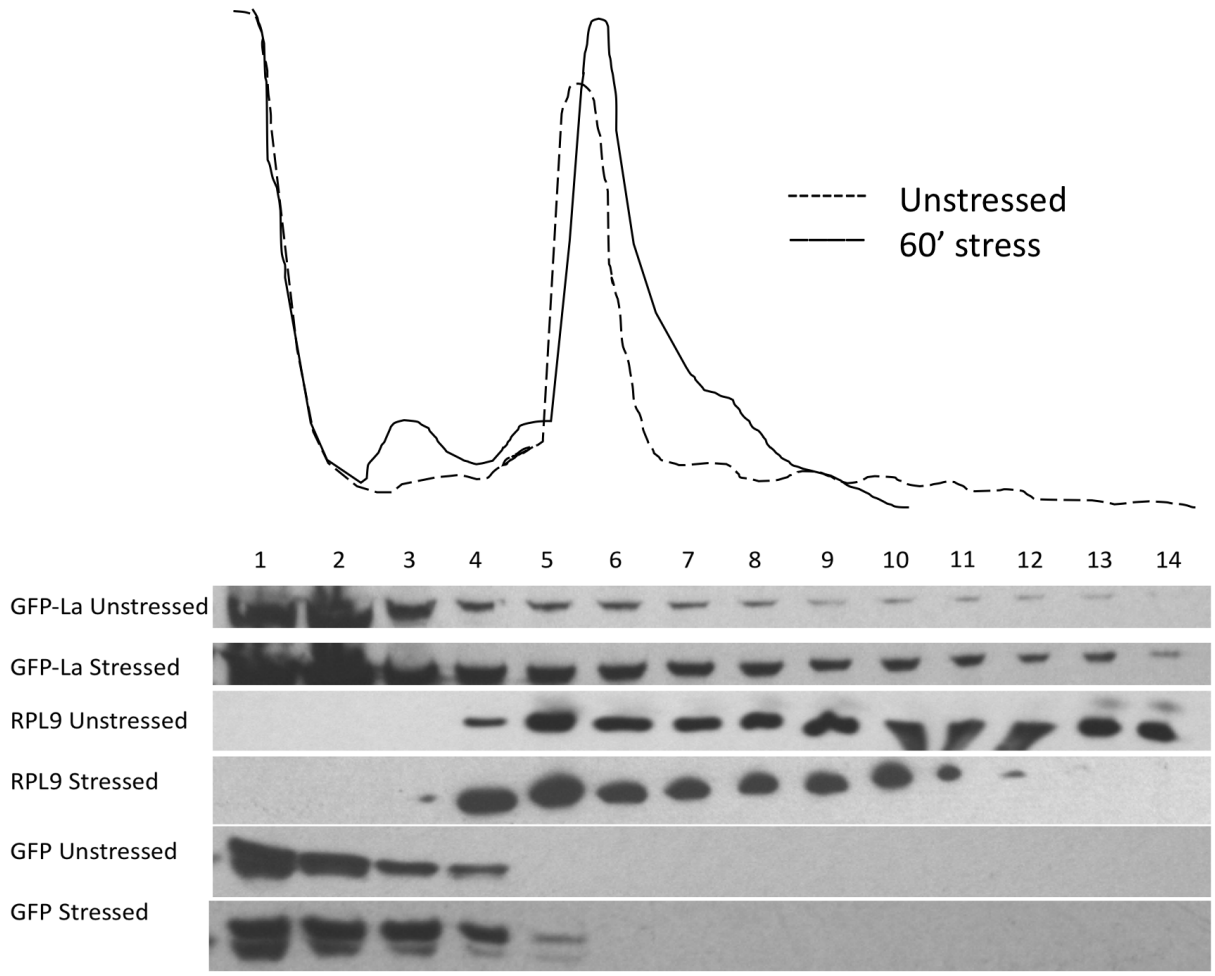


Supplementary Figure 5: Mitochondrial stress induces cytoplasmic relocalization of La

(A) HeLa cells were treated with 20uM clotrimazole to induce stress granules. A time course was performed from 0-120 minutes in 30 minute intervals to visualize localization of La (red). EIF4G was used as a stress granule marker (green). Nuclei were visualized by DAPI (blue). **(B)** Time course of 0-90 mins performed on HEK293T cells and **(C)** U2OS cells.



Supplementary Figure 6: Polysome Analysis of GFP-La

Trace of ribosomes/polysomes fractionated from HEK293T cells untreated and treated with 20uM clotrimazole for 60 minutes. Presence of transfected GFP-La in each fraction was analyzed by western blotting using GFP and Rpl9.

Supplementary Table 2: List of Primers

	Primer Name	Tm	Primer Sequence
Primers for qPCR	CCND1 Forward	60.3	5'- CTC TCC AGA GTG ATC AAG TGT GAC CC-3'
	CCND1 Reverse	60.8	5'- TGT GCA AGC CAG GTC CAC C-3'
	Laminin B1 Forward	58.6	5'- GGC GTC TTC TCC ACT CCT CT-3'
	Laminin B1 Reverse	66.4	5'- ACT CGG GTT CCT GAG CGC GCA C-3'
	MDM2 Forward	54.8	5'- GAA TCA TCG GAC TCA GGT ACA TC-3'
	MDM2 Reverse	55.1	5'- TCT GTC TCA CTA ATT GCT CTC CT-3'
	BiP Forward	55.2	5'- GAA AGA AGG TTA CCC ATG CAG T-3'
	BiP Reverse	56.5	5'- CAG GCC ATA AGC AAT AGC AGC-3'
	Hsp70 Forward	55.3	5'- TTT TAC CAC TGA GCA AGT GAC TG-3'
	Hsp70 Reverse	55.1	5'- ACA AGG AAC CGA AAC AAC ACA-3'
	GAPDH Forward	57	5'- GGA GCG AGA TCC CTC CAA AAT-3'
	GAPDH Reverse	55.7	5'-GGC TGT TGT CAT ACT TCT CAT GG-3'
	eEF1B Forward	61.3	5'-ATA CAG CCG ACA CCA TGG GTT TCG-3'
	eEF1B Reverse	57.4	5'-GAT GTG ATT ATA CCA ACG TAG GGC ATG -3'
	Rpl37 Forward	59.6	5'-ACG AAG GGA ACG TCA TCG TTT GG-3'
	Rpl37 Reverse	59.2	5'-CCT CAT TCG ACC AGT TCC GGT-3'
	BAG 1 Forward	56.8	5'-TGT TAC CTC CCA GCA GGG-3'
	BAG 1 Reverse	53.4	5'-CTG GTC AGC TAT CTT CTC CA-3'
	HIF1-alpha Forward	54.6	5'-GTC AAT GAA TTC AAG TTG GAA TTG GT-3'
	HIF1-alpha Reverse	51.2	5'-AAA AGT GAA CCA TCA TG TTC C-3'
	TP53 Forward	53.2	5'-AGG AAG AGA ATC TCC GCA A-3'
	TP53 Reverse	54.6	5'-GGC TGT CAG TGG GGA A-3'
	Apaf1 Forward	49.7	5'-AAT GGA AAG TCG TTT CGT TA-3'
	Apaf1 Reverse	53	5'-GGT TTT ATC AGC TCC ACA AGA A-3'
	XIST Forward	51.7	5'-TCC CAT TGA AGA TAC CAC G-3'
	XIST Reverse	53.4	5'-AAC CCA TCC AAG TAG ATT AGC T-3'
	YAP1 Forward	58.5	5'-GCT ACA CCC ACA GCT CAG C-3'
	YAP1 Reverse	60.6	5'-TGG GGA GCC AGG GGT G-3'
	HSP90AB1 Forward	53.7	5'-ATT GGC ATG ACC AAA GCT G-3'
	HSP90AB1 Reverse	53.5	5'-TCT TCA TCA TCT TTA TCT TCC TCT TCT-3'
	HSPA1A Forward	57.1	5'-CGA GCT CTT CTC GCG GAT-3'
	HSPA1A Reverse	55.9	5'-TGG TTC TTG GCC GCA TC-3'
	BCL2L2 Forward	61.2	5'-CCT CCC TCT GCC CCC C-3'
	BCL2L2 Reverse	50.7	5'-TAA CCT ACA AAG TCT GCC A-3'
Primers for iCLIP	iCLIP RT1	69.8	5'-/5Phos/NNA ACC NNN AGA TCG GAA GAG CGT CGT GGA TCC TGA ACC GC-3'
	iCLIP RT2	68.8	5'-/5Phos/NNA CAA NNN AGA TCG GAA GAG CGT CGT GGA TCC TGA ACC GC-3'
	iCLIP RT3	68.9	5'-/5Phos/NNA TTG NNN AGA TCG GAA GAG CGT CGT GGA TCC TGA ACC GC-3'
	iCLIP RT4	69.6	5'-/5Phos/NNA GGT NNN AGA TCG GAA GAG CGT CGT GGA TCC TGA ACC GC-3'
	iCLIP RT6	71.8	5'-/5Phos/NNC CGG NNN AGA TCG GAA GAG CGT CGT GGA TCC TGA ACC GC-3'
	iCLIP RT7	68.5	5'-/5Phos/NNC TAA NNN AGA TCG GAA GAG CGT CGT GGA TCC TGA ACC GC-3'
	iCLIP Cut Oligo	60.8	5'- GTT CAG GAT CCA CGA CGC TCT TC/3ddc/ -3'
	iCLIP P3 Solexa	72.4	5'- CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T-3'
	iCLIP P5 Solexa	70.7	5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T -3'
	iCLIP L3 3' Linker	-	5'- /5rApp/AGATCGGAAGAGCGGTTCAG/3ddc/ -3'
	La siRNA 3'	43.1	5' rGrUrA rUrUrA rGrCrA rGrGrU rCrUrG rUrUrU rCTT-3'
	La siRNA 5'	54.8	5'-rGrArA rArCrA rGrArC rCrUrG rCrUrA rArUrA rCTT-3'
	siRNA universal negative control		Proprietary Sequences Cat#SIC001

Chapter 4:

Summary and Future Directions

4.1 General Summary

The La protein, also referred to as Sjogren's Syndrome antigen B (SSB), is an RNA-binding phosphoprotein that shuttles between the nucleus and cytoplasm, interacting with RNA substrates transcribed by both RNA Pol III and Pol II. While hLa's interaction with its nuclear substrates have been well characterized, La's interaction with its cytoplasmic substrates is still under investigation. The objective of this study was to characterize the mechanism by which La recognizes its cytoplasmic targets, and to understand the context and mechanism by which La affects cap independent translation. In this work, we show 1) hLa binds to poly(A) sequences *in vitro*, 2) La binds to the poly(A) tail of mRNA to regulate translation, and 3) La is translocated from the nucleus to the cytoplasm in response to cellular stress in order to mediate cap independent translation during cellular stress.

4.1.1 hLa binds mRNAs through contacts made to the poly(A) tail

In the data presented in Chapter 2, we show that in addition to a sequence specific UUU-3'OH binding mode, hLa exhibits a sequence specific and length dependent poly(A) binding mode and that La may regulate IRES mediated translation via contacts made to the poly(A) tail. La proteins have been linked extensively with the translation of mRNAs with atypical translation initiation contexts; however, it was previously hypothesized that La binds coding transcripts via non-specific recognition of structured RNA elements[41]. Using electromobility shift assays, we demonstrate that hLa has greater affinity for adenylate containing sequences compared to poly(C) or single stranded poly(G), and this occurs in a length dependent manner. Using both hLa point and deletion mutants, we further mapped this poly(A) binding mode to specific amino acids in the winged helix face of the LAM, previously shown to be vacant during uridylate binding, along with the two RRMs.

Given the previous association of La with the translation of coding transcripts and our *in vitro* results suggesting that La displays affinity for poly(A) sequences providing the sequence is extended, we hypothesized that La may function in mRNA translation via contacts made to the poly(A) tail. Using a cross-linking immunoprecipitation assay, we show that while wildtype hLa directly engages and immunoprecipitates poly(A) RNA in human cells, hLa constructs with mutations in the winged helix fold display impaired binding to poly(A) and fail to immunoprecipitate poly(A) RNA, but still immunoprecipitates pre-tRNA Met-e. To assess if the poly(A) binding mode could play an important role in La's capability to enter polysomes and associate with actively translating messages, we performed polysome fractionation. Unlike wildtype hLa, which was found in polysomal fractions, our hLa mutant was found exclusively at the top of the polysome gradient, suggesting a decrease in its ability to enter polysomes.

We then tested the importance of the poly(A) dependent binding mode on La's capacity to promote both cap- dependent and independent translation using a well characterized substrate of La, the IRES of the cyclin D1 mRNA. We observed that overexpression of La resulted in an increase of both cap- dependent and independent translation, and that La mutants displayed diminished ability to drive translation. To further understand if the interaction of La with the poly(A) tail occurs in competition with the cytoplasmic poly(A) binding protein, we overexpressed both PABP and La. Overexpression of PABP resulted in a decrease in La driven cap independent translation, indicating possible competition between La and PABP for the poly(A) tail. The data presented in Chapter 2 provides us with a possible mechanism through which La may interact with its cytoplasmic substrates, functioning to drive cap independent translation via contacts made with the poly (A) tail.

4.1.2 Cellular stress induces the cytoplasmic localization of La and increased association with stress associated mRNAs

In Chapter 3, we report that the hLa protein undergoes subcellular re-localization upon exposure to clotrimazole, an inducer of mitochondrial stress, and displays increased association with messages that are upregulated during stress. Using immunofluorescence to monitor the movement of La upon stress induction using clotrimazole, we show the gradual migration of La from the nucleus to the cytoplasm. While La is observed to be diffuse in the cytoplasm, it does not co-localize with stress granules. Previous reports have shown similar shuttling of nucleoplasmic La to the cytoplasm under conditions of oxidative stress, serum starvation, tumour progression, and viral infection suggesting that this translocation is not specific to mitochondrial stress [56,58,108–110,115].

Given the previous association of La with the translation of coding RNAs that contain atypical translation initiation contexts, which also happen to be upregulated during cellular stress, we postulated that La may shuttle to the cytoplasm during cellular stress to assist in cap-independent translation of stress related mRNAs. HEK293T cells treated with clotrimazole were subject to polysome fractionation and immunoblotting was performed to identify the localization of La and PABP. Our polysome analysis data showed that despite the decrease in the presence of polysomes in stressed cells, an increased association of La with polysomal fractions. Given that cellular stress results in a general decrease of cap dependent translation and an increase in IRES dependent translation, it is probable that La is associating with these upregulated IRES containing messages. Interestingly, we also observed a decrease in the association of PABP with polysomal fractions of stressed cells compared to non-stressed cells. Since PABP is recruited to stress granules during stress to stabilize stored mRNAs and aid in the re-activation of global translation

upon stress granule disassociation, it is plausible that La is recruited to the cytoplasm to assume some roles of PABP. Using iCLIP, we have identified novel substrates of La and mRNAs that are upregulated during clotrimazole induced cellular stress. Interestingly, some of the identified La substrates contain previously established IRES elements in their 5' UTR. Using qPCR analysis, we demonstrated that these messages are not only upregulated during cellular stress, but are also dependent on La for optimal translation during suboptimal cellular conditions. In summary, our data show that hLa is trafficked into the cytoplasm in response to clotrimazole induced cellular stress to associate with messages that rely on alternate mechanisms to promote translation during compromised cellular conditions.

4.2 Future Directions

4.2.1 Mechanistic interplay of La and PABP at the poly(A) tail

Although we provide a potential novel mechanism through which La interacts with its cytoplasmic substrates to regulate translation, at the same time, several questions have emerged from this study that have yet to be answered. We propose that La functions in translation via contacts made directly with the poly (A) tail. For mRNAs where the poly(A) tail is present, our results demonstrate that La may interact with these messages in a manner analogous to which it interacts with its UUU-3'OH RNA Pol III substrates. However, whether La binds the poly(A) tail, remains bound while looping around the RNA to gain access to the 5' UTR and the IRES, or if La is recruited to the poly(A) tail and then translocates to these sites is still unclear. Although previous work has hypothesized the possibility of La simultaneously binding to both the poly(A) tail as well as other regions of mRNA, it has not been experimentally proven [40,41,180]. Similarly, the precise nature by which PABP and La interact at the poly(A) tail is also unknown. In addition, it is still not known if La and PABP always compete for poly (A) tail binding or if certain cellular conditions

cause La to assume the role of PABP. This could occur when PABP has been recruited to stress granules to help stabilize stalled translating messages that occurs with exposure to exogenous stress or viral infection.

Interestingly, several La associated plus-strand viral mRNAs containing IRESs including poliovirus, encephalomyocarditis virus (EMCV) and coxsackievirus B3 (CBV3), have all been identified to harbour a poly(A) tail where its presence has been shown to enhance the translation of viral mRNA [226,227]. In addition, although the Hepatitis C virus does not naturally contain a poly(A) tail, increased IRES driven translation has been observed when a poly(A) tail was artificially engineered to the 3'OH end [226,228]. It has also been proposed that viral IRESs promote translation via different mechanisms and that PABP may not be the only RBP that binds the poly(A) tail of these viral messages. For certain picornaviruses such as EMCV and polio, it has been shown that viral replication and IRES mediated translation occur in cell-free extracts that have been depleted of PABP [227]. Thus, it would be fascinating to test whether La enhances the translation of these viral messages through contacts made with the poly(A) tail.

4.2.2 La-motif mediated poly(A) binding in the LARP families

While genuine La proteins facilitate the processing of both RNA Pol II and RNA Pol III transcripts, the LARP families have evolved to only target one of these classes. Although the LAM remains highly conserved amongst all the LARP families, differences appear in the adjacent linker bridging the LAM and the N-terminal RRM. While this linker in La is long and flexible in its RNA-free form, the corresponding linker in LARP families 4 and 6 (LARP4 and LARP6) appears to be shorter and rigid [42]. It is plausible that the difference in the flexibility and the length of the linker may be the distinguishing factor that allows the La protein to function in the binding of both

the poly(A) and poly (U) transcripts, while the LARP families are restricted to targeting only one of these classes, although this has yet to be tested. Another question that needs to be answered is whether the LARP families also use the highly conserved LAM to regulate translation. Members of the LARP1, LARP4, and LARP6 families are associated with mRNA translation via the direct interaction with PABP. Additionally, human LARP1 and LARP4 have been shown to directly bind poly(A) in a manner parallel to La. It will be vital to perform structural analyses to determine if these LARP families use the same residues in the winged helix fold of the LAM to bind to poly(A).

4.2.3 Alternate cellular cues that induce cytoplasmic localization of La

We show that under certain conditions, La is recruited to the cytoplasm to assist in the translation of stress associated messages; however, several questions emerging from this work are yet to be resolved. While the cytoplasmic redistribution of La has been reported during glucose starvation and oxidative stress (hydrogen peroxide), there are several other forms of stress with the potential to elicit similar responses that have not been tested. Cell exposure to osmotic shock and oxidative stress (e.g., arsenite), as well as non-chemically induced stresses such as hypoxia, heat shock, and UV radiation, would allow us to determine whether the migration of La to the cytoplasm is specific to certain types of stress or if this migration occurs as a general response to global homeostatic disruption.

Unlike stress induced by hydrogen peroxide, oxidative stress that is induced by arsenite results in the formation of stress granules. Consequently, although both these stressors induce the production of reactive oxygen species, key translational machinery is recruited to stress granules with arsenite exposure, but is not with hydrogen peroxide. Therefore, it would be interesting to monitor and compare the similarities and differences of the function of La in the presence of each

of these stressors. It would also be important to detect the movement of La once cells have adapted to or overcome cellular stress and are undergoing recovery. In distinct studies, cell fractionation indicate that La protein expression levels remain the same during both stress and normal conditions, and only the nuclear-cytoplasmic distribution of La is changed [109,115]. Although it still must be tested and proven, this suggests that La may have the potential to return to the nucleus when the cell returns to homeostasis.

4.2.4 Mechanism of La shuttling between nucleus and cytoplasm

An interesting area to explore is the function of La in viruses that stimulate cellular stress, since several La associated viral messages promote translation by inducing stress to recruit cellular machinery. hLa was identified as the first IRES ITAF due to its ability to enhance translation of polioviral RNA[158]. Interestingly, poliovirus can also induce the formation of stress granules in cells [229]. The cytoplasmic distribution of La infected cells is shown to be mediated by cleavage of the peptide bond Gln358 and Gly359, by "3C62" a poliovirus-specific protease [89,173]. Additionally, the well-characterized La associated virus, Hepatitis C, has been shown to induce oxidative stress in infected cells and induce stress granules [111,230]. Although the mechanism has not yet been identified, increased cytoplasmic distribution of La has been observed within infected cells, along with the increased association of La with the HCV IRES [111]. Further studies examining the interaction of La with other picornaviral and HCV-like IRESs under oxidative stress may reveal more information about the mechanism through which La is recruited to the cytoplasm to promote IRES dependent translation and viral replication.

A common mechanism for La recruitment to the cytoplasm is yet to be identified. In apoptotic cells, hLa is dephosphorylated and the nuclear localization signal is cleaved causing the

cytoplasmic distribution of La [205,206]. By contrast, murine La phosphorylated by AKT results in cytoplasmic translocation [224]. Surprisingly, oxidative stress induced by hydrogen peroxide results in no apparent hLa modifications, suggesting that a carrier protein may be involved in mediating nuclear export [109]. This suggests that the nuclear-cytoplasmic redistribution of La appears to be context-specific and can occur through distinct mechanisms. Performing co-immunoprecipitation of La during cellular stress coupled with mass spectrometry will allow us to detect modifications made to hLa and identify possible carrier proteins that mediate nuclear export. One site of interest, located within the NLS of hLa (T389), is known to be phosphorylated by AKT [69]. There also exists the possibility of dysfunction of nuclear import of La during cellular stress resulting in increased accumulation of the protein in the cytoplasm. Additionally, the question of whether La can be co-exported with newly synthesized RNA transcripts to the cytoplasm has yet to be addressed. This can be tested by inhibiting transcription using actinomycin D and/or alpha-amanitin and monitoring the localization of La using immunofluorescence. By inhibiting transcription and examining the localization of La during cellular stress, we will be able to assess if La export is mediated by the ability of La to bind nascent transcribed RNAs, destined for the cytoplasm.

4.3 Conclusion

In this dissertation, we have attempted to further characterize the mechanisms through which hLa recognizes and interacts with its cytoplasmic targets to regulate translation. Our results demonstrate that in addition to a sequence specific UUU-3'OH binding mode, hLa exhibits a sequence specific and length dependent poly(A) binding mode, mapped to the canonical winged helix face of the eponymous LAM. Additionally, we have shown that cytoplasmic La engages poly(A) RNA in human cells and that La promotion of translation from the cyclin D1 IRES occurs

in competition with cytoplasmic PABP. In an effort to understand the cellular conditions during which La interacts with its cytoplasmic substrates we show that stress induced by clotrimazole causes the translocation of La from the nucleus to the cytoplasm and that this translocation is concurrent with the increased association of La with actively translating messages. Using iCLIP, we identify novel mRNAs that are La dependent for translation during cellular stress. Taken together, this data suggests that hLa is trafficked into the cytoplasm in response to cellular stress in order to associate with IRES containing messages, and this interaction may occur through contacts made with the poly (A) tail.

Gene regulation is a fundamental process responsible for the development and survival of an organism. The ability of certain messages to adapt and overcome cellular stress while others remain translationally repressed can have dramatic implications on gene expression. As the ability of cells to maintain active translation in response to cellular stress is vital for its survival, cells have evolved and adopted various mechanisms to adapt and overcome cellular stress. Cellular stress has been associated with the pathogenesis of neurodegenerative diseases, viral replication, and tumor progression. Therefore, understanding the molecular mechanisms by which cells adapt to cellular stress and promote translation using atypical translation initiation contexts during compromised conditions can lead to the discovery of novel therapeutic targets for neurodegenerative diseases, viruses, and cancer.

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Appendix

Selected Materials and Methods

Maintaining Cell Culture

HEK293T, HeLa, and U2OS cells were obtained from ATCC and cultured in DMEM media with serum. Cells were regularly passaged and maintained for 30-40 passages and then discarded.

Reagents

DMEM	GIBCO 11965-092
PBS	MultiCell 311-010-C1
Fetal Bovine Serum	Sigma F1051
Pencillin	ThermoScientific 15140122
Trypsin	Hyclone SV30031.01
DMSO	BioShop DMS555.500

Cell Passaging

1) Aspirate media.
2) Rinse cells with 5-10mLs of 1x PBS.
3) Add 1mL of Trypsin and incubate at 37^{o}C for 2 mins.
4) Inactivate trypsin by adding 9mLs of DMEM.
5) Pipette cells several times to avoid cell clumping.
6) Add 1mL of cells to 9mL of DMEM media containing 10% FBS and 2% antibiotics.

DNA/RNA Transfection

DNA was prepped using Qiagen Plasmid Midi Prep Kit. Transfections were performed either with PolyJet or Lipofectamine 2000. PolyJet was used for DNA transfections. Lipofectamine was used for RNA or DNA/RNA co-transfections. Transfections were scaled up or scaled down according to manufacture's protocol.

Reagents

Plasmid Midi Prep Kit	Qiagen 12143
PolyJet	SignaGen SL100688
Lipofectamine	Invitrogen 11668-019
OPTIMEM	GIBCO 31985-070
DMEM	GIBCO 11965-092

PolyJet transfection

1) Plate cells in plates 18-24hrs pre transfection to a confluency of 70-80% in media with 10% FBS excluding antibiotics.
2) 1 hour prior to transfection, change media for cells.
3) Dilute DNA in serum-free DMEM media and dilute PolyJet in serum-free DMEM media in separate tubes.
4) Immediately add diluted PolyJet to the diluted DNA, pipette up and down to mix DNA with PolyJet and incubate at room temperature for 10-15 mins.
5) Add PolyJet/DNA mixture to cells in a drop wise manner. Incubate at 37^{o}C.
6) Change media 6-8 hrs after transfection.

Lipofectamine transfection

1) Plate cells in plates 18-24hrs pre transfection to a confluency of 70-80% in media with 10% FBS excluding antibiotics.
2) 1 hour prior to transfection, change media for cells.
3) Dilute DNA and/or RNA in Opti-MEM® I and dilute Lipofectamine in Opti-MEM® I in separate tubes.
4) Add diluted DNA to the diluted lipofectamine, pipette to mix DNA/RNA with Lipofectamine and incubate at room temperature for 15-20 mins.
5) Add Lipofectamine/DNA mixture to cells in a drop wise manner. Incubate at 37^{o}C.
6) Change media 6-8 hrs after transfection.

*For knockdown of La, use 100nM of siRNA. Maximum knockdown is seen 48 hours post transfection

In vitro RNA Transcription

Reagents

OneStep RT-PCR Kit	Qiagen 210210
mMESSAGE mMACHINE® SP6 Transcription Kit	Ambion 1340

1) Extract RNA from HeLa cells using Trizol as per manufacturer's protocol.
2) Obtain HeLa poly(A) derived cDNA using the OneStep RT-PCR Kit. 0.5ug of RNA was used as template as per manufactures protocol.
3) Clone IRES into the SalI-BamHI site of the pDL-N dual luciferase construct using restriction enzymes.
4) Use construct as a template for a PCR reaction using primers to add an SP6 promoter upstream of the renilla 5' UTR and a 20A sequence, 40A sequence or a histone mRNA SLBP-binding stem loop after the firefly 3' UTR.
5) Run agarose gel to confirm PCR product.
6) Use 1ug DNA template to transcribe RNA using mMESSAGE mMACHINE® SP6 Transcription Kit.
7) Run reaction overnight at 37^{o}C.
8) Run 1ul transcribed product on agarose gel to confirm transcription reaction.
9) Precipitate RNA using lithium chloride as per manufacture's protocol.
10) Quantify RNA using nanodrop.

Luciferase Assay

Reagents

Dual-Luciferase Reporter Assay System	Promega E1910
White 96-Well Immuno Plates	ThermoScientific 14-245-196Q

1) Wash adherent cells with 1X PBS.
2) Add 20uls of lysis buffer/well (supplied by Promega) in 96 well plate. Dilute lysis buffer 1:5 in ddH_20 and supplement with 1% Protease Inhibitor Cocktail and 1% PMSF.
3) Harvest cells. Collect 15uls and place in an opaque white 96 well plate (Costar)
4) Keep lysate on ice until read using Synergy H4
5) Record numbers for Firefly (Dispenser 1) and Renilla (Dispenser 2)

Synergy H4 Procedure Details:

Dispense: 45 µL, Dispenser 1, Rate 250 µL/sec, Tip prime, 10 µL
Read Luminescence Endpoint
Integration Time: 0:01.00 (MM:SS.ss)
Filter Set 1
Emission: Hole
Optics: Top, Gain: AutoScale
Read Speed: Normal, Delay: 100 msec
Dispense: 45 µL, Dispenser 2, Rate 225 µL/sec, Tip prime, 10 µL
Read Luminescence Endpoint
Integration Time: 0:01.00 (MM:SS.ss)
Filter Set 1
Emission: Hole
Optics: Top, Gain: AutoScale
Read Speed: Normal, Delay: 100 msec

Cross-linking and Immunoprecipitation (CLIP)

Procedure adapted from Abcam

Reagents

Protein G Beads	Thermo Scientific 10003D
Protease Inhibitor Cocktail	Thermo Scientific 78425
RNAse Inhibitor	Ambion AM2696
Sodium Deoxycholate	Sigma-Aldrich D6750
TurboDNAse	Ambion 1340
RNAse I	Ambion AM2295
Proteinase K	Thermo Scientific 100005393
Decade Markers	Ambion AM7778
iBlot Gel Transfer Stack	Invitrogen IB301002
GeneScreen Plus	PerkinElmer NEF1017001PK
Yeast RNA	Thermo Scientific AM7118
Denhardt's Solution	Thermo Scientific 750018
ATP, [γ-P^{32}]	PerkinElmer

Buffers

Lysis buffer (RIPA) - 50 mM Tris-HCl, pH 7.4
100 mM NaCl, 1% NP-40
0.1% SDS
0.5% sodium deoxycholate,
protease inhibitors (1:1000 add fresh each time)
RNAse Inhibitor (1:1000, add fresh each time)

Proteinase K buffer - 100 mM Tris-HCl pH 7.4
50 mM NaCl
10 mM EDTA

Proteinase K urea buffer- 100 mM Tris-HCl pH 7.4
50 mM NaCl
10 mM EDTA
7 M urea

Hybridization buffer-

SSC	6mLs
10% SDS	2mLs
Denhardt Solution	800uls
Yeast RNA	400uls

Fill to 20mLs with ddH20

Wash buffer-

SSC -	10mLs
10% SDS -	1mLs

Fill to 100mLs with ddH20

Stripping buffer- SSC - 500uLs
10% SDS - 1mLs
Fill to 100mLs with ddH20

Co-Immunoprecipitation

1) Transfect HEK293 cells in 15 cm plates with plasmids. Transfect 2 plates per sample with PolyJet
2) 24 hrs post transfection, remove media and wash with 10mLs of ice-cold PBS. Remove PBS
3) Place dish on ice and irradiate with 150 mJ/cm2 at 254 nm using a stratalinker
4) Use 2mLs RIPA buffer to collect cells by pipetting and transfer cells to microfuge tubes
5) Pellet cells (spin at top speed for 10 sec at 4°C), then remove supernatant. Either snap-freeze cell pellets on dry ice and store at -80°C until use or continue working with pellet.
6) To prepare beads, wash beads Protein G beads twice with RIPA buffer. Use 75uls of beads per sample.
7) Re-suspend beads in 100uls of lysis buffer and add 10ug of antibody for beads and rotate tubes at room temperature for 60 mins.
    1) Anti GFP Antibody
    2) Anti cMyc Antibody
    3) NC mouse sera
8) In the meantime, re-suspend cell pellet in 1mL lysis buffer without RNAse Inhibitor. Add 10uls of 1:100 RNase I and 2 uls of Turbo DNase to the cell lysate and incubate for 3 min at 37°C, shaking at 1,100 rpm, then immediately transfer to ice.
9) Spin at 4°C at 14,000 rpm for 20 min and collect the cleared supernatant.
10) Split the supernatant in half for each sample. Half for +Ab and half for -Ab control
11) Remove lysis buffer from the beads, add cell lysate to beads and rotate for 2 hr at 4°C.
12) Discard the supernatant and wash beads 2x with 750uls of RIPA buffer and then 2x with 750uls Proteinase K buffer
13) Re-suspend beads in 200uls of Proteinase K buffer and add 10uls of Proteinase K. Incubate for 30 mins at 37°C, shaking at 1,100 rpm.
14) Add 130uls of Proteinase K Urea buffer and add 10uls of Proteinase K. Incubate for 30 mins at 37°C, shaking at 1,100 rpm.
15) Add 400uls of RNA phenol/chloroform (pH 4.3) to tube and centrifuge at 14,000 rpm at 4°C to separate the phases.
16) Transfer the aqueous layer to a new tube. Add 400uls of chloroform and centrifuge at 14,000 rpm at 4°C.
17) Transfer the aqueous layer into a new tube. Add 1mL of 100% ethanol, 0.5uls glycoblue and 40uls 3M sodium acetate. Precipitate over night at -80°C.

RNA Preparation and Northern Blot Analysis

18) Centrifuge 10 mins, 14,000rpm at 4°C to obtain RNA pellet. Wash again with 70% EtOH.
19) Air dry pellet on ice for 5 mins.
20) Add 10uls of formamide dye and heat sample at 95°C for 10 mins.
21) Immediately place RNA on ice to cool.
22) Load samples on a 15% urea gel. For RNA ladder, prepare Decade Markers as per manufactures protocol
23) Run gel at 100V in 4°C until bromophenol blue is at the bottom of the gel.

24) Label probe while running the gel (see blow).
25) Transfer gel onto a membrane using iblot from Invitrogen. Transfer for 6 minutes.
26) Crosslink RNA to membrane by setting Fotodyne DNA Transfer Lamp UV to 160 J/m^2 for 90 seconds.
27) Place membrane between two Whatman Cellulose Filter Paper and dry membrane for 10mins at 80°C using the gel dryer.
28) Place membrane into glass tube and add 20mLs hybridization buffer. Pre-hybridize membrane for 2 hours at 32°C by rotating end over end in hybridization oven.
29) Heat probe to 90°C for four minutes. Add 4uls of labeled probe to the hybridization buffer. Hybridize γ-P^{32} radiolabeled dT(40) overnight at 32°C.
30) Dispose hybridization buffer in appropriate waste container.
31) Wash membrane 3x by adding 20mls of wash buffer and rotating for 20 minutes.
32) Wrap membrane with saran wrap, place phosphor screen, and expose overnight.
33) Scan membrane using GE typhoon scanner.
34) Strip membrane by adding 20mLs of stripping buffer and rotating at 70°C for 20 minutes. Strip 3x.
35) Reprobe for pre-tRNA Met-e at 37°C.

Labeling Probe

1) Add following components:

a.	25 uM probe	2 uls	
b.	T4 PNK Buffer	2 uls	
c.	dH20	14 uls	
d.	γ-P^{32} ATP*	1 ul	(activity of ~3000Ci/mmol)
e.	T4 enzyme	1 ul	
		20	

*Adjust volume according to activity of γ-P^{32} ATP

2) Incubate at 37°C for 2 hours.
3) Store in radioactive freezer.

Polysome Gradients

Adapted from [231]

Reagents

Sucrose	Fisher Scientific BP220-212
Beckman Coulter Tubes	Beckman Coulter 331372
PBS	MultiCell 311-010-C1
Cycloheximide	Sigma-Aldrich C7698
Protease inhibitor cocktail	Thermo Scientific 78425
RNAse Inhibitor	Ambion AM2696
Triton X-100	Sigma T8787-50mL
Sodium Deoxycholate	Sigma-Aldrich D6750

Buffers

10x Sucrose Gradient Buffer-	200 mM HEPES (pH 7.6) 1 M KCl 50 mM MgCl2 100 μg/ml cycloheximide 1x protease inhibitor cocktail (EDTA-free) 100 units/ml RNase inhibitor
Hypotonic Buffer-	5 mM Tris-HCl (pH 7.5) 2.5 mM MgCl2 1.5 mM KCl 1x protease inhibitor cocktail (EDTA-free)

Gradient Preparation

1) Prepare 50 ml of 60% sucrose solution in MilliQ water and 10ml of 10x sucrose gradient buffer. Filter solutions with 0.22 μm filter.
2) Prepare 10-50% stock sucrose solutions using 60% sucrose.

Final (%)	60% Stock (mL)	water (mL)	10X sucrose gradient
50	8.3	0.7	1
45	7.5	1.5	1
40	6.7	2.3	1
35	5.8	3.2	1
30	5.0	4.0	1
25	4.2	4.8	1
20	3.3	5.7	1
15	2.5	6.5	1
10	1.7	7.3	1

3) Add 1.2 mL of the 50% sucrose solution to a Beckman tube. Flash freeze the gradient in liquid nitrogen or place in -80°C for one hour. Add 1.2mL of the 45% sucrose solution and repeat flash freeze. Continue with each layer until each layer has been flash frozen.
4) Cover tube with parafilm and store at -80°C until use.

RNA Preparation

1) Seed cells into 3 15-cm Petri dishes at 50-60% confluency 18-24 hours prior to transfection.
2) Transfect cells with 12ug DNA.
3) Lyse 24 hours post transfection. Before lysing, incubate cells with cycloheximide at a final concentration of 100 µg/ml for 5 min at 37 °C and 5% CO2.
4) Wash cells twice with 10 ml of ice-cold 1x PBS containing 100 µg/ml cycloheximide.
5) Scrape cells in 5 ml of ice-cold 1x PBS containing 100 µg/ml cycloheximide and collect in a 50 ml tube.
6) Collect cells by centrifugation at 1200rpm for 5 min at 4°C.
7) Discard supernatant and re-suspend cells in 425 µl of hypotonic buffer .
8) Add 5 µl of 10 mg/ml cyclohexamide, 1 µl of 1M DTT, 100 units of RNAse inhibitor and vortex for 5 secs.
9) Add of 25 µl of 10% Triton X-100 (final concentration 0.5%) and 25 µl of 10% sodium deoxycholate (final concentration 0.5%) and vortex for 5 secs.
10) Centrifuge lysates at 14,000 rpm for 15 mins at 4°C and transfer supernatant to a new pre-chilled 1.5 ml tube.
11) Measure OD at 260 nm for each sample using a spectrophotometer.
12) Immediately load RNA onto gradients.

Polysome Fractionation

1) Transfer ultracentrifuge tubes containing sucrose gradients into pre-chilled rotor buckets.
2) Load equal OD 260nm of lysates (10-20 OD). Adjust lysates so that they contain the same OD in 500 µl of lysis buffer and load onto each sucrose gradient.
3) Weigh and balance each gradient before the ultra-centrifugation.
4) Centrifuge at 30,000 rpm, for 4 hr at 4 °C using SW41Ti rotor.
5) When 30 minutes of spin are left, switch on UV lamp.
6) Switch on pump and fraction collector. Fill the tubing with the chasing solution (60% (w/v) sucrose containing 0.02% (w/v) bromophenol blue) until it reaches the needle. Make sure to see at least one drop coming out of the needle, and ascertain that no bubbles are introduced in the pump syringe or tubing.
7) Place the gradient and pierce the tube with the needle by twisting the knob below the tube holder.
8) Set the pump at 1.5 ml/min and collect the fractions. Collect 20 drops per fraction. (~ 500 µl in each fraction for 18 fractions).
9) Stop collecting as soon as the first drop of chasing solution falls in a 2 ml collecting tube.

Immunofluorescence

Reagents

PBS	MultiCell 311-010-C1
Paraformaldehyde	Sigma-Aldrich P6148-500G
Triton X-100	Sigma T8787-50mL
DAPI	Vector H-1200

1) Grow cells on slide.
1) Wash cells 2X with cold PBS.
2) Fix cells with 4% paraformaldehyde in PBS for 10 min at RT.
3) Wash cells 2X with PBS.
4) Permeabilize cells with 0.3% Triton-X in PBS.
5) Block cells with 2% BSA and 0.02% sodium azide in PBS at room temperature for 1 hour.
6) Incubate cells with primary antibody (1:100 – 1:500) at 4°C overnight.
7) Wash cells 3X with PBS.
8) Incubate cells with appropriate TRITC/FITC-conjugated secondary antibody (1:500) for 1 hour at room temperature.
9) Wash cells 3X with PBS.
10) Add one drop of DAPI mounting media and place coverslip on slide.
11) Allow 1 hour at room temperature of DAPI media to dry.
12) Observe slide using Zeiss LSM 700 or store slides in dark at 4^{o}C.