NEW ROLES IN RNA STABILITY AND TRANSCRIPTION
FOR THE RNA EXPORT PROTEIN, REF/ALY

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NEW ROLES IN RNA STABILITY AND TRANSCRIPTION
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Pre-mRNA processing consists of a series of events including capping, splicing, and polyadenylation, which occur co-transcriptionally, are interdependent, and are coupled to other steps in gene expression including transcription and RNA export. For example, in mammalian cells the TREX (transcription-export) complex is deposited on an mRNA in a splicing-dependent fashion, leading to more efficient mRNA export. In yeast, TREX is additionally implicated in RNA quality control pathways. Using factors derived from the Kaposi's sarcoma-associated herpesvirus (KSHV), we established a role for the TREX component, REF/Aly, in nuclear RNA stability. We found that REF/Aly is recruited to the KSHV nuclear noncoding PAN RNA by the viral protein, ORF57, and this recruitment

correlates with ORF57-mediated stabilization of PAN RNA. Additionally, REF/Aly is sufficient to increase the nuclear abundance and half-life of PAN RNA, but is not sufficient to promote its export. REF/Aly appears to protect the poly(A) tail from deadenylation and REF/Aly-stabilized transcripts are further adenylated over time, consistent with previous reports linking poly(A) tail length with nuclear RNA surveillance and supporting a broader conservation of RNA quality control mechanisms from yeast to human. Pre-mRNA processing is also mechanistically coupled to transcription with RNA pol II serving as a platform to recruit RNA processing factors to nascent transcripts. Using an RNA-Seq approach, we suggest a role for REF/Aly in transcription in addition to its well-defined role in RNA export. RNA-Seq analysis identified a subset of transcripts with decreased expression in both nuclear and cytoplasmic fractions upon REF/Aly knockdown. Interestingly, we find that REF/Aly does not affect the stability of candidate transcripts, however, REF/Aly depletion affects pol II density on target genes, implying a role for REF/Aly in pol II recruitment. Furthermore, REF/Aly binds directly to candidate transcripts, supporting a direct effect of REF/Aly on candidate gene transcription. Our data are consistent with the model that REF/Aly is involved in linking splicing with transcription efficiency. Taken together, our data suggest that the importance of REF/Aly is not limited to RNA export, but that REF/Aly is also critical for gene expression at the level of RNA stability and transcription.

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Stubbs, S. H., O. V. Hunter, A. Hoover, and N. K. Conrad. 2012. Viral factors reveal a role for REF/Aly in nuclear RNA stability. Molecular and cellular biology **32:**1260-1270

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4SU	4-thiouridine
ACTB	β-actin
ActD	Actinomycin D
bZIP	basic region-leucine zipper
DEG	Differentially expressed gene
CAR	Cytoplasmic Accumulation Region
CAR-E	Cytoplasmic Accumulation Region element
CBC	Cap Binding Complex
ChIP	Chromatin Immunoprecipitation
CPSF	Cleavage/polyadenylation specificity factor
CTD	C-terminal domain
EBV	Epstein-Barr virus
EJC	Exon Junction Complex
ENE	expression and nuclear retention element
EU	5-Ethynyl Uridine
GTFs	General transcription factors
H3K4me3	Histone 3-Lysine 4 trimethylation
H3K36me3	Histone 3-Lysine 36 trimethylation
hCMV	human cytomegalovirus
HIV	Human immunodeficiency virus
HPV	human papilloma virus

HSV	herpes simplex virus
kb	kilobase
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
MCD	multicentric Castleman's disease
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein
NMD	Nonsense-mediated decay
ORE	ORF57-responsive element
PAN RNA	Polyadenylated nuclear RNA
PAP	Poly(A) Polymerase
PCA	phenol:chloroform:isoamyl alcohol (25:24:1)
PEL	primary effusion lymphoma
PIC	Pre-initiation complex
Pol II	RNA Polymerase II
qRT-PCR	quantitative real-time polymerase chain reaction
RBP	RNA binding protein
ReBD	REF binding domain
RQ	relative quantity
RRM	RNA recognition motif
siRNA	small interfering RNA
snRNP	small nuclear ribonucleoprotein

TCRα	T cell receptor α
Tet	Tetracycline
TetRP	Tetracycline-responsive promoter
TREX	Transcription-export complex
UIF	UAP56-interacting factor
WT	wild-type

CHAPTER ONE

Introduction

In the eukaryotic cell, gene expression is the result of several events including transcription, RNA processing, and RNA export. In the nucleus, a pre-mRNA is produced through transcription by RNA polymerase II (pol II). The pre-mRNA is subject to extensive processing including capping, splicing, and polyadenylation where various protein complexes are deposited onto the RNA to produce a mature messenger ribonucleoprotein (mRNP). Once a mature mRNP has been formed, it is exported from the nucleus to the cytoplasm, where it is used as a template for translation. Integrity of the mRNA is monitored through RNA quality control mechanisms that prevent the export of misprocessed and aberrant mRNPs. Not surprisingly, these events are coupled in the cell through both physical and functional interactions.

Coupling of these events allows for more efficient gene expression, and the importance of coupling is underscored by the fact that pre-mRNA processing is more efficient when coupled to transcription, compared to processing of synthetic full-length pre-mRNAs (8). The RNA polymerase II (pol II) C-terminal domain (CTD) is well suited to facilitate coupling because it is reversibly phosphorylated allowing for coordinated recruitment of RNA processing factors (43, 56). Additionally, coupling allows RNA processing factors to be located close to the elongating polymerase, raising the local concentration of processing factors, and thus allowing more efficient processing of the RNA (6).

In order for a gene to be properly expressed, a pre-mRNA must first be transcribed from its DNA template with pol II. A 5′ cap is added to the nascent RNA emerging from the polymerase, and the cap-binding complex (CBC) is recruited to the 5′ end. The pre-mRNA continues to be processed co-transcriptionally, and introns are removed via splicing and the maturing RNA begins to be decorated with a series of proteins. The exon junction complex (EJC) is recruited in a splicing dependent manner and is deposited ~20 nucleotides (nt) upstream of the exon-exon junction (86) . The EJC and the cap-binding complex promote recruitment of the TREX (transcription-export) complex at the 5´-most exon (40, 145, 146, 171) (22). TREX deposition results in recruitment of TAP/NXF1, which along with its partner, p15, facilitates export of the mature transcript to the cytoplasm (44, 70).

Like other RNA processing events, mRNA export from the nucleus to the cytoplasm is coupled to other steps in mRNA synthesis. RNA export is mediated by a repertoire of proteins that comprise a protein complex called the TREX complex. The TREX complex is recruited to the 5′ end of nascent RNA in a splicing dependent manner (22, 111). Although TREX is conserved in yeast, recruitment is dependent on transcription and 3′ end formation rather than splicing (50, 78, 87). One component of the TREX complex is the RNA export adaptor, REF, also called Aly. REF/Aly binds the RNA and recruits TAP/NXF1 to the mature mRNP so that TAP/NXF1 can export the RNA. REF/Aly is conserved from yeast to humans, and the yeast REF/Aly, Yra1, is essential for bulk mRNA export (145, 149) . Although REF/Aly is now a well-established RNA export factor, it was first implicated in transcription enhancement (14, 160). Because nuclear events in gene expression are coupled, it makes sense for proteins involved in these processes to be multifunctional thus allowing

for more efficient usage of proteins. REF/Aly appears to be a good candidate for a multifunctional protein because it is recruited co-transcriptionally and interacts with the elongating polymerase as well as with the TREX complex (145, 146, 166). Based on these observations of REF/Aly, we wanted to further define roles of REF/Aly and determine if this RNA export protein is in fact a multifunctional protein that works at several steps in mRNA biogenesis.

The work performed in these studies reveals additional roles for REF/Aly aside from its well-established role in RNA export. We found that REF/Aly is involved in nuclear RNA stability as well as transcription. Specifically, we found that REF/Aly stabilizes the viral RNA, PAN RNA, a Kaposi's sarcoma-associated herpesvirus nuclear non-coding RNA, in a fashion that is separable from its role in mRNA export. Specifically, we determined that REF/Aly is recruited to PAN RNA by the viral protein, ORF57, and that REF/Aly binds directly to the 5′ end of the transcript. Additionally, REF/Aly is both necessary and sufficient to increase PAN RNA stability. Abrogation of the REF/Aly:ORF57 interaction inhibits ORF57-mediated stabilization of PAN RNA, demonstrating that increases in PAN RNA are a result of stabilization by REF/Aly. REF/Aly is sufficient to stabilize PAN RNA when it is artificially tethered to the RNA, and increased stability is not caused by export of the RNA to the cytoplasm. Taken together, our data suggest a role for REF/Aly in nuclear RNA stability and suggests that RNA export factors are able to protect RNA from degradation in the nucleus.

Our studies using viral factors revealed that REF/Aly is capable of stabilizing at least one RNA, and we were interested to determine if REF/Aly affects cellular RNAs early in

their biogenesis, upstream of its function in RNA export. Using an RNA-Seq approach, we identified transcripts whose levels were altered upon REF/Aly depletion. While transcripts were seen to be both up and down regulated, we chose to focus on a group of transcripts that were down regulated in nuclear and cytoplasmic fractions when REF/Aly was depleted. We reasoned that transcripts whose expression was decreased in both fractions might be transcripts that were affected by REF/Aly upstream of mRNA export. Our experiments revealed that REF/Aly depletion affects the steady-state levels of transcripts identified in the RNA-Seq, and that REF/Aly binds directly to those transcripts. Newly transcribed bulk poly(A) RNA levels did not seem to be affected in the absence of REF/Aly, supporting a role for REF/Aly in a specific group of transcripts. Using metabolic labeling assays, chromatin immunoprecipitation (ChIP) assays, and examining decay rates of transcripts, we determined that REF/Aly does not influence the stability of candidate genes but that it is important for the recruitment of poll II to genes.

Taken together, these studies show that the importance of REF/Aly is not limited to RNA export, but that REF/Aly is also critical for gene expression at the level of transcription and RNA stability. In agreement with other studies, our work suggests that gene expression events are intimately connected, and suggest a role for REF/Aly in linking transcription efficiency with splicing.

CHAPTER TWO

Review of the Literature

Gene Expression

In the eukaryotic cell, nuclear events in gene expression are intimately coupled. Effective synthesis of an mRNA requires several steps including transcription, RNA processing, and RNA export. Processing of a pre-mRNA involves addition of a 5′ cap, intron removal via splicing, and 3′ end formation including the addition of a poly(A) tail (Figure 1). A great deal of communication between these processes occurs, and it is not surprising that many RNA processing events actually happen co-transcriptionally. Interestingly, pre-mRNA processing has been demonstrated to be more efficient when coupled to transcription, compared to processing of a full-length, synthetic pre-mRNA (8). Processing of a pre-mRNA, uncoupled from transcription can take greater than 20 minutes, whereas processing coupled to transcription can occur within 30 seconds of the polymerase reaching the end of the gene (6, 164). Coupling between transcription and processing is advantageous for several reasons. Coupling increases the local concentration of mRNA processing factors at the site of transcription, positioning them close to the nascent transcript. Additionally, the rate of transcript elongation can greatly influence RNA folding, the assembly and recruitment of RNA binding proteins, and can affect alternative splice site choice (35, 64). Finally, coupling between processes results in interactions of processing factors and the pol II elongation complex, allowing the elongation complex to allosterically activate or inhibit recruitment and activities of mRNA processing factors (6, 60). Following processing, if a

mature mRNP is formed, the RNA is exported from the nucleus to the cytoplasm for translation. If an export incompetent mRNP is formed, it is retained in the nucleus and degraded by RNA quality control machinery.

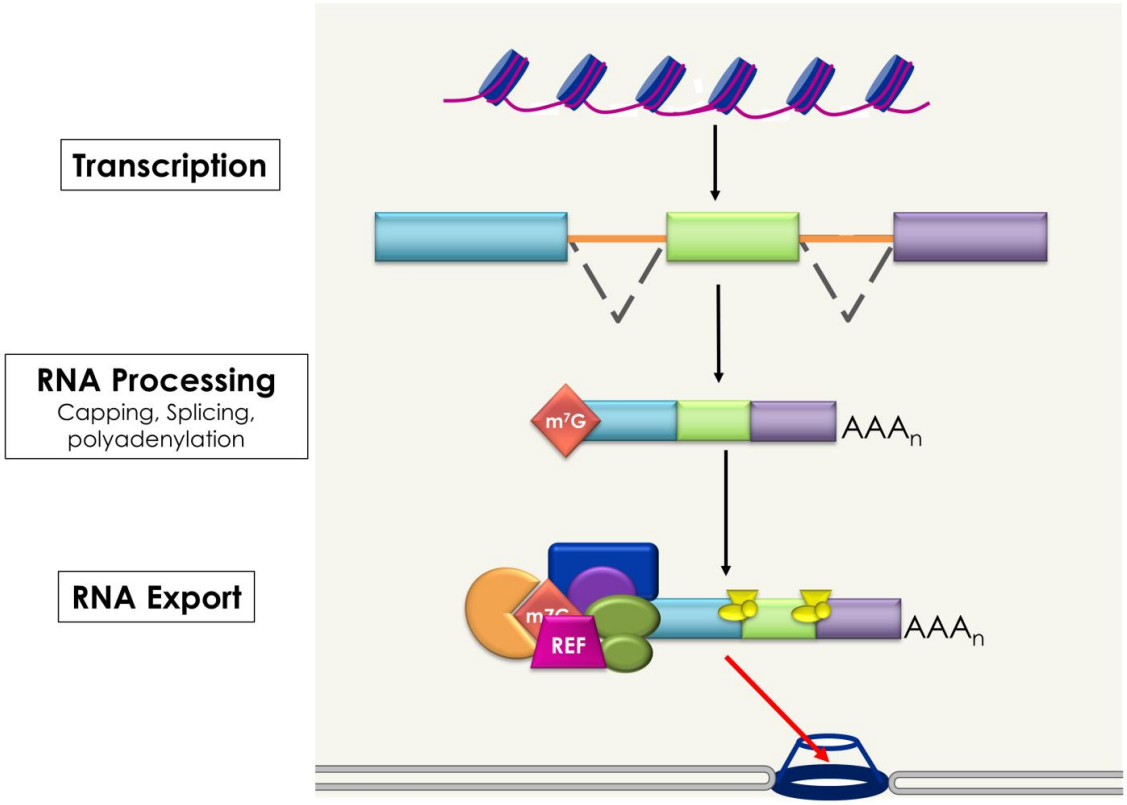

Figure 1. Nuclear Events in Gene Expression. Effective production of a mature messenger RNA (mRNA) involves a cascade of events. This includes transcription by RNA polymerase II to produce a pre-mRNA that contains introns (denoted by orange lines) and exons (denoted by boxes). As the RNA is transcribed, the nascent transcript is capped at the 5′ end (green diamond), and the spliceosome (not illustrated) is recruited to excise introns and join together exons. Splicing recruits the Exon Junction Complex (EJC) which is deposited ~20 nt upstream of the exon junction (yellow shapes). As the transcript is terminated, the 3′ end is cleaved and polyadenylated. Additionally, splicing triggers recruitment of the TREX complex to the 5′ end of the RNA (colored shapes). Once an export competent mRNP is formed, the export receptor TAP/NXF1 interacts with the TREX complex and carries the transcript to the nuclear pore (blue basket) to be exported to the cytoplasm. Note that while gene expression events are drawn sequentially, these events actually occur co-transcriptionally.

Transcription

RNA Polymerase II and the CTD

RNA Polymerase II (pol II) is composed of 12 proteins and transcribes all protein-coding genes (messenger RNAs, mRNA), as well as some non-coding RNAs in eukaryotes. The largest subunit of pol II, RPB1 contains a C-terminal domain (CTD) that helps to coordinate many transcription and processing activities (13). The CTD acts as a recruitment platform for transcription and processing proteins, allowing for integration of processing events throughout the transcription cycle (61, 65). The CTD is conserved from fungi to humans, and the mammalian CTD is composed of 52 heptad repeats, 21 that are consensus, and 31 that are non-consensus (13, 19, 31, 98). The consensus sequence of the pol II heptad is N-Tyr 1-Ser 2-Pro 3-Thr 4-Ser 5-Pro 6-Ser 7-C, and non-consensus heptads can vary at one or more positions. Truncation of the CTD results in reduced cell viability (5, 97, 163) and severe defects in 3′-end processing and splicing (112, 113). The CTD is reversibly modified throughout the transcription cycle, and these modifications are critical for coordinating RNA processing proteins and transcription-coupled modifications (43, 56, 65). Although phosphorylation of the CTD has been studied the most extensively, other modifications including glycosylation, ubiquitinylation, and methylation have also been observed (81, 143).

In order for a gene to be transcribed, hypophosphorylated pol II is recruited to the promoter along with general transcription factors (GTFs), forming the preinitiation complex (PIC). In order to escape from the promoter, pol II is phosphorylated on Serine-5 by the kinase CDK7, a component of TFIIH, part of the PIC. Phosphorylation of Serine-5 on the

CTD allows the polymerase to escape the promoter and enter into abortive transcription, where the polymerase falls off of the gene and must reinitiate, or the elongation phase. Approximately 10-50 nucleotides downstream of the start site, pol II reaches intrinsic pause sites, where it is stopped by negative elongation factors (13). Phosphorylation of Serine-2 by CDK9 (PTEF-b) is required to bypass the pause site and continue into productive elongation throughout the body of the gene. Therefore, as seen in Figure 2, a general model emerges for CTD phosphorylation where Serine-5 phosphorylation is enriched at the beginning of the gene, and decreases throughout the gene body while phosphorylated Serine-2 increases in the gene body and toward the end of the gene (13, 65). While these patterns represent the general trend for Serine-2 and Serine-5 phosphorylation, they are not observed at every gene. Other residues, including Serine-7 and Threonine-4 can be phosphorylated, however, their significance and prevalence has been less well studied (56).

Histone Modifications and Chromatin Remodeling

Modification of the pol II CTD also recruits factors that result in histone modifications and chromatin remodeling. Posttranslational modifications to histones include methylation, phosphorylation, ubiquitination, acetylation, ADP-ribosylation, and sumoylation of lysine. Certain modifications are present during active transcription, such as trimethylation of lysine 4 on histone 3 (H3K4me3) and H3K36 trimethylation (H3K36me3), and are referred to as euchromatin modifications (Figure 2). Conversely, histone modifications such as H3K9 or H3K27 methylation are localized to inactive gene regions and are commonly referred to as heterochromatin modifications (91). For the work presented

here, we will focus on the euchromatin modification H3K36me3. Multiple methylases have been identified to mono- or dimethylate H3K36. Trimethylation of H3K36 however is carried out by the specific enzyme SetD2, an essential enzyme that interacts with Serine-2 phosphorylated pol II (151). Because of this, H3K36me3 is found preferentially at the 3′ end of genes (3). Important for these studies, H3K36me3 marks have been linked to RNA processing factors and intron-exon boundaries (63, 66, 83). Additionally, the pol II-interacting protein, Iws1, which is required for H3K36me3 on some genes, binds to the TREX complex member REF/Aly (166, 167).

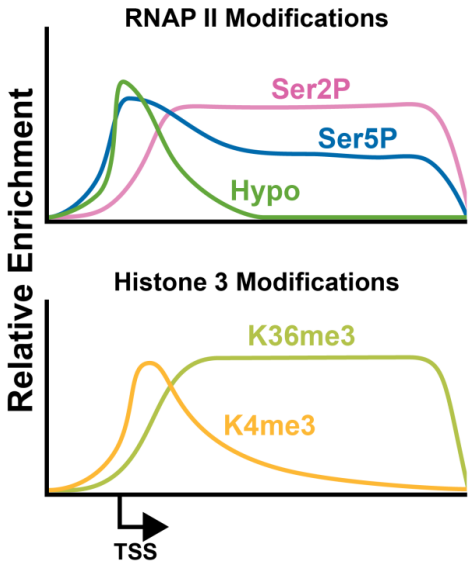

Figure 2. RNA pol II and Histone 3 modifications. Generally, both pol II and H3 posttranslational modifications exhibit a pattern across each gene. Hypophosphorylated pol II is found primarily at the 5′ end of the gene and peaks near the TSS (dark green). Ser-5 becomes phosphorylated at the switch to elongation, peaks close to the TSS and decreases toward the 3′ end of the gene (blue line). Conversely, Ser-2 phosphorylation peaks after the TSS and is maintained to the 3′ end (purple line). Similar to pol II, H3 modifications exhibit a pattern where H3K4me3 peaks at the 5′ end of the gene (yellow line) and H3K36me3 peaks in the gene body and is maintained until the end of the gene (light green line). Modified from Brookes and Pombo, 2009.

RNA Processing

As soon as nascent RNA emerges from the polymerase it is coated with RNA binding proteins (RBPs) that aid in various steps in processing. While many RBPs have been well characterized, two recent studies identified more than 700 RBPs, with a large number having no previous association as an RNA binding protein (2, 17, 119). Numerous RBPs act as processing factors and are recruited co-transcriptionally, many through interaction with the pol II CTD. For the RNA to be processed into a mature transcript, it must undergo capping, splicing, and 3′-end processing. An mRNA must be fully processed in order to be export competent, and this process involves many protein complexes such as the EJC and TREX complex. Following processing, the mature mRNP is able to be transported to the cytoplasm for translation.

Capping

The first step in pre-mRNA processing is the addition of a 5′ 7-methyl guanosine cap. In mammalian cells, the γ-phosphate is first removed from the terminal nucleotide with an RNA triphosphatase. Next, guanylyltransferase transfers GMP to the first nucleotide forming an unusual 5′-5′ triphosphate linkage. Finally, a methylation at the N7 position of the transferred GMP occurs via 7-methyltransferase. In humans the RNA triphosphatase and guanylyltransferase activities are carried out by a single polypeptide while the methyltransferase is encoded by a separate polypeptide. In yeast, the RNA triphosphatase and guanylyltransferase activities are carried out by two different enzymes, Cet1 and Ceg1, respectively (61). As the RNA begins to emerge from the polymerase, the CTD helps to

recruit the capping machinery to add the cap to the 5′ end of the RNA (4). Serine-5 phosphorylation of the CTD aids in recruitment of the capping machinery, and deletion of the CTD results in highly inefficient capping of transcripts (23, 112, 168). Capping is tightly coupled to early events in transcription through interaction of capping enzymes to the CTD and loss of Serine-5 phosphorylation is correlated with release of the capping enzyme (61). The requirement of Serine-5 phosphorylation ensures that only transcripts that are being productively elongated become capped. It is also thought that capping enzymes may help to relieve transcriptional repression, suggesting an added layer of regulation during transcription, and further evidence for coupling between the processes (108). The cap binding complex (CBC) is recruited to the capped RNA and binds to the 7-methyl guanosine cap, and is composed of two subunits, CBP20 and CBP80. Additionally, the CBC is thought to help protect the mRNA from nuclear 5′-3′ exonucleases (130).

Splicing

As the transcript continues to emerge from the polymerase, introns begin to be removed by co-transcriptional splicing. Splicing is carried out by the multi-subunit complex, the spliceosome. The spliceosome is composed of the small nuclear RNPs (snRNPs) U1, U2, U4, U5, and U6 as well as additional proteins. Removal of introns is a critical step in maturation of an mRNA for several reasons. Perhaps most importantly, introns disrupt coding regions, and it is therefore necessary to remove them to allow proper protein expression. Additionally, introns quite often contain premature stop codons which if exported and translated would result in truncated proteins. Furthermore, splicing recruits

important protein complexes such as the Exon Junction Complex (EJC), which is necessary for downstream maturation and translocation of the mRNP. Moreover, splicing greatly contributes to protein diversity. It is thought that 92-94% of human transcripts are alternatively spliced. This allows one gene to encode more than one protein, which significantly adds to the diversity of the proteome while optimizing the genome (61).

Coupling between splicing and transcription is supported by several lines of evidence. First, pol II has been shown to interact with SR (Serine/Arginine) splicing factors and components of the splicing machinery (82, 118, 169). Second, truncation of the pol II CTD results in low splicing efficiency in vivo (113), and exogenous expression of CTD peptides or addition of anti-CTD antibodies results in an accumulation of unspliced transcripts (169). Third, splicing is considerably less efficient in vitro when uncoupled from transcription (8, 164). Alternative splicing is also thought to be kinetically coupled to transcription through changes in pol II elongation rates. A good example of this is the fibronectin EDI exon studies performed by de la Mata et al. Using a mutant pol II (C4 pol II) that is less processive and cannot efficiently read through intrinsic elongation blocks, they showed that when the elongation rate of the polymerase is lower, an alternative exon is more likely to be included (35).

Splicing triggers recruitment of the EJC, which is deposited approximately 20-24 nucleotides upstream of exon-exon junctions (86), and marks previous splice events. Furthermore, the EJC aids in efficient RNA export since it recruits the TREX complex. Direct comparison of two RNAs, one that had a 5′ exon long enough to bind an EJC and one 5′ exon that was too short to bind an EJC, revealed that the longer 5′ RNA that was able to

bind the EJC was more efficiently exported (85). Additionally, all core components of the EJC are involved in nonsense mediated decay (NMD) (9). The EJC stays associated with the RNA into the cytoplasm where it is aids in downstream processes including the pioneer round of translation (61).

3′-end Formation

Formation of the 3′ end involves cleavage of the transcript and addition of a poly(A) tail. The cleavage machinery, particularly CPSF (Cleavage/polyadenylation specificity factor), recognizes the polyadenylation signal AAUAAA, and endonucleolytically cleaves the transcript 10-30 nucleotides downstream of the signal. In addition to the AAUAAA sequence element, a G/U rich sequence called the downstream sequence element (DSE) is recognized by cleavage stimulating factor (CtsF). CPSF and CtsF along with cleavage factor I (CFI) and CFII, promote efficient cleavage of the pre-mRNA. Once the transcript has been cleaved it is polyadenylated at the 3′ end by the poly(A) polymerase (PAP) which adds ~200-250 adenosines in a template independent manner. In the cytoplasm the poly(A) tail is important for stability of the RNA, as it protects the transcript from decay, and it is important for translation efficiency (47). Emerging evidence also suggests that the poly(A) tail plays an important role in RNA metabolism in the nucleus. For example, poly(A) tails have now been shown to stimulate degradation of the transcript in the nucleus, and this is dependent on the poly(A) binding protein PABPN1 (12).

As with all other steps in maturation of the pre-mRNA, 3′-end formation is closely coupled to transcription and the CTD. Like splicing, truncation of the CTD results in

defective transcription termination (113). However, purified pol II and the CTD are able to stimulate cleavage activity, even in absence of transcription (59), and several 3′ end formation proteins are associated with the CTD (130). Similar to capping, the phosphorylation status of the CTD is important for directing 3′ end formation. The yeast CFI subunit, Pcf11 preferentially binds to serine 2 phosphorylated CTD, and yeast strains that are defective in Serine-2 phosphorylation show decreased crosslinking of 3′ formation factors to the end of the gene (1, 94).

Coupling between RNA processing steps

As implied above, coupling of RNA processing steps to transcription is widespread. In addition to coupling to transcription, many RNA processing events are linked to one another. The addition of a 5′ cap increases splicing efficiency in mammalian cells, and depleting the CBC results in decreased splicing efficiency (61, 71). Additionally, the CBC is thought to enhance the cleavage reaction (130), demonstrating a role for coupling between capping and splicing or 3′ end formation. Furthermore, the TREX complex interacts with the CBC, supporting a role for coupling between capping and export (79). Splicing and polyadenylation also appear to be coupled through the splicing factor U2AF65, which interacts with the poly(A) polymerase (PAP) (122, 123, 158). As seen in Figure 3, coupling between RNA processing steps and coupling of RNA processing to transcription allow for more efficient gene expression and lead to the idea of an "mRNA factory," where each step in gene expression is not independent, but rather a smaller part to a large machine that results in the production of a mature mRNP.

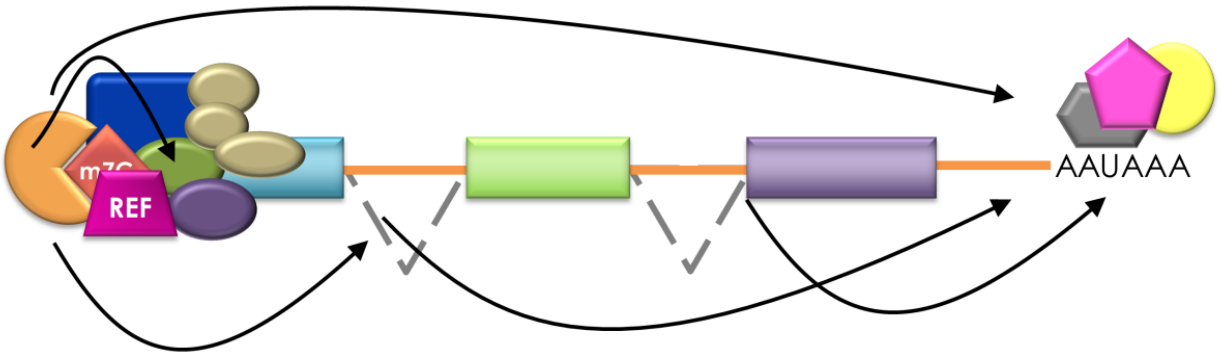

Figure 3. Coupling of RNA processing. Coupling between capping, splicing, and 3′ end formation all allow more efficient recruitment of protein complexes as well as more efficient processing of the RNA. For example, capping and the CBC enhance splicing efficiency as well as 3′ end formation.

RNA Export

Export of RNA from the nucleus to the cytoplasm involves a series of steps including transcription, assembly of the mature mRNP, localization of the mRNP to the nuclear pore, and translocation to the cytoplasm for translation. While multiple export pathways exist to export different classes of RNAs, most mRNAs are exported in a TAP/NXF1 dependent pathway, which will be the focus of these discussions.

Transcription-Export (TREX) Complex

The major protein complex responsible for export of the mature mRNA is the Transcription-Export, or TREX Complex. Like other steps in RNA processing, recruitment of export factors is coupled to steps in RNA processing and transcription. TREX is a highly conserved, multi-protein complex consisting of REF/Aly, UAP56, CIP29, and the THO

complex (Hpr1, TEX1, Thoc2, Thoc5, Thoc6, Thoc7) as seen in Figure 4. Recently, several additional putative TREX complex members were identified that appear to be unique to the mammalian TREX complex. They include ZC11A, PDIP3 and Chtop (18, 45).

Much of the information known about the TREX complex was first discovered from studies using *Saccharomyces cerevisiae*. Although the yeast TREX complex contains fewer proteins, many are conserved from yeast to humans (see Table 1). The yeast TREX complex consists of Yra1 (REF/Aly), Sub2 (UAP56), Tex1, and the THO complex (Tho2, Hpr1, Mft1, and Thp2) (16). A similar protein to the human CIP29 protein, Tho1, exists in yeast, and has been shown to function in mRNA export and mRNP biogenesis. However, Tho1 was not originally identified as a THO/TREX component (76, 129). Because most yeast genes lack introns, TREX recruitment is linked with transcription and 3′ end formation rather than splicing and capping (50, 78, 87). Thus, while TREX recruitment in yeast is directly linked with transcription, TREX recruitment in humans is indirectly linked with transcription through splicing.

In mammalian cells, the TREX complex is recruited to nascent RNA in a capping and splicing dependent manner (22, 111). Two TREX complex members, UAP56 and REF/Aly co-localize with splicing factors in nuclear speckle domains (175). In fact, studies from the Reed lab suggested that splicing actually occurs within speckles, and that UAP56 and REF/Aly aid in the release of transcripts from speckles for export to the cytoplasm (36). However, a more recent study using FRET argues that assembly of TREX members with TAP/NXF1 occurs outside of speckles even though TREX components are found to be localized to speckles (153). In either case, EJC deposition near splice junctions, and binding

of the CBC results in recruitment of the TREX complex to the 5′ end of the RNA (22, 40, 145, 146, 171). Based on this model, the TREX complex is thought to be poorly recruited to transcripts lacking either a 5′ cap or the EJC (22, 175). This presents a problem for naturally intronless genes; however, recent work has identified alternative mechanisms of TREX recruitment that will be discussed in a later section.

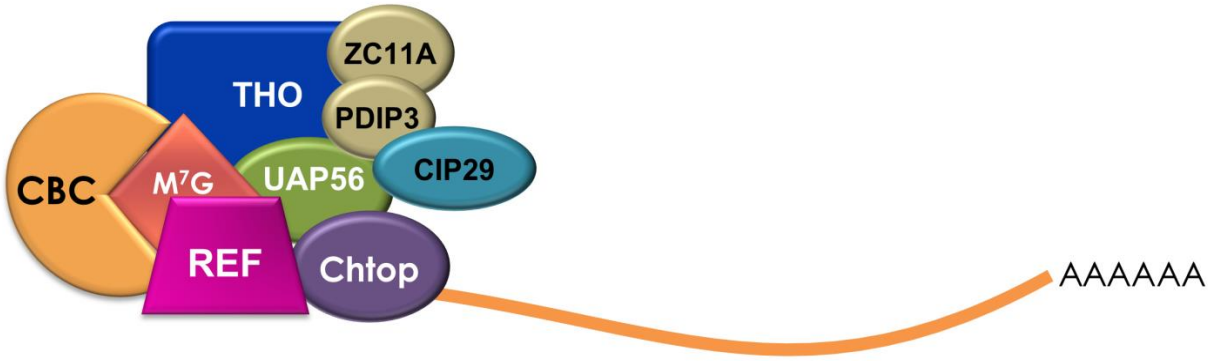

Figure 4. The Mammalian TREX Complex. The mammalian TREX complex is composed of REF/Aly, UAP56, CIP29, and the THO complex. Newer studies have expanded the repertoire of mammalian TREX members to include Chtop, PDIP3, and ZC11A although their functions remain less well characterized. UIF, a redundant export adaptor can also be found in the TREX complex but its recruitment to TREX requires the histone chaperone FACT.

Once a pre-mRNA is capped and EJCs have been deposited, the THO complex and the RNA helicase UAP56 are recruited to the 5′ end of the pre-mRNA. In an ATP-dependent manner, UAP56 then assembles with CIP29 and REF/Aly (40). REF/Aly binds directly to the CBC component CBP80 and UAP56, with UAP56 serving to bridge the interaction between REF/Aly and the THO complex. Therefore, REF/Aly is closest to the cap and

UAP56 and THO are found further away from the 5′ end (111). Upon completion of transcription and processing, the mRNP is ready to be exported. REF/Aly recruits the export receptor TAP/NXF1 and its partner p15/NXT1. TAP recruitment to the RNA results in displacement of UAP56, the RNA is "handed off" from REF/Aly to TAP which is facilitated by arginine methylation of REF/Aly (54, 67). The RNA binding site of REF/Aly overlaps with the TAP interaction domain, and REF binding to TAP stimulates TAP's RNA binding activity (54). TAP and p15 then carry the mature message to the nuclear pore where it interacts with nuclear pore proteins that facilitate export to the cytoplasm (44, 70).

Human TREX Component	Alternative Name	*S. cerevisiae* Ortholog	*Drosophila* Ortholog
UAP56	BAT1, DDX39B	Sub2	UAP56
DDX39	URH49, DDX39A	Sub2	UAP56
REF/Aly	Ref, Alyref, Thoc4, Bef	Yra1	REF1
CIP29	HCC1, Tho1, Sarnp	Tho1	
UIF	FYTTD1		
Chtop	SRAG, CAO77 FOP		
PDIP3	SKAR, PolDIP3		
ZC11A	ZC3H11A		
THO			
Hpr1	Thoc1, p84	Hpr1	HPR1
Thoc2	Tho2	Tho2	THO2
Thoc5	fSAP79, Fmip		THOC5
Thoc6	fSAP35, WDR58		THOC6
Thoc7	fSAP24		THOC7
Tex1	Thoc3	Tex1	TEX1
		Mft1	
		Thp2	

Table 1. TREX Complex Components. Core members of the TREX complex are conserved from yeast to humans. However, metazoans possess several additional TREX components.

Splicing-independent recruitment of TREX

While most human pre-mRNAs contain introns, a small portion of pre-mRNAs (~5%) do not (142). This presents a problem for naturally intronless pre-mRNAs because the export machinery is conventionally thought to be recruited as a result of splicing. So far, two alternative mechanisms of TREX recruitment have been proposed. Recently, studies from the Reed laboratory have proposed an additional mode of recruitment of TREX members to intronless RNAs (88). Using three naturally intronless mRNAs (HSPB3, IFN-α1, and IFN-β1), they identified a region within the coding region of each mRNA termed a CAR element, or Cytoplasmic Accumulation Region that range from 162 to 285 nucleotides (nts). CARs conferred stability to the RNA and allowed accumulation of the transcript in the cytoplasm. Further work identified a 10 nt consensus element (CAR-E) that promotes cytoplasmic accumulation of intronless mRNAs (89). Mutation of the polyadenylation signal resulted in accumulation of the intronless mRNAs in the nucleus, consistent with other data demonstrating a role for 3′ end formation in RNA export. Depletion of the TREX components UAP56, THOC2, or TAP blocked the export of the intronless mRNAs (88). Furthermore, CAR-E RNA was shown to associate with the TREX complex components THOC2, THOC5, THOC6, THOC3, THOC1 and UAP56 (89). Taken together, these data support the hypothesis that at least some intronless mRNAs are capable of recruiting the TREX complex independent of splicing because they contain a CAR element. In further support of this hypothesis, the naturally intronless transcripts did not localize to nuclear speckle domains (88), which they propose is the location of splicing (36)

Another alternative mechanism of TREX recruitment has been proposed by the Jones lab. Yoh et. al demonstrated a direct link between transcription, RNA processing and export through the transcriptional elongation factor Spt6 (166). Specifically, their studies showed that Spt6 binds directly to Serine-2 phosphorylated pol II and that a mutant Spt6 that is unable to bind phosphorylated pol II results in transcripts with splicing defects that accumulate in the nucleus. When Iws1, an Spt6 binding protein, was depleted, similar defects were seen. Iws1 interacts with REF/Aly and is thought to facilitate the recruitment of REF/Aly to Spt6-dependent genes. Thus REF/Aly recruitment through Iws1 provides an alternative mechanism to recruit TREX to newly formed mRNPs.

REF/Aly

Of particular interest in these studies is the TREX component REF/Aly. Ref/Aly is the canonical RNA adaptor protein that binds nascent RNA and acts to recruit the export receptor TAP/NXF1. REF/Aly's importance is underscored by the fact that it is conserved from yeast to humans (145, 149). One REF gene exists in humans (REF/Aly/THOC4) and *S. cerevisiae* (Yra1), while multiple are found in *Drosophila melanogaster*, *Caenorhabditis elegans*, and mouse. REF/Aly is essential for cell viability in *Drosophila* and humans, although phenotypes among the two vary when REF/Aly is depleted. In both *Drosophila* and *C. elegans*, no significant block of export is observed upon REF/Aly depletion (48, 99). Conversely, in yeast, deletion of Yra1 prevents bulk mRNA export. Differences in phenotype are likely due to the presence of redundant factors in higher eukaryotes that are not present in yeast (161). Even among studies in human cells, poly(A) accumulation

phenotypes vary. Strong nuclear accumulation of poly(A) RNA is observed in some studies while others suggest a more modest retention of poly(A) RNA in the nucleus after knock down (38, 55, 80, 124). A redundant export adaptor, UIF has recently been identified, and is upregulated in the absence of REF/Aly (55). No work has been done demonstrating the relative expression of UIF among cell types, which may account for differences in observed phenotypes.

REF/Aly interacting proteins

REF/Aly is found in association with a variety of proteins. In fact, REF/Aly was first proposed to be involved in transcription based on the finding that it interacts with the transcription factors AML-1 and LEF-1 (14), as well as bZIP containing proteins (160). Additionally, REF/Aly interacts with the transcription elongation factor Spt6 though Iws1 (166), perhaps supporting an alternative mechanism for recruitment of RNA processing factors to nascent transcripts. As mentioned previously, REF/Aly also associates with the RNA processing and export proteins including the CBC member CBP80, UAP56, and TAP/NXF1.

REF/Aly domain structure

REF/Aly has three main domains, an N-variable region, a central RNA recognition motif (RRM), and a C-variable region, as seen in Figure 5. Among the REF protein family the N- and C-variable regions are flanked by REF-N and REF-C motifs that are around 12 and 17 amino acids, respectively. The N- and C-variable regions range from 0-95 amino

acids and vary based on species. The solution structure of the mouse Aly RRM has been determined, as well as the N-region and RRM of mouse REF2-1 (49, 128). Structural and biochemical studies revealed that REF/Aly associates with RNA through arginine-rich regions within the N- and C-terminal domains (amino acids 1-73 and 155-218, respectively), and through weak interactions with the RRM (amino acids 74-154) (49, 54). In addition, methylation of arginine in the arginine-rich regions promotes displacement of REF/Aly from RNA (67).

TAP/NXF1 interactions with REF/Aly are mediated through amino acids 16-36 and through weak interactions with the RRM (54). UAP56 binds both the N- and C-terminal peptides of REF/Aly, and deletion of both domains is necessary to abrogate UAP56 binding to REF/Aly (49, 55, 102). UAP56 and TAP/NXF1 binding domains of REF/Aly partially overlap, implying a mutually exclusive interaction of UAP56 and TAP/NXF1 with REF/Aly. This model is based on several lines of evidence. First, binding of TAP to REF/Aly causes displacement of UAP56 (54, 67). Second, in yeast, the UAP56 and TAP interaction domains of REF/Aly are overlapping, suggesting that these interactions are unlikely to occur at the same time (147). Third, based on structural data of REF/Aly, Golovanov et al. suggest that human UAP56 and TAP are unlikely to bind REF/Aly at the same time (49). Based on the fact that RNA, UAP56, and TAP interaction domains are in close proximity to one another, these studies support a mechanism where binding of RNA, UAP56, and TAP with REF/Aly occurs sequentially.

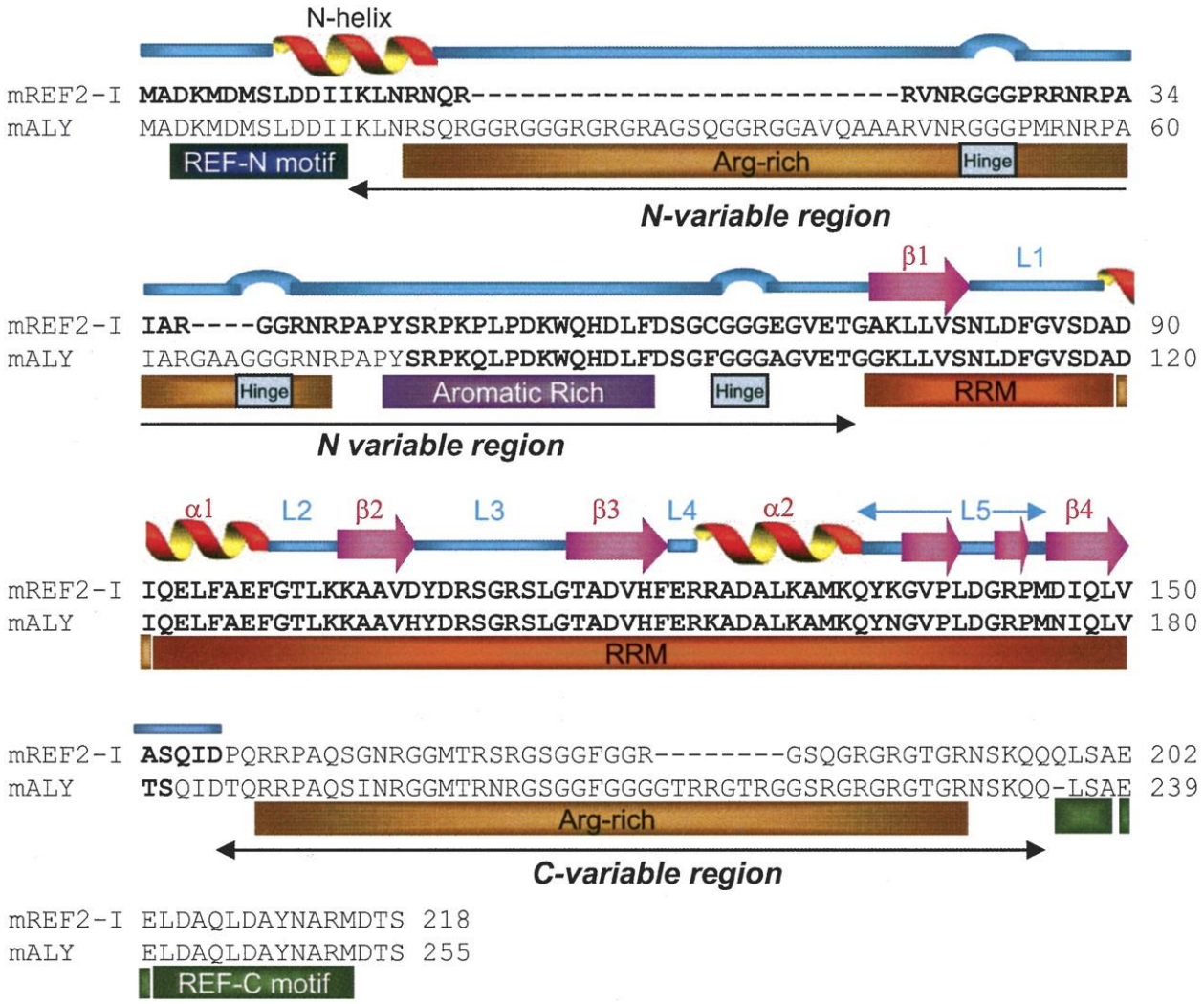

Figure 5. Primary and secondary structure of mouse Aly and REF2-1. NMR techniques were used to determine the structure of murine REF2-1. Human Aly aligns well with REF2-1 and both possess conserved N-and C-terminal domains (also called a REF-N and REF-C Motif), as well as an N-variable region, central RNA Recognition Motif, and a C-terminal region. Taken from Golovonov et al. 2006.

UIF, a redundant RNA export adaptor

UAP56-interacting factor, or UIF, was recently discovered as an export adaptor protein, similar to that of REF/Aly (55). UIF was identified by searching a basic local alignment search tool (BLAST) database to identify proteins that had peptides similar to the N-terminal REF/Aly peptide that interacts with UAP56. Interestingly, the UAP56-interacting region is the only conserved region between UIF and REF/Aly. UIF appears to be conserved among vertebrates, but no homolog has been identified in *C. elegans* or *Drosophila*. Similarly to REF/Aly, UIF is localized to the nucleoplasm and speckles, and binds in a mutually exclusive manner with UAP56 and TAP/NXF1. Knock down of REF/Aly or UIF does not significantly alter export or cell viability. However, double knock down of REF/Aly and UIF results in a dramatic export block and reduction in cell viability (55).

RNA Quality Control

In the nucleus, the integrity of a mature mRNP is maintained using RNA quality control (QC) pathways. Quality control processes ensure the destruction of RNAs that are unfolded, misprocessed, or that do not assemble into a mature ribonucleoprotein particle (RNP). Aberrant mRNPs arise from defects in splicing, polyadenylation, or misassembly of RNA associated complexes. RNA QC is well studied in yeast, but remains poorly characterized in humans. Defective RNAs can be exported to the cytoplasm for degradation, retained in the nucleus, or degraded in the nucleus (38). Degradation in the nucleus is carried out by the nuclear exosome in association with the nucleases Rrp6 and Dis3 (139). RNAs defective in splicing or 3′ end formation seem to be retained in the nucleus near the gene (32,

58, 74, 154) and can be hyperadenylated by the TRAMP complex, which stimulates degradation of the RNA by the exosome (38). Defects in RNA export factors that cause export blocks result in hyperadenylation of transcripts and retention at the site of transcription (58, 74, 135). Perhaps most interestingly, the yeast homolog of REF/Aly, Yra1 has been linked to nuclear RNA quality control machinery. Yra1 ubiquitination leads to a release of Yra1 from the mRNP and the authors suggest that this is a part of a nuclear RNA quality control mechanism that only promotes the export of mature mRNPs (69).

Most work studying the links between RNA processing and RNA quality control have been performed in yeast, and significantly less is known about mammalian RNA quality control links with RNA processing. One recent study however, hints at similar links in processing and quality control in mammalian cells. X Qu et al. showed that blocking mRNA export by knockdown of TAP/NXF1 results in a hyperadenylation of transcripts, reminiscent of that observed in yeast (131). Additionally, work from our lab demonstrates that nuclear transcripts can be hyperadenylated by the canonical poly(A) polymerases (PAP), which is dependent on PABPN1, and degraded by the exosome (12).

Viral factors interact with cellular export machinery

In order to facilitate production of their own genes, many viruses usurp host machinery, including the cellular export machinery. In fact, several herpesviruses including Kaposi's sarcoma-associated herpesvirus (KSHV), human cytomegalovirus (hCMV), and herpes simplex virus (HSV) have been shown to modulate the host export machinery. The majority of herpesvirus genes are transcribed from single-exon genes, which presents a

problem for export since the TREX complex is recruited in a splicing-dependent manner. Herpesviruses are thought to overcome this requirement for splicing by recruiting the TREX complex to the RNA through a multifunctional protein known as ORF57 in KSHV, and ICP27 or UL69 in HSV and hCMV, respectively (10, 20, 21, 96, 107). These multifunctional proteins are a part of the ICP27 family of proteins and bridge the interactions between the viral RNA and RNA export machinery factors.

KSHV ORF57

ICP27 family members have been implicated in additional processes including transcription, splicing, export, and translation. In fact, KSHV ORF57, also called Mta, is thought to function in transcriptional control, splicing regulation, translation, RNA stability and mRNA export (11, 26, 106, 152). Of particular interest to these studies is ORF57's involvement in RNA stability and export. ORF57 interacts directly with REF/Aly and viral mRNAs, and is able to increase the export efficiency of at least some RNAs (10, 105, 107, 120). While ORF57 is essential for viral replication (53, 104), ORF57 binding with REF/Aly is dispensable for lytic replication (92). However, an increase in UIF is observed when REF/Aly is depleted (55), and UIF interacts with ORF57 (72), thus in the absence of REF/Aly, ORF57 can interact with UIF to provide an alternative route for viral RNA export.

Previous work from our lab has also shown that ORF57 acts to stabilize the viral, nuclear non-coding polyadenylated RNA, PAN RNA (136, 150, 174). ORF57 stabilizes PAN RNA through a 300 nt ORF57 responsive element (ORE) in the 5′ end of the transcript that is necessary for ORF57 binding to PAN RNA (136). Further analysis defined a minimal

ORE of 30 nt where ORF57 binds directly to PAN RNA. The core ORE is sufficient to induce ORF57-responsiveness in a reporter construct, and is necessary for ORF57-responsiveness of PAN RNA. The core ORE is predicted to form a stem loop and mutational analysis identified a 9 nt sequence as a potential ORF57 binding site (140).

PAN RNA

PAN RNA is a noncoding, highly abundant viral RNA that is expressed during KSHV lytic replication. The function of PAN RNA remains elusive; however it accumulates to extremely high levels and accounts for ~80% of the polyadenylated RNA in the cell during the lytic phase (144, 150). As seen in Figure 6, PAN RNA is approximately 1.1 kb in length and has a 5′ cap and 3′ poly(A) tail, causing it to resemble an mRNA (Song et al., 2001; Sun et al., 1996)(174). PAN RNA contains a 3′ 79-nt stability element called the ENE, or expression and nuclear retention element that confers increased stability of the RNA (28, 30). The ENE protects PAN RNA from deadenylation and decay by sequestering the poly(A) tail and forming a triple helix between the ENE and poly(A) tail (28, 29, 117). Because PAN RNA is nuclear, capped and polyadenylated, it serves as a useful tool to examine nuclear events in gene expression uncoupled from downstream processes such as RNA export and translation.

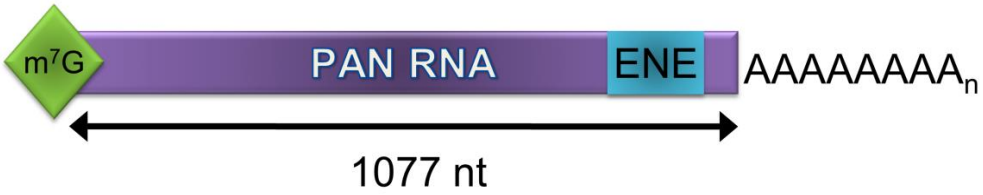

Figure 6. PAN RNA. Polyadenylated nuclear RNA, or PAN RNA is a nuclear, noncoding viral RNA that is approximately 1 kb long. PAN RNA possesses a 5′ cap and a 3′ poly (A) tail, making it resemble an mRNA. Because of this, PAN RNA is a useful tool to uncouple upstream processes in gene expression from downstream processes such as export and translation.

Kaposi's sarcoma-associated herpesvirus

ORF57 and PAN RNA are two components from the virus Kaposi's sarcoma-associated herpesvirus (KSHV). KSHV is the causative agent of Kaposi's Sarcoma (KS), as well as the lymphoproliferative disorders multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL) (15, 39, 114). KSHV is a member of the herpesvirus subfamily Gammaherpesvirinae, and is a double stranded DNA virus. The KSHV genome is approximately 165 kb and encodes at least 90 viral products. Additionally, KSHV exhibits a high level of sequence homology with the prototype gammaherpesvirus herpesvirus Saimiri (HVS).

KSHV infects primarily B cells and endothelial cells and upon infection exists in either a latent or a lytic state. During the latent phase of infection the viral genome is maintained as a circular episome in the nucleus. Few viral proteins are expressed during the latent phase, with the exception of Kaposins A, B, and C, as well as vFLIP, vCyclin, LANA and LANA-2. LANA is the main product expressed during latency and it is thought to

maintain the viral episome by tethering it to the chromosome. Reactivation from latency is initiated by the protein ORF50 (also called Rta), and it is sufficient to drive the switch to lytic gene expression where progeny virions are produced that can go on to infect other cells (46).

In the United States, KS is primarily associated with transplant patients undergoing immunosuppression regimens and immunocompromised AIDS patients. KS is the most common AIDS-associated cancer, particularly because immune suppression induced by HIV infection makes patients more susceptible to diseases caused by oncogenic viruses like Epstein-Barr virus (EBV), human papilloma virus (HPV), and KSHV (138). With the advancement of antiretroviral treatment for HIV in the developed world, KS is mostly controlled. However, in underdeveloped countries, particularly those in sub-Saharan Africa where HIV is rampant and endemic KS exists, KS is the most prevalent cancer in men and second most prevalent cancer in women (126).

CHAPTER THREE

Methodology

Antibodies

All antibodies were purchased commercially from the following sources: REF (Sigma, clone 11G5), Pol II (Covance, clone 8WG16), H3K36me3 (Abcam, ab9050), hnRNP C1/C2 (clone 4F4).

Plasmids

With the exception of TetRP-PANΔENE-6MS2 described below, all PAN RNA and ORF57 expression plasmids have been previously described as well as the MS2 alone (pcNMS2-NLS-Flag) construct (28-30, 136). To create TetRP-PANΔENE-6MS2, 6xMS2 binding sites were cut from MS2-PANΔ79 using NcoI and ligated to Trp-PANΔ79 cut with NcoI. The Fl-ΔReBD expression plasmid, pc-Flag-ORF57II ΔReBD, was created using SOEing PCR techniques (62). Twenty-eight of the 35 amino acids in the putative REF-binding domain were deleted. Primers NC576 (5´ gagcaattgtccgaacccgc 3´) and NC577 (5´ gggacgtgggatggtggggcgctcgcaggagtctgagtt 3´) were used to generate the 5´ fragment and primers NC578 (5´ aactcagactccctgcgagcgccccaccatcccacgtccc 3´) and NC579 (5´ tgctcttatgagagcggtga 3´) were used to create the 3´ fragment. pc-Flag-ORF57II was used as the template. The resulting PCR amplicons were used as template for PCR using primers NC576 and NC579 to generate the ORF57 fragment containing the REF-binding domain

deletion. The resulting PCR product was cut with KpnI and EcoRV and inserted into pc-Flag-ORF57II cut with the same restriction enzymes.

Cell Culture

HEK293 cells were grown in Dulbecco's Modified Eagle's Medium (Sigma) containing 10% fetal bovine serum (FBS), 1x penicillin-streptomycin (Sigma), and 2mM L-glutamate. 293TOA and 293A-TOA were grown in DMEM that was supplemented with 100 µg/mL G418 (Fisher Bioreagents), 10% Tetracycline-free FBS, 1x penicillin-streptomycin, and 2mM L-glutamate.

Transfection

HEK293, 293TOA, and 293A-T0A cells were transfected using TransIT-293 (Mirus) according to manufacturer instructions. UV cross-linking experiments in chapter four were performed using HEK293 cells in 10-cm plates, with a total of 12 µg plasmid DNA. A typical transfection consisted of 1.5 µg PAN-WT, 3.0 µg ORF50, 3.0 µg ORF57, and 3.0 µg Fl-REF. Because ORF57 increases PAN RNA levels, we increased the amount of PAN-WT plasmid to 6.0 µg in the samples lacking ORF57 expression to yield roughly equivalent PAN RNA expression levels. All control samples were balanced with matched empty vector plasmids so that each sample contained an equal amount of transfected DNA. MS2-REF and ORF57 titrations were performed in 12-well tissue culture plates with 0.7-0.8 µg plasmid DNA. Transfections were set up as previously described (136). Total RNA was harvested 18-24 hours after transfection using TRI Reagent (Molecular Research Center). UV

Crosslinking experiments described in chapter five were performed using 293A-TOA cells in 10-cm plates with 10µg Flag-REF2-1 plasmid DNA (103) using Transit29 (Mirus).

For knockdown experiments presented in chapter five, 293A-TOA cells were transfected with a final concentration of 16nM siRNAs using Lipofectamine RNAiMax (Invitrogen) according to manufacturer instructions. For siREF samples, cells were transfected with a pool of two (8nM each) *Silencer* Select siRNAs targeting REF (Ambion). Control samples were transfected with *Silencer* Select Negative Control #2 siRNA. Cells were transfected with siRNAs for 24 hr before replating on larger plates. For example, for RNA-Seq and ChIP sample preparation, 293A-TOA cells were initially plated on 6-well plates for siRNA transfection. Twenty-four hr after transfection cells were trypsinized, re-plated on 10-cm plates, and allowed to grow for 72 more hours, 96 hr total. We found that efficient knockdown required cellular doublings, not just additional time, presumably because the REF/Aly protein is stable.

Northern Blotting

RNA was analyzed by standard northern blotting techniques (24, 30). For northern blots with oligonucleotide probes (Figure 13D), the probes were 5´-end labeled with T4 polynucleotide kinase using standard procedures. Oligonculeotide NC29 (5´ atcggcggcaccaatgaaaaccagaagcggcaagaaggca 3´) and NC31 (gcacgttaaattgtcaaaagtataacatgtttttccaata 3′) were used to detect the PAN RNA 5´ and 3´ ends, respectively. Hybridization was performed for 4-18 hr in ExpressHyb (Clontech) at 42°. In situ hybridization experiments were performed as previously described (30, 136).

RNase H digestion

To remove poly(A) tails, total RNA was incubated for 1 hour at 37°C with 0.375 units of RNase H (Promega), 10 U RNasin (Promega), and 1.25 µM oligonucleotide dT_{40}, in RNase H Buffer (20mM Tris pH 7.5, 100mM KCl, 10 mM DTT, 10mM $MgCl_2$) in a final volume of 20µl. To cleave PAN RNA and resolve changes in poly(A) tail length (Figure 13), RNase H digests were identical, except NC581 (5´ aatccaatgcaataacccgcaagg 3´) replaced the dT_{40} oligo. Differences in poly(A) tail length were determined by running a 0.1-2kb RNA ladder (Invitrogen) next to treated samples. A standard curve was generated by measuring the migration of each band from a fixed point and plotting the mobility by $\log_{10}$ of molecular weight of the ladder. Mean poly(A) tail lengths, as defined by the midpoint of the band, were determined based on the standard curve.

RT-qPCR

All RNA analyzed by RT-qPCR was treated with RQ1 DNase (Promega) before the reverse transcription (RT) reaction. RT reactions were performed using SuperScript™ II RT (Invitrogen) or M-MuLV-RT (New England Biolabs) and random hexamers (Sigma) or dT_{20} (fractionation experiments) as per the manufacturer's protocol. cDNAs were then treated with RNase A and RNase H for 30 min at 37°C and purified with phenol:chloroform:isoamyl alcohol (25:24:1; PCA). cDNAs were resuspended in 20 µl H_2O and diluted in an assay dependent manner. Primer sequences and efficiencies are described in Table 2. Relative quantities (RQ) were determined based on amplification efficiency and Ct value. To determine the relative expression of candidate transcripts after fractionation, single

knockdown, or knockdown over time, RQ values were normalized to RQ values of 7SK RNA. RNA levels in decay assays presented in chapter five were performed using a one-step qRT-PCR reaction with MultiScribe Reverse Transcriptase (Invitrogen).

Cross-linking Experiments

UV cross-linking experiments were performed as previously described (27). However, two different wash conditions were used in UV cross-linking experiments. RIPA washes were performed by washing beads five times in 1mL RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150mM sodium chloride, 50mM Tris-HCl [pH 8.0], 2mM EDTA) and nutating at room temperature for 3 minutes. In some cases we used a more stringent washing procedure as follows: 1) 1ml RIPA, 2) 500µl RIPA-U Plus (RIPA supplemented with 1M Urea, 0.2mg/ml poly(U) RNA, 1mg/ml torula yeast RNA), 3) 500µl RIPA-U Plus, 4) 1mL low salt wash (0.1% SDS, 1% TritonX100, 2 mM EDTA, 20 mM TRIS (pH 8), 150 mM NaCl), 5) 1mL high salt wash (0.1% SDS, 1% TritonX-100, 2 mM EDTA, 20 mM TRIS (pH 8), 500 mM NaCl), 6) 1 mL TE. To ensure that both conditions were suitable for cross-linking experiments described in chapter four, protein was taken from Inputs and Pellets, run on a western blot and probed for ORF57 (Figure 8). A lack of ORF57 signal in pellets indicates that both wash conditions were suitable to dissociate REF from ORF57.

To determine the position of REF binding (Figure 9), UV cross-linking assays were modified and real-time PCR was performed as previously described to include a partial RNase digestion (140). Primer amplification efficiencies were determined to be 92% (50-

124), 91% (293-372), 82% (642-728), and 83% (994-1064). C_t values and amplification efficiencies were used to determine the relative quantities (RQ) of Inputs and Pellets. RQ values of the no-RT controls were subtracted from plus RT samples and percent immunoprecipitation was determined by calculating the pellet/input ratio. Samples were then normalized to the +UV/+Fl-REF samples to compare between experiments, and all values were represented relative to the 5´-most amplicon. UV crosslinking experiments performed in chapter five were carried out in 293A-TOA cells as previously described using stringent wash conditions (27, 148) and immunoprecipitated RNA was analyzed by RT-qPCR. RQ values of -reverse transcriptase samples were subtracted from +reverse transcriptase RQ values, and immunoprecipitation efficiency was determined by calculating the pellet-to-input ratio. All samples were expressed relative to signal from ACTB to control for experimental variation.

Decay Assays

Decay assays in chapter four were performed as previously described (28) with the following exceptions. 293TOA cells were plated on 12-well plates and transfected with 0.3µg TetRP- PANΔENE-6MS2 and 0.4µg of the appropriate MS2-expression construct per well. At given time points, total RNA was harvested using TRI Reagent. The endogenous 7SK RNA served as a loading control.

To assess relative stability of candidate transcripts in chapter five, cells transfected with REF or Control siRNAs were treated with 1 µg/ml Actinomycin D to inhibit transcription. Cells were harvested at various time points from 0-4 hr by addition of Tri-

Reagent. RNA was extracted and analyzed by qRT-PCR to determine RNA abundance after transcription shut-off.

Nucleo-cytoplasmic Fractionation

For RNA-Seq sample preparation, 293A-TOA cells were transfected with 16 nM of siREF (pooled) or siControl and grown on three 10-cm plates per siRNA for 96 hr. After knockdown, cells were harvested from one 10-cm plate, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in 500 µl Buffer I (0.32 M Sucrose, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM Tris pH 8.0) which was immediately added to 5 ml Tri-Reagent (Molecular Research Center); this is the "total" fraction. Cells were then harvested and combined from the other two 10-cm plates and resuspended in 1 ml Buffer I with fresh 1 mM DTT, 0.04 U/µl RNaseIN, and 0.5% TritonX-100, and incubated on ice for 5 min. Samples were then centrifuged at 500xg for 5 min at 4°C. To obtain the "cytoplasmic" fraction, the supernatant was removed and added to 10 ml Tri-Reagent. The pellet was then resuspended in 1ml Buffer I and added to 10 ml Tri-Reagent; this is the "nuclear" fraction.

RNA-Seq

Fractionated RNAs were selected two times using the Poly(A) Purist Kit (Ambion) to enrich for polyadenylated RNA following manufacturer specifications. Analysis using an Agilent Bioanalyzer indicated that rRNA contamination in samples was minimal. Two biological replicates were prepared and sequenced using the SOLiD system. Sequencing

data was analyzed using TopHat with Bowtie and differentially expressed genes (DEGs) were identified using Cufflinks (156).

4-thiouridine Labeling and Click-IT Nascent RNA Capture

For 4-thiouridine (4SU) labeling experiments (37, 170), 293A-TOA cells were pulsed for 2 hr with 2.5 µM 4sU. Cells were then harvested with Tri-Reagent and collected RNA was treated with RQ1 DNase. 15 µg RNA was biotinylated by addition of buffer containing 10 mM Tris pH 7.5, 1 mM EDTA, 0.2 mg/ml Biotin-HPDP (Pierce) in a final volume of 125 µl. The reaction was incubated at room temperature for 3 hr in the dark. Next, the sample volume was brought to 250 µl in 1 M ammonium acetate and extracted with 250 µl Chloroform:isoamyl-OH (24:1). The aqueous phase was added to 700 µl 100% ethanol and precipitated overnight at -20°C. Dynal MyOne Streptavidin T1 beads (10 µl per sample) were washed three times in MPG-I (1M NaCl, 10 mM EDTA, 100 mM TrisHCl pH 7.5, 0.1% Igepal) and resuspended in 170 µl MPG-I. Precipitated RNA from the biotinylation reaction was washed with 70% ethanol, resuspended in 30 µl H_2O, and heated at 65°C for 5 min. RNA was added to the washed Dynal Streptavidin T1 beads for a final volume of 200 µl and incubated at room temperature for 30 min with nutation. Beads were washed 8 times using the following washes: 1) MPG-I, 2) 65°C MPG (1M NaCl, 10 mM EDTA, 100 mM TrisHCl pH 7.5), 3-6) MPG-I, 7) MPG without NaCl, 8) MPG diluted 1:10. Selected RNAs were eluted by first adding 200 µl elution buffer (MPG 1:10 + 5% β-mercaptoethanol) and incubated at room temperature for 5 min. Eluted RNA was moved to a new tube and beads were resuspended in another 200 µl elution buffer and incubated at 65°C for 10 min. Eluted

fractions were combined and RNA was PCA extracted and ethanol precipitated. Relative expression of RNAs was measured by RT-qPCR.

Analysis of bulk poly(A) RNA with EU was performed as previously described (51) using 1.5 µg RNA. Selected RNA was eluted from beads by addition of RNase T1 after the final wash. 30 µl of RNA/beads were incubated with 20 mM Tris pH 6.8, 8 U RNasin, and 1000 U/ul RNase T1 for 15 minutes at 37°C. The reaction was stopped by addition of 170 µl G-50 Buffer (20 mM Tris pH 7.5, 0.25% SDS, 0.3 M sodium acetate, 2 mM EDTA) containing 0.1 mg/ml Proteinase K and 1.5 µl glycoblue. RNA was extracted with an equal volume of PCA and ethanol precipitated. The entire sample was then assayed by northern blot with a 1.8% agarose gel and a 5´-end labeled dT_{40} probe.

Chromatin Immunoprecipitation (ChIP)

293A-TOA cells were grown on 6-well plates and transfected with 16 nM siControl or pooled siREF. Twenty-four hr after transfection, cells were replated on 10-cm plates and allowed to grow until REF/Aly was depleted for 96 hr. To crosslink cells, 0.75% formaldehyde was added to each plate and plates were rocked for 10 min at room temperature. Formaldehyde was quenched by addition of 125 mM glycine. Cells were harvested from plates, washed 3 times with PBS, and resuspended in 500 µl RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 50 mM Tris-HCl [pH 8.0], 2 mM EDTA) containing protease inhibitors. Cells were sonicated for 45 cycles (30 seconds On and 30 seconds Off) at 4°C using a Digenode Bioruptor on the high setting to shear DNA to approximately 200-300 base pairs. Sheared chromatin was centrifuged at max

speed for 10 min at 4°C and then DNA was measured using a NanoDrop Spectrophotometer. Samples were normalized and ~200µg DNA was added to protein A beads conjugated with either 8WG16 (4 µl) or H3K36me3 (3 µg) antibodies. DNA was immunoprecipitated for at least 2 hr at 4°C, and then washed with 1 ml of the following washes: 1) RIPA, 2) low salt wash (0.1 SDS, 1% TritonX100, 2 mM EDTA, 20 mM Tris (pH 8.0), 150 mM NaCl), 3) high salt wash (0.1 SDS, 1% TritonX100, 2 mM EDTA, 20 mM Tris (pH 8.0), 500 mM NaCl), 4) LiCl wash (0.25 M LiCl, 1% NP40, 1% NaDeoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0), 5) T.E. buffer (10 mM Tris pH 8.0, 1 mM EDTA), 6) T.E. buffer. Chromatin was eluted by addition of 200 µl elution buffer (100 mM $NaHCO_3$, 1% SDS) and incubation at 65°C for 15 min. To reverse protein crosslinks and degrade protein, chromatin was incubated at 65°C overnight in elution buffer containing 20 mg/ml Proteinase K and 40 µl Tris pH 6.8. Chromatin was purified using PCA and ethanol precipitation and DNA was analyzed by qPCR. ChIP amplicon primer sequences and efficiencies can be found in Table 3.

Table 2. Real Time PCR Primers

Gene	Span	Amplicon Length	Primer Sequence		Primer #	Efficiency
FXYD6	695-793	99	For:	ACCGCCAATGCAACAGA	IDT	89%
			Rev:	GCATCCAAAGGTTCAAGCAG	IDT	
TLCD1	354-471	118	For:	GGTCACTTTCTGGCTATTTGC	NC1559	96%
			Rev:	ACGTGATGGACAAGGTATTCC	NC1554	
THTPA	1254-1396	143	For:	TGCATGAGGAGGCTGAAGTA	NC1319	89%
			Rev:	CAGGCGCTGATAGTCTTGAG	NC1320	
loc100128881	484-585	102	For:	TCTGAGGCCAGGAGCTT	IDT	95%
			Rev:	GCCGTGTATACTCCGATGAAG	IDT	
7SK	1-108	108	For:	TAAGAGCTCGGATGTGAGGGCGATCTG	NC1164	94%
			Rev:	CGAATTCGGAGCGGTGAGGGAGGAAG	NC1165	
ACTB	1047-1176	130	For:	ACCCAGCACAATGAAGATCA	NC1224	91%
			Rev:	CTCGTCATACTCCTGCTTGC	NC1225	
18S	5233-5381	149	For:	AACCCGTTGAACCCCATT	NC1597	96%
			Rev:	CCATCCAATCGGTAGTAGCG	NC1598	

Table 3. ChIP Primers

Gene	Span	Amplicon Length	Primer Sequence		Primer #	Efficiency
TLCD1 #1	(-802)-(-742)	61	For:	GGGCAGGCTGACACCTAGTG	NC1196	93%
			Rev:	GCTCTCCAAGCCGGTAGCTT	NC1197	
TLCD1 #2	(-635)-(-570)	66	For:	CGGCCCCAGGAGTTTAAGAG	NC1218	86%
			Rev:	GCACTTGGGAACTGCTGAGTAA	NC1219	
TLCD1 #3	113-176	64	For:	AGCATGCCCCGACTGCT	NC1220	91%
			Rev:	CCCGGAAGGTCAGCGTG	NC1221	
TLCD1 #4	808-869	62	For:	TGCCCCATCCCTCCACTA	NC1200	91%
			Rev:	TGTCCGCTAGCCACGATGT	NC1201	
TLCD1 #5	1261-1323	63	For:	AACTTCCAGGAAAGCGTCTG	NC1282	100%
			Rev:	TGGCCTAGAGGGAAGAAAGA	NC1283	
THTPA #1	(-1239)-(-1148)	92	For:	ATACTGGTGAGAGGGCAGGT	NC1417	94%
			Rev:	GAACCGTGGCTCTTAACTCC	NC1418	
THTPA #2	(-96)-(-11)	86	For:	TAGGTAGAGTACCGCAGGCA	NC1376	100%
			Rev:	GTTCCAAAGGGAGACTTTGC	NC1377	
THTPA #3	969-1058	89	For:	CTGGAGCAGCAGGTGTCTTA	NC1378	94%
			Rev:	GCAGCACCTTACAGAGTTGG	NC1379	
THTPA #4	2010-2107	98	For:	GCATGATTTGGCAGATTTGT	NC1419	95%
			Rev:	ACTGCAGGGCTGTAAGGC	NC1420	
THTPA #5	2878-2946	69	For:	GGAACTCTGGGTCTAACGGA	NC1382	93%
			Rev:	TGTCAGAAGGGACACTGAGG	NC1383	
ACTB #1	(-1152)-(-1088)	65	For:	GCCCACCCGGTCTTGTG	NC1252	100%
			Rev:	CCTGTCCTTGTCACCCTTTCTT	NC1253	
ACTB #2	(-72)-(-14)	59	For:	CCGAAAGTTGCCTTTTATGG	NC1226	100%
			Rev:	GCCGCTGGGTTTTATAGGG	NC1227	
ACTB #3	1264-1328	65	For:	AGGCATCCTCACCCTGAAGTAC	NC1254	96%
			Rev:	TCTCCATGTCGTCCCAGTTG	NC1255	
ACTB #4	1897-1965	69	For:	CTTCAACACCCCAGCCATGT	NC1230	100%
			Rev:	CCAGAGGCGTACAGGGATAGC	NC1231	
ACTB #5	2832-2891	60	For:	TCGTCCACCGCAAATGC	NC1232	99%
			Rev:	TCAAGAAAGGGTGTAACGCAACT	NC1233	
GAPDH #1	(-775)-(-716)	60	For:	GCCGCTGGTGCACACA	NC1256	89%
			Rev:	CCCTGTAGCCTGGACCTGATAA	NC1257	
GAPDH #2	28-82	55	For:	CTCTCTGCTCCTCCTGTTCGA	NC1234	100%
			Rev:	CACCTGGCGACGCAAAA	NC1235	
GAPDH #3	325-389	65	For:	CCACATCGCTCAGACACCAT	NC1236	100%
			Rev:	AGCCACCCGCGAACTCA	NC1237	
GAPDH #4	795-849	55	For:	TAGAGCGGCCGCCATGT	NC1238	99%

			Rev:	TTCCTAACGGCTGCCCATT	NC1239	
GAPDH #5	1968-2026	59	For:	TCCCCTCCTCATGCCTTCTT	NC1240	97%
			Rev:	CCAGGCGCCCAATACG	NC1241	

CHAPTER FOUR

Viral factors reveal a role for REF/Aly in nuclear RNA stability

Introduction

Like many RNA processing events in the eukaryotic cell, mRNA export from the nucleus to the cytoplasm is coupled with other steps in mRNA synthesis (125, 132, 139). In mammalian cells, the protein complex TREX associates with the 5´ end of transcripts as a result of splicing and TREX subsequently promotes the export of the mature mRNA (22, 111). The mammalian TREX complex contains REF/Aly, UAP56, hTEX1, and CIP29 as well as components of the THO complex (hHpr1, hTho2, fSAP35, fSAP79, and fSAP24), and many of these proteins are conserved from *Saccharomyces cerevisiae* to humans (40, 111, 133, 146, 149, 172). However, in yeast, TREX recruitment is linked with transcription and 3´ end formation rather than splicing (50, 78, 87). TREX deposition subsequently recruits TAP/NXF1 and the mRNA is "handed off" from REF/Aly to TAP/NXF1 (54). TAP/NXF1, with its partner p15/NXT1, is the receptor responsible for bulk polyadenylated RNA export (44, 70).

A role for the TREX component REF/Aly in mRNA export is supported by several lines of evidence. The human REF/Aly protein is conserved from yeast to humans, and the yeast homolog, Yra1, is essential for bulk mRNA export (145, 149). REF/Aly interacts directly with both UAP56 and TAP/NXF1, two factors required for bulk mRNA export (40, 111, 145, 146, 149). REF/Aly increases mRNA export efficiency in *Xenopus* oocyte systems (102, 134) and artificial tethering of REF/Aly increases the export efficiency of otherwise

inefficiently exported transcripts (30, 40, 55, 165). Small interfering RNA (siRNA)-mediated knockdown of REF family members decreases bulk mRNA export to various degrees in metazoan cells. In some cases, little or no nuclear poly(A) accumulation is observed, while other studies report an accumulation of poly(A) RNA in the nucleus (36, 48, 55, 80, 99, 124). These distinct phenotypes may be due to redundancy with other adaptors (161), compensatory changes in gene expression (55), and/or differences in experimental procedures. Knockdown of REF is toxic to both *Drosophila* and human cells (48, 55), but in *Drosophila* cells, no bulk poly(A) accumulation is observed. This observation suggests that *Drosophila* REF is necessary for export of only a subset of essential mRNAs, or that it may have an additional essential function(s) (48). Indeed, REF/Aly has previously been implicated in transcriptional control (14, 160) and here we propose a role for REF/Aly in nuclear RNA stability.

RNA surveillance or RNA quality control pathways are the processes that destroy transcripts that are misprocessed or unfolded, and/or do not assemble into a suitable ribonucleoprotein particle (RNP) (38, 139). In yeast, Yra1 is linked to the RNA quality control machinery. Iglesias and colleagues demonstrated that Yra1 ubiquitination leads to its release from the nuclear messenger RNP (mRNP) and proposed that this is part of a nuclear RNA surveillance mechanism that selectively promotes export of mature mRNPs (69). More generally, export factors are physically or genetically linked to the nuclear RNA decay machinery involved in transcript surveillance (34, 57, 73, 93, 101, 159, 171). For example, defects in mRNA export factors lead to hyperadenylation and retention of transcripts at the site of transcription. Moreover, this retention depends on Rrp6, an exonuclease that otherwise

degrades aberrant RNAs, but the precise mechanism of retention remains unknown (58, 74, 135). Significantly less is known about the interrelationships between mRNA export, polyadenylation, and RNA surveillance in mammalian nuclei, but recent work has shown that inhibition of mRNA export by TAP/NXF1 knockdown leads to a hyperadenylation phenotype similar to that observed in yeast (131) and one factor, ZC3H3, has been proposed to link regulation of polyadenylation with export in *Drosophila* and human cells (68).

Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a multifunctional regulator of gene expression called ORF57 (Mta) that has been implicated in transcriptional control, splicing regulation, translation and mRNA export (11, 26, 106, 152). ORF57 interacts directly with both REF/Aly and viral mRNAs which, in some cases, increases the export efficiency of the mRNA (10, 105, 107, 120). Because most KSHV mRNAs are transcribed from single-exon genes, it has been proposed that ORF57 recruits the TREX complex to viral mRNAs to promote splicing-independent export (10, 107). In addition, we have recently shown that ORF57 binds and stabilizes the KSHV polyadenylated nuclear (PAN) RNA (136), a 5´-capped, RNA polymerase II-transcribed, polyadenylated noncoding RNA that is retained in the nucleus (136, 150, 174). Thus, ORF57 promotes the nuclear stability of transcripts independently of its role in mRNA export.

Because of its mRNA-like characteristics and nuclear localization, PAN RNA serves as a useful tool for examing nuclear events in gene expression uncoupled from downstream processes such as mRNA export or translation. The present studies of ORF57 and PAN RNA reveal that REF/Aly stabilizes RNA in a fashion that is separable from its role in mRNA export. ORF57 recruits REF/Aly to PAN RNA where it binds directly to the transcript. The

REF/Aly association with PAN RNA displays a 5′ bias, reminiscent of the placement of REF/Aly on spliced mRNAs (22, 111). Deletion of the REF/Aly binding domain from ORF57 abolishes its stabilization function, supporting the model that REF/Aly is an essential co-factor for ORF57-mediated nuclear RNA stabilization. Artificial tethering of REF/Aly to PAN RNA in the absence of ORF57 leads to higher PAN RNA levels by increasing the PAN RNA half-life and maintaining longer poly(A) tail lengths. Perhaps surprisingly, REF/Aly tethering is not sufficient to promote PAN RNA export from the nucleus. Taken together, our results strongly support a role for REF/Aly in nuclear RNA stability and suggest that binding of export factors actively protects nuclear transcripts from degradation by the RNA quality control machinery in the mammalian cell nucleus.

Results

REF/Aly binds to PAN RNA in an ORF57-dependent manner

ORF57 binds viral mRNAs and recruits REF/Aly to the mRNP presumably to facilitate viral mRNA export. However, ORF57 also binds to PAN RNA, but does not lead to its export. It is possible that ORF57 binding does not promote PAN RNA export because REF/Aly is not recruited to PAN RNA. Alternatively, REF/Aly recruitment by ORF57 may not be sufficient for PAN RNA export. To distinguish between these possibilities, we employed a UV irradiation cross-linking RNA immunoprecipitation approach (27, 121). We transfected HEK293 cells with expression constructs for PAN RNA, ORF57, and/or an N-terminally Flag-tagged version of the murine REF2-I protein (Fl-REF), which is 74% identical to human REF/Aly but has shorter variable regions (149). The following day,

transfected cells were exposed to UV light to covalently cross-link protein with RNA. Immediately following UV treatment, the cells were lysed under stringent conditions, Fl-REF was immunoprecipitated with anti-Flag antibodies, and the presence of PAN RNA in the immunoprecipitates was examined by northern blot. Figure 7A shows that PAN RNA was co-immunoprecipitated with Fl-REF in the presence of ORF57 (lane 11), but it was nearly undetectable in the absence of ORF57 (lane 8). Importantly, neither the no-cross-linking (Figure 7A, lanes 7 and 10) nor the no-Fl-REF controls (lanes 9 and 12) showed significant immunoprecipitation of PAN RNA. Quantification of the data demonstrated a statistically significant increase in Fl-REF cross-linking to PAN RNA when ORF57 was expressed (Figure 7B). Western blot analysis of the proteins verified that Fl-REF was efficiently immunoprecipitated under the stringent conditions used for UV cross-linking experiments (Figure 7C, lanes 7-12). Additionally, inspection of the REF/Aly levels in the cell lysate using a REF/Aly antibody showed that the exogenously expressed Fl-REF levels were only slightly above the endogenous levels. Moreover, there was no dramatic up-regulation of Fl-REF upon co-expression of ORF57 (compare lanes 1, 2 to lanes 4, 5). As an additional control, we verified that the immunoprecipitation conditions used were stringent enough to disrupt the REF/Aly interaction with ORF57 (Figure 8). From these data, we conclude that ORF57 promotes a direct interaction between REF/Aly and PAN RNA. Because live cells were exposed to UV in this experiment, the absence of signal in the no-UV controls (Figure 8, lanes 1,4) indicates that the interaction occurs in cells, and is not due to reassortment of protein-RNA complexes post-lysis (27, 116). Furthermore, UV irradiation is a zero-length cross-linker, so UV-dependent cross-linking can be interpreted as the result of a direct

interaction between a protein and an RNA (27, 121). Thus, these data show that Fl-REF is recruited to the PAN RNP in an ORF57-dependent fashion and that, upon recruitment, Fl-REF interacts directly with the RNA.

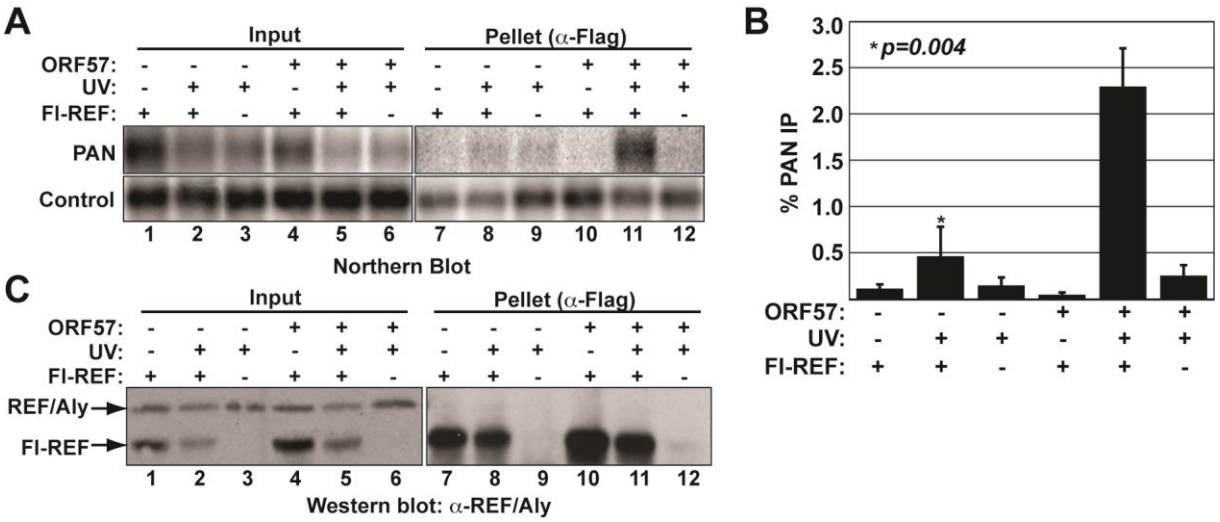

Figure 7. REF/Aly binds PAN RNA in an ORF57-dependent fashion. (A) UV cross-linking shows that Fl-REF binds PAN RNA directly. Cells transfected with PAN RNA, Fl-REF, and ORF57 expression constructs as indicated were exposed to UV light ~24 hrs after transfection or were left untreated. Lysates from the transfected cells were subjected to immunoprecipitation with anti-Flag agarose. Northern blotting was performed to detect PAN RNA in the input and pellets; different exposures of the input and pellets are displayed. The control panels show an exogenously added β-globin transcript used to normalize RNA recovery and loading. **(B)** Quantification of UV cross-linking experiments. PAN RNA signals were first normalized to the β-globin control then the pellet values were divided by input values to determine the percent PAN RNA immunoprecipitation. Average values were plotted for each of the samples; error bars are standard deviation (*n=3*). The p-value is an unpaired Student's t-test of the data sets for the +Fl-REF/+UV samples. **(C)** Western blot analysis to confirm immunoprecipitation in UV cross-linking experiments. Input and pellet protein samples were run on a gel, transferred to nitrocellulose, and probed with a monoclonal anti-REF/Aly antibody that recognizes Fl-REF as well as the endogenous REF/Aly. The murine Fl-REF migrates at a faster mobility due to its shorter non-conserved variable regions (149). The input and pellet panels are shown at different exposure times.

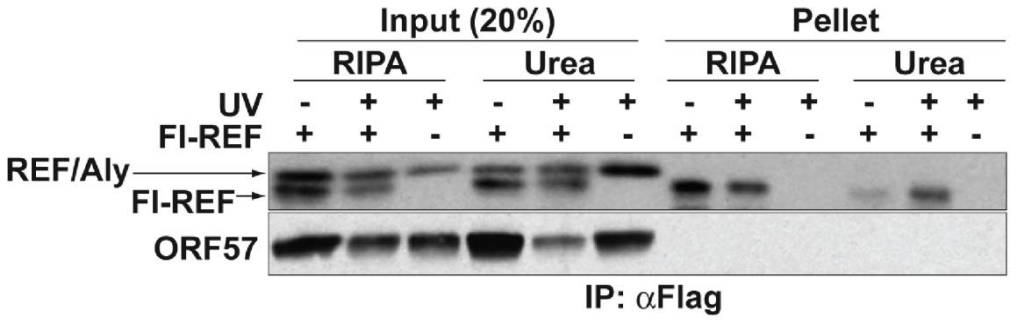

Figure 8. UV cross-linking immunoprecipitation conditions abrogate the ORF57-REF interaction. UV cross-linking and immunoprecipitation with anti-Flag antibodies was performed as in Figures 1 and 2. The presence of Fl-REF (top) or ORF57 (bottom) was examined in the inputs and pellets by western blot. Two different wash procedures were used as described in the Materials and Methods. One protocol included a wash step containing urea while the other was performed with RIPA buffer alone. Fl-REF is immunoprecipitated under both of these conditions, but ORF57 is not coimmunoprecipitated.

PAN RNA contains an ORF57-responsive element (ORE) in its 5´-most 300 nucleotides (nt) that is sufficient for ORF57 binding (110, 136, 140). In addition, deletion of this element from PAN RNA dramatically decreases the efficiency of ORF57 binding to PAN RNA. Therefore, it seems likely that ORF57 binding to the ORE recruits REF/Aly to the PAN RNP. Alternatively, it is possible that ORF57 activates REF/Aly binding independent of the ORF57 association with PAN RNA. Indeed, accessibility of the REF/Aly RNA-binding domain (RBD) to RNA is regulated by interactions with other proteins (49). To distinguish between these models, we performed UV cross-linking experiments in which we expressed an ORE deletion (PANΔORE; Figure 9A) instead of full-length PAN RNA. Because ORF57 binding to PANΔORE is significantly reduced (136, 140), the recruitment model predicts that the loss of ORF57 binding to PAN RNA will lead to loss of Fl-REF

recruitment in the presence of ORF57. Consistent with this prediction, PANΔORE RNA was co-immunoprecipitated significantly less efficiently than wild-type PAN RNA (Figure 9A and 9B), even though ORF57 was co-expressed. Some residual binding was still observed, but this was also observed in the case of ORF57 (140). Thus, efficient REF/Aly binding to PAN RNA depends on the presence of both ORF57 (Figure 7) and the ORE (Figure 9), strongly supporting the model that ORF57-binding to PAN RNA recruits REF/Aly to the PAN RNP.

REF/Aly binds to the 5´ ends of spliced cellular RNAs in a splicing and cap-binding complex (CBC)-dependent fashion (22). The data above show that ORF57 recruits REF/Aly to PAN RNA independently of splicing, but efficient recruitment depends on a 5´ RNA element. To determine whether REF/Aly binds to the 5´ end of PAN RNA directly, we modified the UV cross-linking protocol to include a partial RNase digestion prior to immunoprecipitation. The immunoprecipitated fragments were then analyzed by quantitative reverse transcription PCR (qRT-PCR) to approximate the region of REF/Aly interaction. Four primer sets spanning PAN RNA were used for the qRT-PCR analysis (Figure 9C). As expected, we observed a UV-dependent and Fl-REF-dependent enrichment for PAN RNA in the immunoprecipitates. In addition, we saw higher immunoprecipitation efficiency at the 5´ end of the molecule. However, the 5´ bias is not absolute; REF/Aly associated with fragments further downstream on PAN RNA. Importantly, using the same assay, ORF57 was shown to bind to PAN RNA with a nearly identical trend (140). The observed downstream binding may be due to insufficient RNase treatment, or it is possible that ORF57 and REF/Aly bind at more than one site on PAN RNA. Because these data mirror those seen with

ORF57, they strongly support the conclusion that ORF57 recruits REF/Aly to PAN RNA at its 5´ end, but the interaction may occur at other sites on the transcript as well.

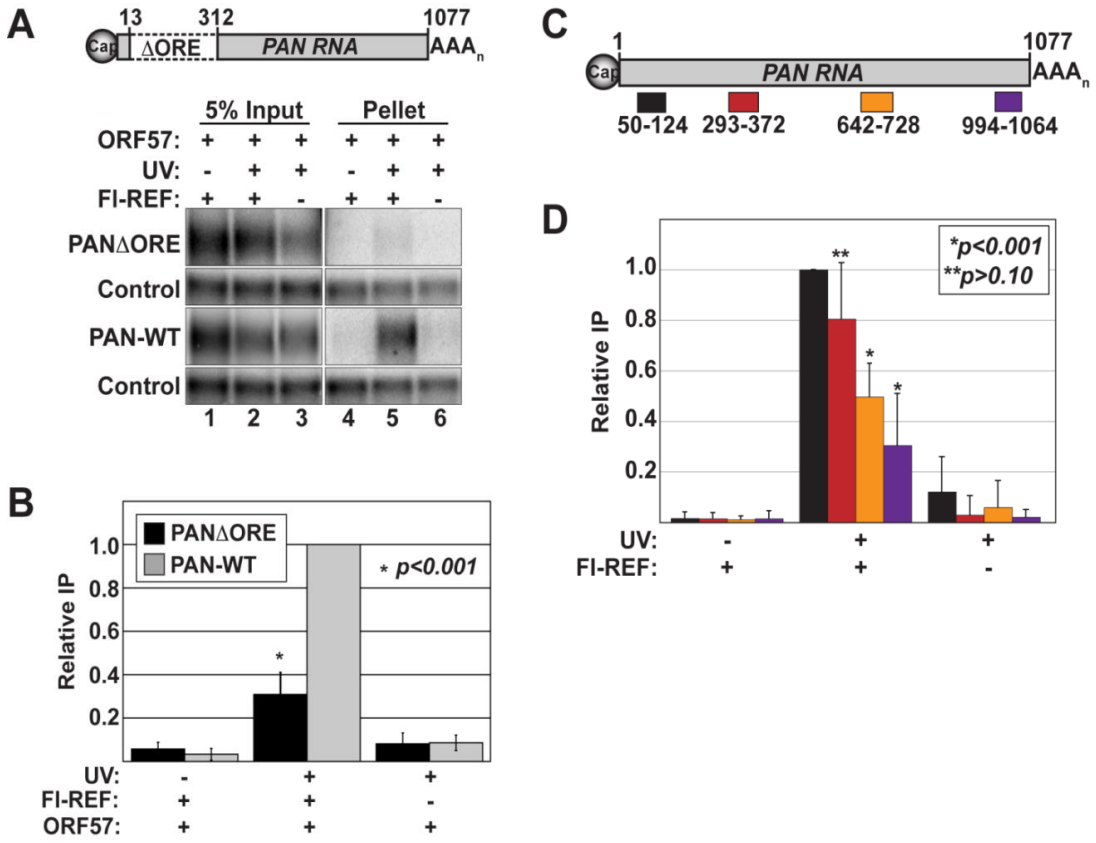

Figure 9. REF/Aly recruitment is enhanced by the ORE and its binding is enriched at the PAN RNA 5´ end. (A) *Top,* Diagram of PANΔORE RNA. The numbers represent PAN RNA nt sequence relative to its transcription start site (69). *Bottom*, Representative northern blots from a UV cross-linking co-immunoprecipitation experiment with a wild-type PAN-WT and PANΔORE RNAs. Details are as in Figure 1. **(B)** Quantification of data from PANΔORE cross-linking experiments. Error bars represent standard deviation and p-values are unpaired Student's t-test of the PAN-WT and PANΔORE data sets from the +UV/+Fl-REF/+ORF57 samples (*n=4*). **(C)** Diagram of the amplicons used for qRT-PCR. **(D)** Results from UV-cross-linking qRT-PCR experiments. The data are plotted for each of the indicated samples and controls. The colors used in the bar graphs match the amplicons as depicted in (C). The bars are the mean percent immunoprecipitation values relative to the 5´-most amplicon and the error bars are standard deviation (*n=3*). Determination of primer efficiencies were described previously (63, 66). P values are two-tailed, unpaired Students' t-test comparing each data set to that of the 5´-most amplicon.

The ORF57 REF/Aly interaction domain is necessary for its nuclear stabilization function

To determine if REF/Aly is an essential co-factor for ORF57-mediated increases in RNA stability, we generated a flag-tagged ORF57 expression construct that lacks 28 amino acids essential for the interaction of REF/Aly-with ORF57 (Fl-ΔReBD) (107, 120). This deletion does not alter the localization of ORF57 (105, 120), nor does it have a substantial effect on ORF57 protein levels (Figure 10A, lanes 1 and 2). To verify the loss of interaction between Fl-ΔReBD and REF/Aly, we immunoprecipitated Fl-ORF57 or Fl-ΔReBD using anti-flag agarose beads and looked for co-immunoprecipitation of endogenous REF/Aly (Figure 10A lanes 3-8). While REF/Aly was co-immunopreciptiated with the wild-type Fl-ORF57, co-immunoprecipitation of REF/Aly with Fl-ΔReBD was undetectable (lanes 7,8). No immunoprecipitation was observed when ORF57 was untagged (lane 6).

We next tested whether deletion of the REF-binding domain abolished the ability of ORF57 to increase PAN RNA levels. Consistent with previously published results (120), Fl-ΔReBD was strongly compromised in its ability to up-regulate PAN RNA (Figure 10B, compare lanes 1-3 with 7-9 and Figure 10C). We previously showed that ORF57's ability to stabilize PAN RNA was at least partially redundant with the cis-acting element called the ENE (136). The ENE is a 79-nt element near the 3´ end of PAN RNA that interacts with the poly(A) tail to stabilize PAN RNA in cis. Stabilization by the ENE is not dependent on ORF57, nor does ORF57 sequence-specifically bind this region (136, 140). However, the ENE and ORF57 likely protect PAN RNA from the same decay pathway. As a result, deletion of the ENE causes significant decreases in PAN RNA levels due to its destabilization in the absence of ORF57 (28-30). As a result, an ENE deletion mutant of

PAN RNA (PANΔENE) provides us with a broader dynamic range to detect increases in stabilization by Fl-ΔReBD. Even in this case, Fl-ΔReBD did not significantly increase the levels of PANΔENE RNA (Figure 10B lanes 4-6, 10-12 and Figure 10C). These data show that when ORF57 is compromised for REF/Aly binding, it is unable to contribute to PAN RNA stability. One caveat to these experiments is that the deletion may affect interactions with other proteins or activities of ORF57 unrelated to REF/Aly function. Optimally, we would assess ORF57 activity in cells in which REF/Aly is knocked down with RNAi. Unfortunately, when we knockdown REF/Aly using RNAi in HEK293 cells, we concomitantly decrease exogenous ORF57 expression, so it is technically not feasible to assay for loss of ORF57 stabilizing function in the absence of REF/Aly. Even so, these data are consistent with the model that ORF57 recruits REF/Aly to PAN RNA where it functions to stabilize the transcript.

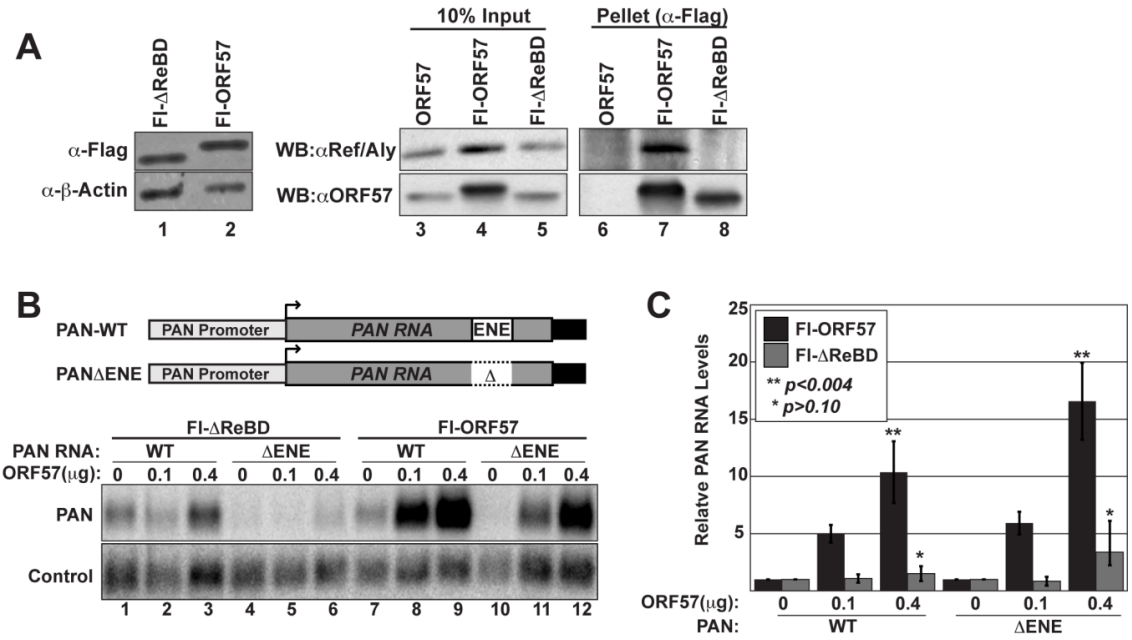

Figure 10. REF/Aly-binding correlates with ORF57 activity. (A) Immunoprecipitation of REF/Aly is abrogated in the Fl-ΔReBD mutant. Lanes 1 and 2 are a western analysis demonstrating similar expression levels of the Fl-ORF57 and Fl-ΔReBD proteins; endogenous b-actin serves as a loading control. Lanes 3-8 are western blot analysis of 10% input and 100% pellets from a co-immunoprecipitation experiment. Whole cell extracts from HEK293 cells transiently expressing either Fl-ORF57, Fl-ΔReBD, or an untagged ORF57 were used for immunoprecipitation with anti-Flag agarose in the presence of RNase. Western blotting (WB) was performed with REF/Aly or Flag antibodies as indicated. **(B)** *Top*, Schematic diagram of the PAN-WT and PANDENE expression constructs. Transcription is driven by the PAN RNA promoter and constructs contain the PAN RNA polyadenylation signal (black box). *Bottom*, Northern analysis comparing the effects of Fl-ORF57 or Fl-ΔReBD on the accumulation of full-length PAN RNA (WT) or PANΔENE (ΔENE). Membranes were probed with PAN RNA or control probes as indicated. The loading control detects RNA from a co-transfected plasmid that expresses the murine mgU2-19/30 scaRNA (11, 76) **(C)** Quantification of the northern blot data. Each PAN RNA value was first normalized to the loading control and all values are given relative to the corresponding no-ORF57 signal. The error bars are standard deviation (*n=3*); the p-values are two-tailed, unpaired Students' t-test of the asterisked data set with the matching no-ORF57 data set.

REF/Aly is sufficient to increase nuclear PAN RNA levels

REF/Aly is recruited to PAN RNA by ORF57 and it appears to be a necessary co-factor for ORF57-mediated increases in PAN RNA stability. We next tested whether REF/Aly recruitment was sufficient to confer increased accumulation of PAN RNA. To address this experimentally, we employed a tethering assay to promote an interaction between REF and PAN RNA in HEK293 cells (25). We expressed PAN RNA from plasmids containing six binding sites for the bacteriophage MS2 coat protein that either included or excluded the ENE (Figure 11A). We transiently co-expressed these transcripts with a fusion protein containing REF2-I fused to the MS2 coat protein at its C-terminus (REF-MS2) and examined the effects of tethering REF-MS2 on PAN RNA accumulation (Figure 11B, lanes 1-3). As controls, we tested PAN RNA lacking MS2 binding sites or we replaced REF-MS2 expression with MS2 coat protein alone (lanes 4-9). The MS2 protein contains a simian virus 40 (SV40) nuclear localization sequence to ensure its translocation to the nucleus. Quantification of the data showed that tethering of REF-MS2 to PAN RNA increases its accumulation in a dose-dependent fashion (Figure 11C) and this increase is not observed in the absence of MS2-binding sites or when MS2 coat protein alone is expressed. Thus, recruitment of REF-MS2 to PAN RNA independently of ORF57 is sufficient to increase PAN RNA accumulation.

The decay machinery in the nucleus differs from that in the cytoplasm, so it is possible that the observed increases in PAN RNA accumulation were an indirect result of REF-mediated export of PAN RNA to the cytoplasm. In addition, because PAN RNA is a nuclear transcript, the hypothesis that ORF57 recruits REF/Aly to PAN RNA predicts that

REF/Aly recruitment is insufficient for PAN RNA export. Therefore, we performed fluorescence in situ hybridization (FISH) to determine the localization of PANΔENE RNA upon REF-MS2 tethering (Figure 12). As expected, in the controls lacking either REF-MS2 (middle row) or MS2-binding sites (bottom row), PANΔENE RNA displayed punctate nuclear staining (136). Most importantly, PANΔENE remains localized exclusively to the nucleus in the presence of REF-MS2 (top row). In this case, both the number of cells showing signal above background and the intensity of the signal in individual cells were increased compared to controls. We conclude that the REF-dependent increases in PAN RNA levels reflect an increase in nuclear RNA abundance, demonstrating that REF/Aly is sufficient to increase the nuclear accumulation of PAN RNA but is not sufficient to promote its export.

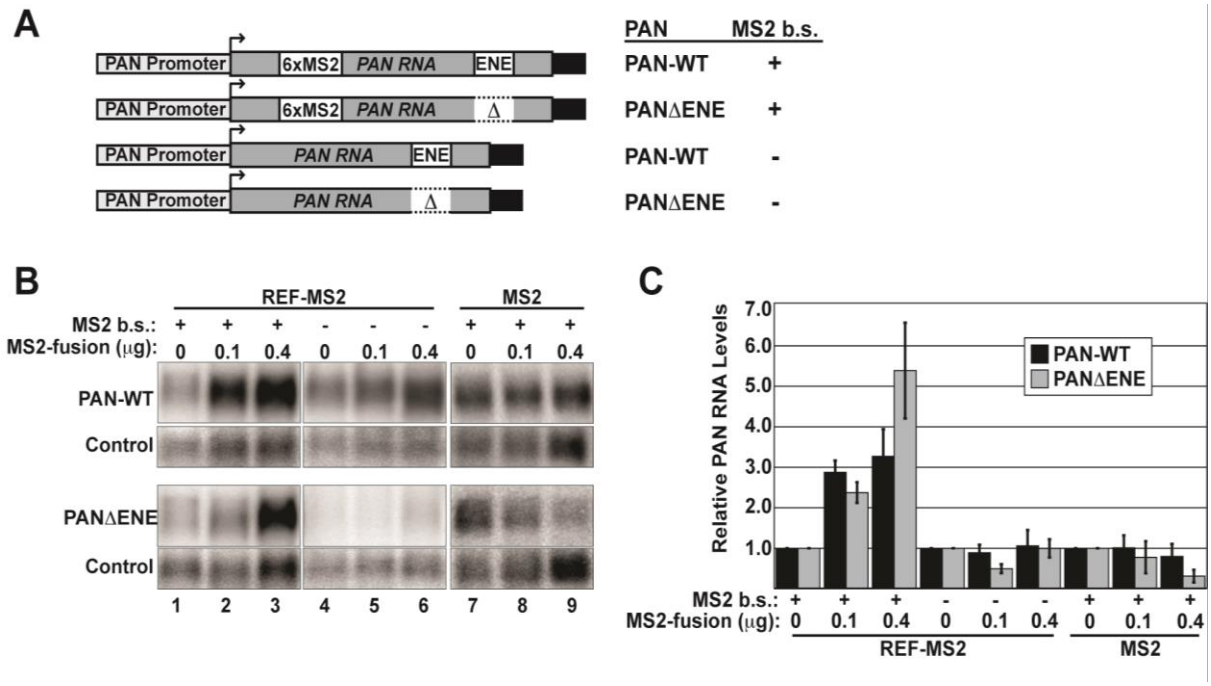

Figure 11. REF/Aly is sufficient to increase PAN RNA abundance. (A) Diagram of the PAN RNA expression constructs used in the tethering experiments. PAN RNA is driven by its own promoter and has its own polyadenylation signals (black rectangles). The ENE is present (ENE) or absent (Δ) as indicated as are the six MS2 binding sites (b.s.). **(B)** Representative northern blots from tethering experiments. The presence or absence of MS2 binding sites and the amounts of co-transfected MS2 construct are shown above the panels. To the left of the panels the probe and PAN RNA expression construct are indicated. Panels differ in their exposures to best display the given signal, but relative quantification is shown in (C). The loading control detects RNA from a co-transfected mgU2-19/30 plasmid that expresses a previously described murine scaRNA (11, 76) **(C)** Quantification of the data from multiple REF-MS2 tethering assays. The data are presented as mean values relative to the matched no REF-MS2 or no MS2 control. The error bars are standard deviation (*n=3*).

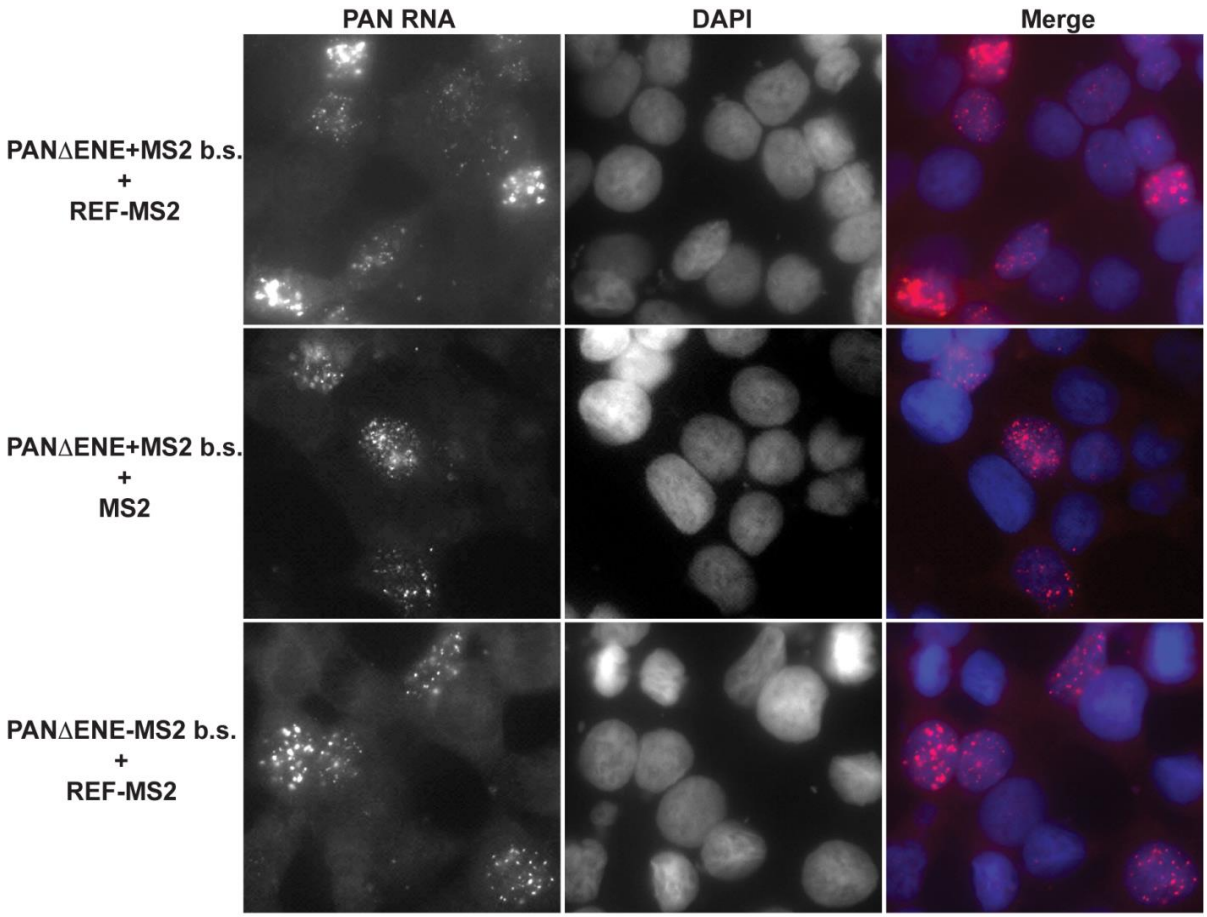

Figure 12. REF/Aly tethering does not promote PAN RNA export. In situ hybridization of PANΔENE RNA containing MS2 binding sites (top, middle) or lacking MS2 binding sites (bottom). MS2 or REF-MS2 were co-transfected as indicated. Left panels are probed for PAN RNA, the middle panels are DAPI-stained nuclei and the right panels are merged signal. The PAN RNA plus REF-MS2 panels are shown at a lower exposure to avoid saturation of the signal.

REF/Aly increases the half-life of PAN RNA and affects poly(A) tail lengths

Next we addressed the cellular mechanism underlying the REF-mediated increases in nuclear PAN RNA abundance. To directly test whether REF/Aly stabilizes PAN RNA in the nucleus, we determined the half-life of PANΔENE RNA in the presence or absence of REF-MS2 tethering. PAN RNA lacking the ENE, but containing six MS2 binding sites, was

cloned behind the tetracycline-responsive promoter (TetRP; Figure 13A). We co-transfected HEK293 tet-off advanced (293TOA) cells with this construct and either the REF-MS2 or the MS2 coat protein expression construct. 293TOA cells allow us to inhibit TetRP-driven transcription by adding doxycycline (dox) to the medium. Approximately 18 h after transfection, doxycycline was added to the medium, RNA was harvested at given intervals, and expression levels were compared by Northern blotting (Figure 13A). Quantification of the Northern blotting data showed that the apparent half-life was extended from ~0.5hr to ~3hr when REF-MS2 was tethered to PAN RNA (Figure 13B). Taking these results with those reported above, we conclude that REF/Aly association with PAN RNA is sufficient to increase the nuclear half-life of PAN RNA.

Inspection of the Northern blot data revealed that in the absence of REF-MS2, the length of PAN RNA shortened over time. In contrast, little or no shortening was observed when REF-MS2 was tethered to the RNA (Figure 13A). The differences in mobility were more readily seen when samples from the 0- and 1-h time points were run on the same gel (Figure 13C). Upon treatment of these samples with RNase H and oligo(dT) to remove poly(A) tails, each of the RNAs had the same mobility (lanes 5-8), which was slightly higher than that for the 1-hr MS2-only samples (compare lane 3 with 5-8). Thus, the shortened forms of PAN RNA were due to decreases in poly(A) tail length, consistent with the previous report that PAN RNA undergoes deadenylation prior to its destruction (28). We interpret these data as indicating that REF/Aly recruitment protects the transcripts from deadenylation and/or promotes ongoing re-adenylation that counterbalances nuclear deadenylation. Interestingly, we noticed a subtle, but reproducible trend that PAN RNA mobility decreases

over time in the presence of REF-MS2 after transcription shut-off (Figures 13A and 13C). To better resolve the relative lengths of the transcripts, we cleaved samples from a REF-MS2 transcription shut-off experiment with RNase H and the DNA oligonucleotide NC581 (Figure 13A, top) prior to northern blot analysis. This treatment shortens the transcripts making changes in gel mobility due to poly(A) tail extension more obvious. We then detected the 3´ or 5´ fragments using an oligonucleotide probe specific for each fragment (Figure 13A, top). This analysis showed that the 3´ fragments lengthened over time after transcription shut off, while the 5´ fragment mobility remained constant (Figure 13D). We estimated the increase over the 8-h period to be ~50 nt, extending an ~150 nt poly(A) tail to ~200 nt. Thus, when REF-MS2 is recruited to PAN RNA in the nucleus, the transcript is subject to poly(A) extension subsequent to the initial cleavage and polyadenylation step.

It is worth noting that when we performed the decay experiments using short (2-hr) transcription pulses, we saw no differences between PAN RNA decay rates of tethered MS2 and REF-MS2. Previous studies of PAN RNA decay kinetics reported diminution of the effects of stabilizing factors using shorter transcription pulses that are due to the multiple pathways involved in PAN RNA destruction (28, 136). An additional contributing factor here may be that the levels of transcript are not sufficient to drive binding to REF-MS2 after only a short transcription pulse. Alternatively, it remains formally possible that REF/Aly may play a role in transcription as has been previously proposed (14, 160) and that some of the effects on steady-state levels are transcriptional. Even so, the decay data presented in Figure 13 as well as the changes in poly(A) tail length clearly indicate a post-transcriptional role for REF/Aly in PAN RNA accumulation.

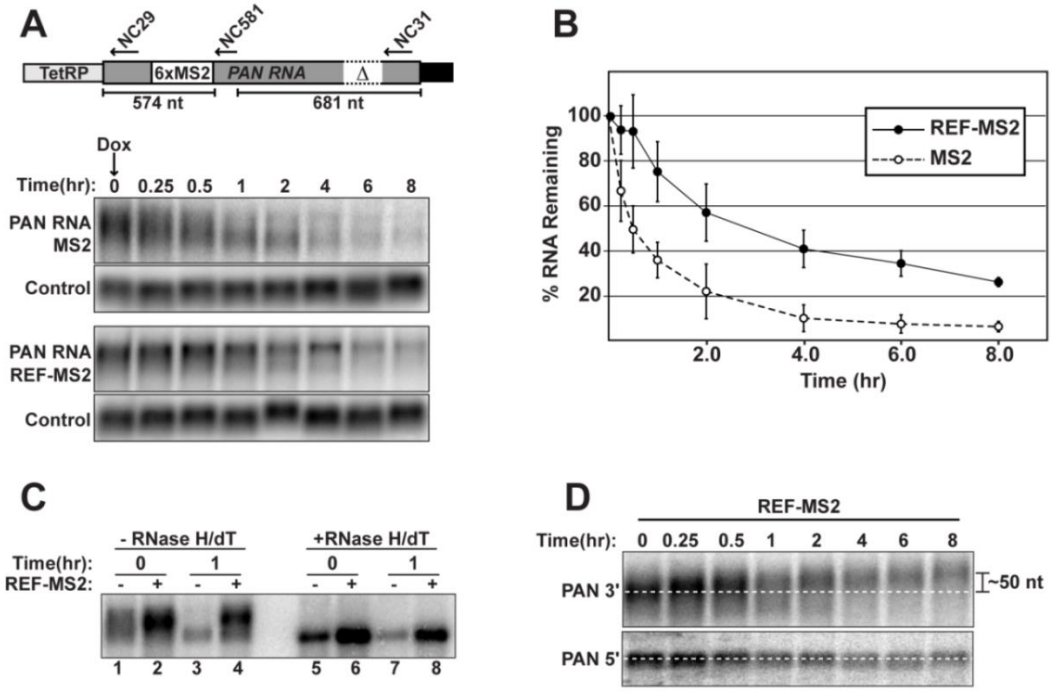

Figure 13. REF/Aly promotes PAN RNA stability. (A) *Top* Schematic diagram of the TetRP- PANΔENE-6xMS2 construct. Position of oligonucleotides (arrows) for RNase H-targeted cleavage (NC581) as well as probes for detection of the 5´ (NC29) and 3´ (NC31) ends of PAN RNA are shown. The schematic is not to scale. *Bottom* Northern blots from an in vivo RNA decay experiment. 293TOA cells were co-transfected with TetRP-PANΔENE-6MS2 and MS2 (upper panels) or REF-MS2 (lower panels). After transcription shutoff by addition of dox to the media, RNA was collected at given intervals. Blots were probed first for PAN RNA then for an endogenous loading control (7SK RNA). **(B)** Decay curve of PANΔENE with or without REF-MS2. PAN RNA signals were first normalized to the loading control and expressed as a percentage of the time-zero samples. Each data point is the average of three experiments with the error bars representing the standard deviation. **(C)** Northern blot of 0- and 1-hour samples from an RNA decay experiment with either MS2 alone (-) or REF-MS2 (+) as indicated. The same samples were treated with RNase H and oligo dT to remove the poly(A) tails and run on the same gel. **(D)** REF-MS2 promotes PAN RNA hyperadenylation subsequent to transcription shut off. Total RNA from a REF-MS2 transcription shut-off experiment was cleaved with RNase H and NC581 and subjected to northern blotting. In the top panel, the 3´ end of the cleaved PAN RNA was detected with probe NC31. The bottom panel shows the 5´ PAN RNA fragment detected on the same blot using probe NC29. The dashed white lines serve as a guide to show that the mean length of the 3´ end increases while the 5´ end remains constant. The mean poly(A) tail lengths were estimated to be ~150 nt at t=0 and ~200 nt at t=8 (data not shown). The positions of the oligonucleotides used for RNase H cleavage and RNA detection are depicted in (A).

Discussion

The data presented here are consistent with two primary conclusions. First, we conclude that the cellular REF/Aly protein stabilizes transcripts in the nucleus in a manner that is separable from its role in nuclear export. We show that recruitment of REF/Aly is sufficient to increase the abundance of PAN RNA in the nucleus (Figures 11-12), so the effect cannot be attributed to its previously defined role in RNA export. Rather, binding of REF/Aly to PAN RNA leads to an increase in transcript stability, apparently by directly or indirectly protecting the poly(A) tail (Figure 13). Second, we conclude that the KSHV ORF57 protein recruits REF/Aly to PAN RNA to promote the stabilization of the transcript. KSHV ORF57 binds an element in PAN RNA near its 5´-end, increasing the PAN RNA half-life (136, 140). Here, a stringent UV cross-linking technique, we showed that ORF57 recruits REF/Aly to PAN RNA (Figures 7 and 9) resulting in a direct interaction between REF/Aly and the 5´ end of PAN RNA in vivo. Three observations support the conclusion that ORF57-mediated stabilization is a result of REF/Aly activity: (i) REF-MS2 is sufficient to stabilize PAN RNA in the nucleus when recruited to the transcript independent of ORF57 (Figures 11-13), (ii) The REF-interaction domain of ORF57 is necessary for its stabilization function (Figure 10)(120), and (iii) both REF-MS2 tethering and ORF57 lead to stabilization apparently by affecting poly(A) tail metabolism (Figure 13) (136). Thus, these studies have uncovered a cellular co-factor for the KSHV ORF57 protein's RNA stability function and have identified a novel function for a cellular export factor.

A role for REF/Aly in nuclear RNA surveillance?

Our data indicate that the stabilization function of REF/Aly can be separated from its role in nuclear export. When REF/Aly is recruited to PAN RNA either by ORF57 or by direct tethering, PAN RNA is not exported from the nucleus, however its half-life increases. This observation has interesting implications for RNA biogenesis. At least in some cases, intronless transcripts are less stable than their spliced counterparts (28, 30, 173) and inefficiently exported transcripts tend to be less abundant presumably due to nuclear decay (for example, see reference(55). Given the promotion of export by splicing, it is reasonable to propose that stabilization is an indirect consequence of a shorter exposure to nuclear decay factors. However, our data support a more direct role for REF/Aly in stabilizing nuclear transcripts. Specifically, transcripts that associate with REF/Aly resist nuclear decay even upon increased nuclear dwell-times. Further supporting this idea, a recent report showed that TREX components lead to the nuclear stabilization of naturally occurring intronless mRNAs (88). These data are consistent with the idea that assembly of export factors protects transcripts from a nuclear RNA surveillance machinery that degrades “non-exportable” mRNPs.

We propose that the upstream events in mRNA export, specifically REF/Aly recruitment, retain an mRNP that is not fully export-competent in a state in which it is protected from nucleolytic attack. We can imagine two broad non-mutually exclusive models for protection of the RNA. First, the changes in stability may result from alterations in the mRNP that render it refractory to the exonucleases. For example, the ENE stabilizes transcripts by limiting access of the poly(A) tail to the degradation machinery (28, 29).

Perhaps TREX similarly protects the poly(A) tail by promoting interactions between the 3´ and 5´ ends of the transcript. Indeed, Yra1 interacts with the nuclear poly(A)-binding protein in yeast (69) and one can imagine that interactions between REF/Aly at the 5´ end of a transcript with factors at the 3´ end may promote circularization and stabilization of the transcripts in the context of the mRNP. Second, we can envision a model in which the REF/Aly complex maintains transcripts at a particular subnuclear locale that is devoid of nuclease activity. Similar models in which hyperadenylated transcripts accumulate at the sites of transcription where they are relatively stable, have been proposed for yeast (135). Further studies are necessary to distinguish between these models.

Another observation consistent with the idea that REF/Aly protects PAN RNA from the nuclear RNA surveillance machinery is that, upon REF/Aly binding, poly(A) tails appear to be protected from deadenylation and subject to further adenylation. In yeast and mammalian cells, hyperadenylation occurs as a consequence of compromised Mex67 or TAP/NXF1 function, respectively. While one can imagine that this hyperadenylation is an indirect consequence of export blockage, recent work suggests that this may be due to a direct role of Mex67 in poly(A) tail metabolism (131). In cleavage and polyadenylation assays, extracts from Mex67 temperature-sensitive strains generated significantly longer poly(A) tails than their wild-type counterparts (131). Moreover, the changes in length were due primarily to the lack of poly(A) trimming in the Mex67-deficient extracts. Yeast TREX components including Yra1 are necessary to release transcripts from the cleavage and polyadenylation machinery prior to export (69, 131). Dias and colleagues showed that in mammalian cells, REF/Aly and UAP56 are necessary for release of RNAs from nuclear

speckle domains (36) and that TREX components are recruited to naturally occurring intronless transcripts to stabilize them (88). Taking these observations with our data, we propose that REF/Aly is recruited early in mRNA biogenesis and that this recruitment renders the transcript resistant to deadenylation and decay by affecting mRNP structure or localization. After REF/Aly recruitment, further maturation of the mRNP leads to release from retention, poly(A) tail trimming, and export. Interestingly, REF/Aly primarily binds to polyadenylated transcripts that are in insoluble nuclear material (115), consistent with the proposed role for REF/Aly upstream of transcript release.

What is the role of REF/Aly in KSHV RNA metabolism?

We used the unique PAN RNA to probe the mechanisms of ORF57 and REF/Aly in nuclear RNA metabolism. However, the present study also provides mechanistic information regarding ORF57-mediated stabilization of PAN RNA. While the function of PAN RNA remains unknown, ORF57 promotes its accumulation in both transfected and infected cells (53, 84, 104, 120, 136). It seems reasonable to propose that its function depends on its extraordinarily high abundance: nearly 70-80% of the poly(A) RNA in lytically reactivated cells is PAN RNA (144, 150). Several factors contribute to this abundance. First, the PAN RNA promoter has robust activity in the lytic phase, driven by the KSHV RTA protein (162). Second, the ENE stabilizes PAN RNA in cis. Third, ORF57 binds to PAN RNA and promotes its stability. Our present results strongly suggest that the cellular REF/Aly protein is a necessary co-factor for ORF57-mediated stabilization. Thus, assuming the high

abundance of a PAN RNA is necessary for its function in KSHV, REF/Aly may promote this function by increasing PAN RNA half-life.

ORF57 is a member of the herpes simplex virus (HSV) ICP27 family of herpesvirus proteins that have been implicated in nearly every step of RNA biogenesis from transcription through decay, (26, 52, 106, 137, 141, 155). While the mechanisms of these proteins differ in some respects, one common theme is that each family member interacts with the cellular mRNA export machinery. For example, ICP27 binds to REF/Aly and TAP/NXF1 directly (20, 21), while human cytomegalovirus (hCMV) UL69 interacts directly with UAP56 (96). KSHV ORF57 binds to REF/Aly directly, but only weak direct binding to TAP/NXF1 has been observed (107). In all cases, the prevailing model is that ICP27 family members provide a link between viral mRNAs and the cellular export machinery. Herpesvirus mRNAs are presumed to require this function because their single-exon genes are not subject to splicing-coupled mechanisms for export factor recruitment. However, the extent to which this mechanism is used by each herpesvirus remains an area of significant interest, as is the determination of whether this export mechanism is utilized by the majority of viral mRNAs or only a subset.

In nearly every case examined, ICP27 homologs are multifunctional and essential for viral replication. However, whether the functions driven by interaction with export factors are essential for viral replication remains unclear. In HSV-infected cells, TAP/NXF1 is essential for viral mRNA export, whereas REF/Aly is dispensable (77). This may be due to REF/Aly redundancy with other cellular factors like UIF, which has recently been reported to be redundant with REF/Aly in both KSHV-infected and uninfected mammalian cells (55,

72). Interestingly, while REF/Aly may not be required for HSV mRNA export, ICP27 recruits REF/Aly, but not TAP/NXF1, to sites of viral transcription (20). Therefore, consistent with our observations with ORF57, ICP27 appears to recruit REF/Aly to transcripts early in their biogenesis to perform a function independent of its role in mRNA export. In light of these results, it will be interesting to determine whether ORF57 and REF/Aly are co-transcriptionally transferred to nascent transcripts to promote herpesvirus evasion of host RNA quality control pathways.

CHAPTER FIVE

Depletion of REF/Aly alters gene expression and reduces RNA Polymerase II recruitment

Introduction

In the eukaryotic cell, a pre-mRNA must undergo multiple processing events to generate a mature mRNA. Many of these nuclear pre-mRNA processing steps, including capping, splicing, and 3´-end formation occur co-transcriptionally (6, 125, 132, 139). In fact, pre-mRNA processing is not only temporally linked to RNA synthesis, but it is also more efficient when coupled to transcription (6). RNA polymerase II (pol II) is uniquely suited to facilitate co-transcriptional pre-mRNA processing largely through its repetitive carboxyl-terminal domain (CTD) that recruits various RNA processing factors throughout the transcription cycle (65). Reversible phosphorylation of multiple residues of the CTD facilitates the recruitment and activities of RNA processing factors (43, 56). As a result, truncation of the CTD results in severe defects in 3′-end processing, splicing, (112, 113) and cell viability (5, 97, 163), thereby demonstrating the importance of coupling between transcription and RNA processing.

Much work has been done demonstrating that cells link transcription with downstream events in RNA processing, but recent investigations suggest that RNA processing can, in turn, modulate transcription rates. For example, several aspects of pre-mRNA splicing have been associated with transcription. Splicing efficiency and splice site mutations have been shown to impair transcription activity by decreasing assembly of the PIC complex (33) and repositioning the active transcription marker, H3K36me3 (83).

Furthermore, first exon length is an important determinant of the active chromatin signatures H3K4me3 and H3K9ac, as well as transcription factor density (7). Not only are splicing elements within the gene important for determining transcription activity, but splicing proteins are also linked to transcription activity. Depletion of the splicing factor SC35 causes accumulation of pol II in the gene body and reduces elongation efficiency (95). Additionally, SC35 associates with the 7SK complex at gene promoters and facilitates release of P-TEFb from the 7SK complex to enable transcription elongation (75). Other steps in pre-mRNA processing aside from splicing have also been linked to transcription activity. The Cap Binding Complex (CBC) interacts with P-TEFb (Cdk9 and Cyclin T1) and affects Ser-2 phosphorylation (90). In yeast, deletion of the CBC results in decreased recruitment of the Bur and Ctk complexes, causing lower Ser-2 phosphorylation and H3K36 methylation (63). Disruption of 3′-end processing results in decreased TFIIB and TFIID at promoters and causes reduced transcription (109). These data provide evidence that cross-talk between gene expression events is bidirectional, and suggest an added layer of complexity between transcription and mRNA processing. However, little is known about the mechanisms and factors involved.

Pre-mRNA splicing changes ribonucleoprotein (RNP) composition to facilitate downstream events in gene expression. Subsequent to intron removal by the spliceosome, the exon junction complex (EJC), is deposited ~20 nucleotides (nt) upstream of the exon-exon junction (86). The EJC and the CBC promote recruitment of the TREX (transcription-export) complex to the 5´-most exon (22, 40, 145, 146, 171). The TREX complex is a highly conserved multi-protein complex composed of REF/Aly, UAP56, CIP29, and the THO

complex (Hpr1, TEX1, Thoc2, Thoc5, Thoc6, Thoc7). Recently, several additional putative TREX complex members were identified that appear to be unique to the mammalian TREX complex, including ZC11A, PDIP3, and Chtop (18, 45). Deposition of TREX results in recruitment of TAP/NXF1 to the mRNA where it binds REF/Aly, displacing UAP56 and triggering the transfer of the mRNA from REF/Aly to TAP/NXF1 (54, 67). TAP/NXF1 and its partner p15 interact with the nuclear pore to assist in export of the mature transcript to the cytoplasm (44, 70).

One TREX component, REF/Aly, has been implicated in multiple processes including RNA export, nuclear RNA stability, and transcription. REF/Aly's importance is underscored by the fact that it is conserved from yeast to humans (145, 149), but depletion of REF/Aly homologs results in a variety of phenotypes. Export of bulk mRNA is not significantly affected when REF/Aly is knocked down in *C. elegans* and *D. melanogaster* (48, 99). However, deletion of Yra1p in yeast prevents bulk mRNA export (145, 149). Depletion of REF/Aly in human cells also displays a variety of phenotypes. Some reports show a strong nuclear accumulation of poly(A) RNA upon REF/Aly depletion, while other studies suggest a more modest retention of poly(A) RNA in the nucleus (38, 55, 80, 124). Although export phenotypes vary, REF/Aly is essential for cell viability in both *Drosophila* and humans. Differences in phenotype may be explained by the presence of redundant export factors (161), up regulation of other export factors (55), or experimental procedures. Nevertheless, these data suggest that REF/Aly functions in the export of at least some specific mRNAs.

Prior to its definition as an mRNA export factor, REF/Aly was implicated in transcriptional control. REF/Aly interacts with AML-1 and LEF-1, two transcription factors

that are involved in activation of the T cell receptor α gene (TCRα) enhancer (14). REF/Aly increases the binding of AML-1 and LEF-1 to DNA, and overexpression led to an increase in the activation of the TCRα enhancer complex. Additionally, REF/Aly exhibits chaperone functions by promoting dimerization of basic region-leucine zipper (bZIP) transcription factors which increases the association of bZIP proteins to DNA (160). Furthermore, REF/Aly associates with Iws1, a protein that interacts with the elongation factor, Spt6 (166), perhaps linking REF/Aly with transcription elongation. Thus, REF/Aly may provide additional functions in RNA synthesis besides its function in RNA export.

REF/Aly is also known to stabilize at least one RNA, PAN RNA, a Kaposi's sarcoma-associated herpesvirus nuclear non-coding RNA (148). Artificial tethering of REF/Aly to PAN RNA is sufficient to increase the half-life of PAN RNA. However, the transcript remains in the nucleus, demonstrating that the REF/Aly export function is not required for the stabilization activity. In addition, tethering of REF/Aly increases the nuclear abundance of an inefficiently spliced intron-containing reporter (55). Furthermore, the TREX complex appears to increase the nuclear stability of endogenous intronless mRNAs (88, 89). These data suggest that TREX components, particularly REF/Aly, are capable of stabilizing nuclear transcripts, independent of their roles in RNA export.

Here, we aimed to identify endogenous RNAs affected by REF/Aly early in their biogenesis. In particular, we looked for transcripts that were destabilized or had decreased transcription in the absence of REF/Aly. Using an RNA-Seq approach, we identified transcripts whose levels were altered upon REF/Aly depletion and focused on several candidates that were down regulated in the absence of REF/Aly in both the nuclear and

cytoplasmic fractions, suggesting that the changes in expression were not driven solely by mRNA export defects. Perhaps surprisingly, half-life determinations, metabolic labeling assays, and chromatin immunoprecipitation (ChIP) assays showed that REF/Aly likely enhances the transcription of target genes. ChIP assays revealed that depletion of REF/Aly causes a significant decrease in pol II recruitment to candidate genes. Additionally, we show that REF/Aly binds directly to candidate transcripts, consistent with a direct effect of REF/Aly on the candidates. Our data suggest that the importance of REF/Aly is not limited to RNA export, but that REF/Aly is also critical for gene expression at the level of transcription and may be involved in linking transcription efficiency with splicing.

Results

Depletion of REF/Aly alters RNA expression

Our goal was to find specific mRNAs that were affected by REF/Aly early in their biogenesis, particularly at the levels of nuclear RNA stability or transcription. Therefore, we wanted to exclude RNAs that were affected by REF/Aly primarily at the level of mRNA export. To identify candidate transcripts, we employed an RNA-Seq strategy coupled to siRNA-mediated REF/Aly knockdown. In principle, if REF/Aly is necessary for the export of a transcript, its total levels may decrease upon REF/Aly knockdown if the transcript is more rapidly degraded when sequestered in the nucleus. Alternatively, the transcripts may accumulate in the nucleus leading to higher nuclear levels upon export block. In contrast, we reasoned that if REF/Aly is directly involved in promoting nuclear stability or transcription of specific mRNAs, these transcripts should display decreased levels in both nuclear and

cytoplasmic compartments upon REF/Aly depletion. Therefore, in our RNA-Seq experiments we examined the effects of REF/Aly depletion on unfractionated cells, nuclear and cytoplasmic fractions, and focused on transcripts reproducibly down-regulated in all three samples.

To determine the proper conditions for siRNA-mediated knockdown of REF/Aly, 293A-TOA cells (136) were transfected with siRNAs targeting REF/Aly or a control siRNA. Cells were counted and protein was harvested every 24 hours for 5 days to monitor changes in cell growth and to determine the length of time required to deplete REF/Aly protein levels. REF/Aly protein levels diminished gradually over time and became nearly undetectable after approximately 4 days. Cell growth began to slow at 3 days but cells did not stop doubling until after 4 days (Figure 14A), consistent with previously published observations (55). Cell morphology was not appreciably affected by REF/Aly depletion until after 96 hr (data not shown). To maximize REF/Aly depletion and minimize changes in cell growth, we chose to perform RNA-Seq experiments following a 96-hr depletion of REF/Aly.

To prepare samples for RNA-Seq, cells were treated for 96 hr with siRNAs targeting REF/Aly or with a control siRNA. Polyadenylated RNA was collected from unfractionated cells (total), nuclear or cytoplasmic fractions. Two biological replicates were sequenced, mapped, and differentially expressed genes (DEGs) were identified using Cufflinks (156). We found 2234 genes that were differentially expressed in the total sample, 3886 in the cytoplasmic fraction, and 4859 in the nuclear fraction (Figure 14B). In order to select for transcripts whose expression is enhanced by REF/Aly upstream of export, we focused on transcripts that were down regulated in all samples upon REF/Aly depletion and found 646

DEGs (Figure 14C). Gene ontology analysis using GOrilla (41, 42) did not identify any enriched terms for candidates identified in the RNA-Seq analysis. Four candidate RNAs, FXYD6, TLCD1, THTPA, and loc100128881, were chosen for further analysis because they were decreased greater than fourfold upon REF/Aly depletion (Figure 14B, orange).

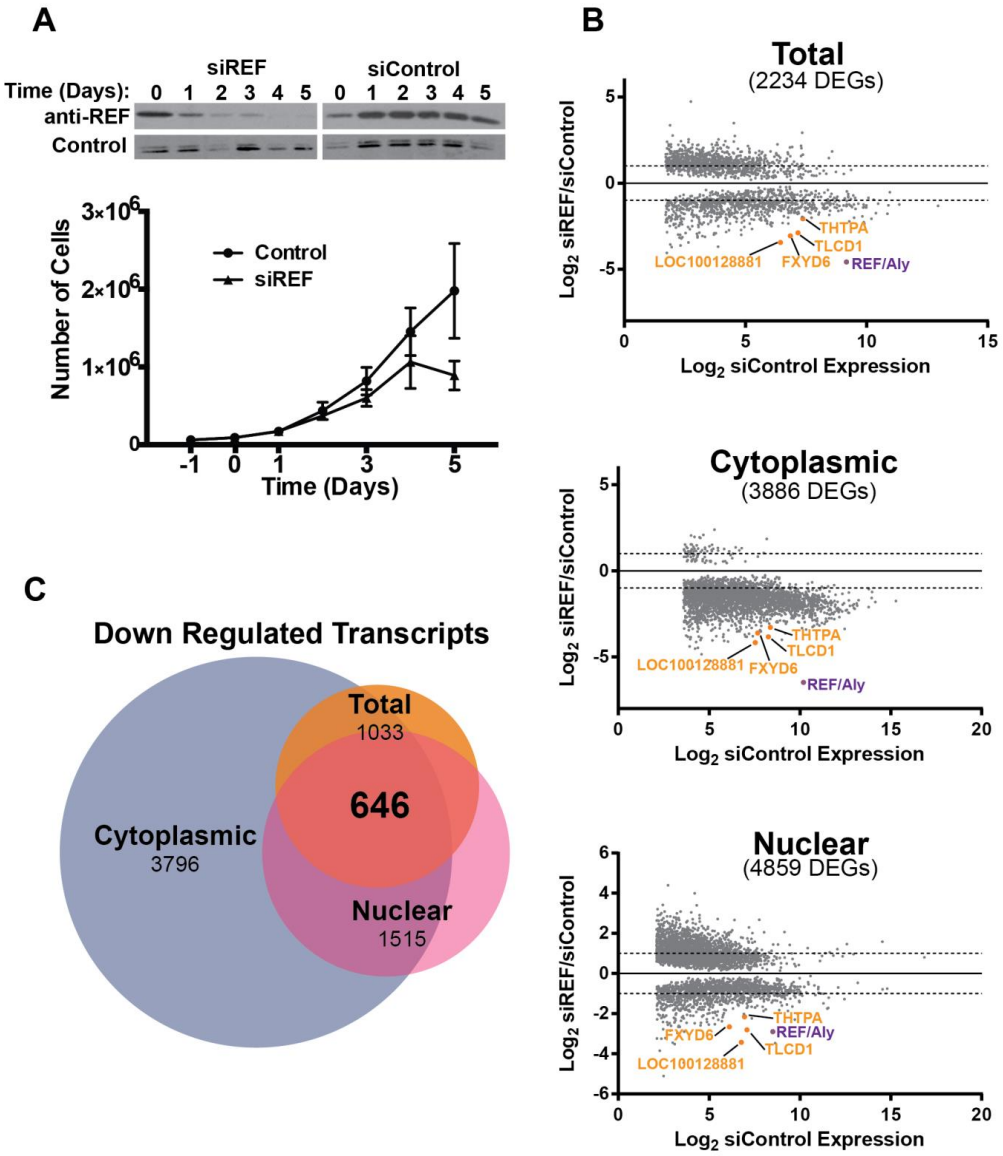

Figure 14. REF/Aly depletion alters RNA expression profiles. (A) *Top* Representative western blots of REF/Aly depletion over time. Western blots were performed on whole cell extracts from cells depleted with either REF/Aly or control siRNAs for the indicated times. The blot was probed with REF/Aly antibodies and hnRNP C1/C2 as a control. *Bottom* Growth curves of cells treated with REF or control siRNAs. Day 0 indicates the day of siRNA transfection. Error bars are standard deviation from the mean ($n=3$) (B) Scatter plots showing only the DEGs from each of the three samples as indicated. The y-axis is the differential of RNA levels between REF and control siRNAs, while the x-axis is the expression levels of the control siRNA sample. Note that the genes with the lowest 20% expression were omitted. (C) Venn diagram comparing the overlap of transcripts down-regulated upon REF/Aly depletion in the cytoplasmic (blue), total (orange) and nuclear (pink) samples.

Validation of RNA-Seq candidates

As expected, visual inspection of the mapped reads showed larger peaks in the control sample compared to the siREF/Aly sample, corresponding with the DEGs identified through Cufflinks (Figure 15A). To confirm RNA-Seq results, REF/Aly was knocked down in 293A-TOA cells for 96 hr, cells were fractionated, and RNA was analyzed by RT-qPCR. In accordance with RNA-Seq results, expression of candidate transcripts was decreased in the total, cytoplasmic, and nuclear fractions when REF/Aly was depleted (Figure 15B). Our RNA-Seq analysis used a two-siRNA pool targeting REF/Aly mRNA. To ensure that changes in expression levels were not a result of off-target effects from either of the siRNAs, each transcript was assayed for expression by RT-qPCR following depletion of REF/Aly with a single siRNA. All four candidate transcripts showed decreased RNA levels with single siRNA knockdown (Figure 15C). Importantly, decreased expression closely resembled knockdown with the siRNA pool, strongly supporting the conclusion that the candidate RNA levels are decreased as a result of REF/Aly depletion and are not due to off-target effects of either siRNA. We conclude that the RNA-Seq analysis uncovered transcripts whose abundance is dictated by REF/Aly. In addition, because the transcripts decrease in the cytoplasm and the nucleus, our data suggest that the diminished expression is not solely due to inefficient mRNA export.

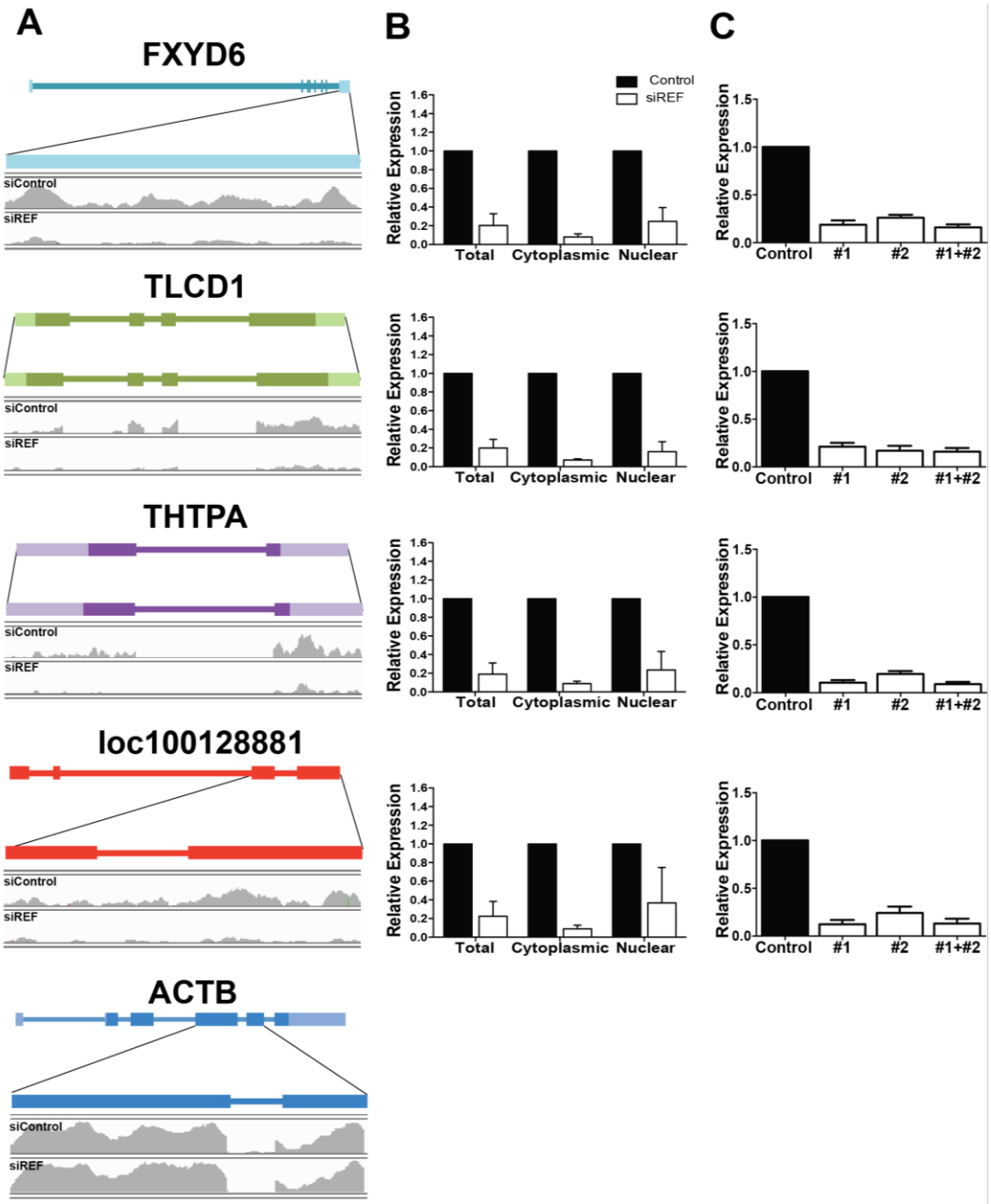

Figure 15. Validation of RNA-Seq candidates. (A) Diagram of candidate genes identified from RNA-Seq analysis. Mapped reads are shown for samples treated with siControl (top) or siREF (bottom) for area of gene indicated above traces. (B) RT-qPCR validation of candidate expression level following REF/Aly depletion and fractionation into nuclear and cytoplasmic fractions. (C) Relative expression of candidate transcripts measured by RT-qPCR following REF/Aly depletion with individual siRNAs (siREF #1 or siREF #2) or pooled siRNAs (siREF #1+2). RQ values in (B) and (C) were normalized to 7SK RNA expression; error bars are standard deviation from the mean (*n*=3).

Changes in RNA levels are not due to a decrease in RNA stability

As described above, we hypothesized that REF/Aly acts early in biogenesis of our selected target mRNAs. However, examination of steady-state RNA levels can be misleading because some transcripts might have been generated prior to functional knockdown of REF/Aly. In order to look at changes in RNA accumulation over a brief window subsequent to REF/Aly knockdown, we metabolically labeled RNAs using the modified uridine analog, 4-thiouridine (4SU), to capture only newly made RNAs (37, 170). Upon addition to the media, 4SU is taken up by cells and incorporated into nascent transcripts (Figure 16A). The analog-containing RNA can then be biotinylated using a thiol-disulfide exchange reaction with HPDP-biotin, captured on streptavidin beads, and detected by RT-qPCR. This assay allows for RNAs made during the 2-hr pulse to be isolated from RNAs made before the pulse. By performing the pulse after REF/Aly knockdown, we can ensure that the transcripts we are monitoring have been generated subsequent to functional knockdown of REF/Aly. The accumulation of our candidate transcripts was significantly lower when REF/Aly was depleted in these 4SU labeling assays (Figure 16B), mirroring the steady-state analyses (Figure 15B). Importantly, RNA was not recovered in the no-4SU control sample, demonstrating that we are assaying 4SU-containing RNAs rather than background signal. These data can be interpreted in two ways. First, REF/Aly may be involved in the synthesis of these transcripts. Second, REF/Aly may protect transcripts from a rapid decay process that functions within two hours. In either case, these data show that REF/Aly effects on candidate transcripts occurs relatively early in their biogenesis.

To determine if changes in the abundance of candidate transcripts upon REF/Aly depletion is a result of decreased RNA stability, we performed transcription inhibition experiments using the transcription inhibitor Actinomycin D (ActD). Following a 96-hr depletion of REF/Aly, cells were treated with 1 µg/ml ActD and RNA was harvested at time points from 0-4 hours after transcription shut-off. Comparison of the decay rates of specific RNAs in cells transfected with control siRNA or with REF/Aly siRNAs revealed no differences in RNA stability when REF/Aly was depleted (Figure 16C). We therefore conclude that changes in transcript abundance were not due to changes in RNA stability, at least over a 4-hour period. Because the 4SU labeling experiments (Figure 16A and 16B) demonstrated that the effects of REF/Aly are observed within 2 hr, we conclude that REF/Aly depletion most likely decreases the transcription rates of the candidate mRNAs.

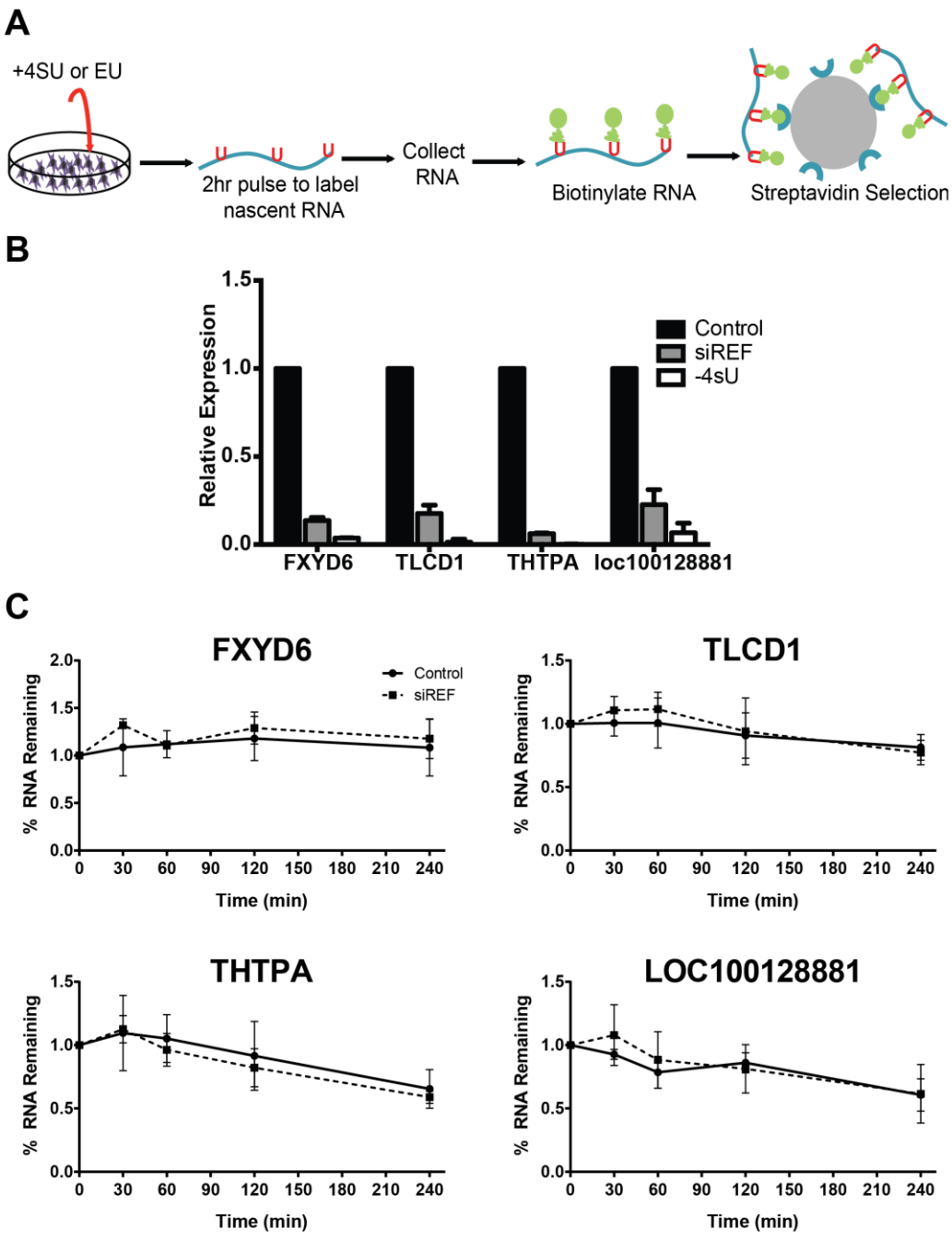

Figure 16. Decreases in RNA levels are not due to decreased stability. (A) Schematic of 4SU labeling experiments; see text for details. (B) Analysis of candidate transcript expression by RT-qPCR in a 4SU labeling experiment. -4SU samples were transfected with siControl but no 4SU was added during the pulse. (C) Decay curves of candidate transcripts following transcription inhibition with 1 µg/ml ActD. Dashed lines represent cells treated with siREF and solid lines represent cells treated with siControl. All experiments were performed three times and error bars represent standard deviation from the mean.

REF/Aly depletion leads to diminished pol II recruitment to target genes

To more directly assess REF/Aly's role in transcription, we employed ChIP to look at polymerase density on our genes of interest. Following a 96-hr knockdown, ChIP was performed with a pol II antibody that preferentially recognizes a hypophosphorylated form of the CTD (8WG16) (127). Five amplicons spanning the TLCD1 and THTPA loci were designed, as indicated in Figure 17A. Additionally, an amplicon located on chromosome 17 in a region with no annotated gene was used as a negative control. Pol II signal peaked in the amplicons closest to the transcription start site for both TLCD1 and THTPA, as expected for recognition of the hypophosphorylated CTD (13) (amplicons 3 and 2, respectively, see black bars in Figure 17B). Interestingly, when REF/Aly was depleted, a significant decrease in pol II signal was observed for both TLCD1 and THTPA. These data demonstrate that depletion of REF/Aly results in a decrease in pol II density on two genes that were identified in the RNA-Seq as having decreased expression levels when REF/Aly is depleted. We could not examine changes in transcription for FXYD6 or loc10012881 because both of these genes overlapped with neighboring genes, making experimental design of ChIP amplicons difficult. However, taken with the Act D and 4SU experiments (Figure 16), these data support the idea that REF/Aly is important for transcription of at least two candidate genes, TLCD1 and THTPA.

Trimethylation of lysine 36 on histone 3 (H3K36me3) is a marker for active chromatin found preferentially at the 3´ends of genes (3). H3K36me3 marks are linked to RNA processing factors and intron-exon boundaries (63, 66, 83). In addition, REF/Aly binds to Iws1, which is required for H3K36 trimethylation on at least some genes (166, 167).

Therefore, we reasoned that REF/Aly might be important for the H3K36 methylation of our candidate genes. To test this, we employed ChIP using an antibody that recognizes H3K36me3. As expected, H3K36me3 signal peaked in the 3´-most amplicon for both TLCD1 and THTPA (Figure 17C). However, when REF/Aly was depleted, no changes in H3K36me3 patterns were seen. Thus, even though REF/Aly depletion changes pol II density, it does not alter H3K36 trimethylation patterns on our candidate genes. Together, our data suggest that REF/Aly is necessary for efficient pol II loading, but its absence does not lead to a fully "closed" chromatin state.

Candidates are down-regulated soon after REF/Aly depletion

While REF/Aly depletion affects the steady-state levels of specific transcripts, we cannot conclusively state that these are due to direct effects of REF/Aly. For example, changes in RNA levels and transcription of our candidate transcripts could result from cell toxicity or other indirect effects. If so, we would expect direct effects of REF/Aly to be temporally linked to its depletion; that is, direct targets should be diminished relatively quickly upon REF/Aly depletion. To test this, we depleted REF/Aly for 48, 72, or 96 hr and measured RNA levels by RT-qPCR. As seen in Figure 18A, by 48 hr, candidate RNA levels are reduced by approximately 50% even though residual amounts of REF/Aly were still present (see Figure 14A) and no changes in growth rate (Figure 14A) or cell morphology (data not shown) were observed at this time. RNA levels continue to diminish over time correlating with REF/Aly depletion (Figure 18A). While this observation is by no means a

proof of a direct effect of REF/Aly, it is consistent with the idea that changes in RNA levels are a direct result of REF/Aly depletion and not a result of toxicity.

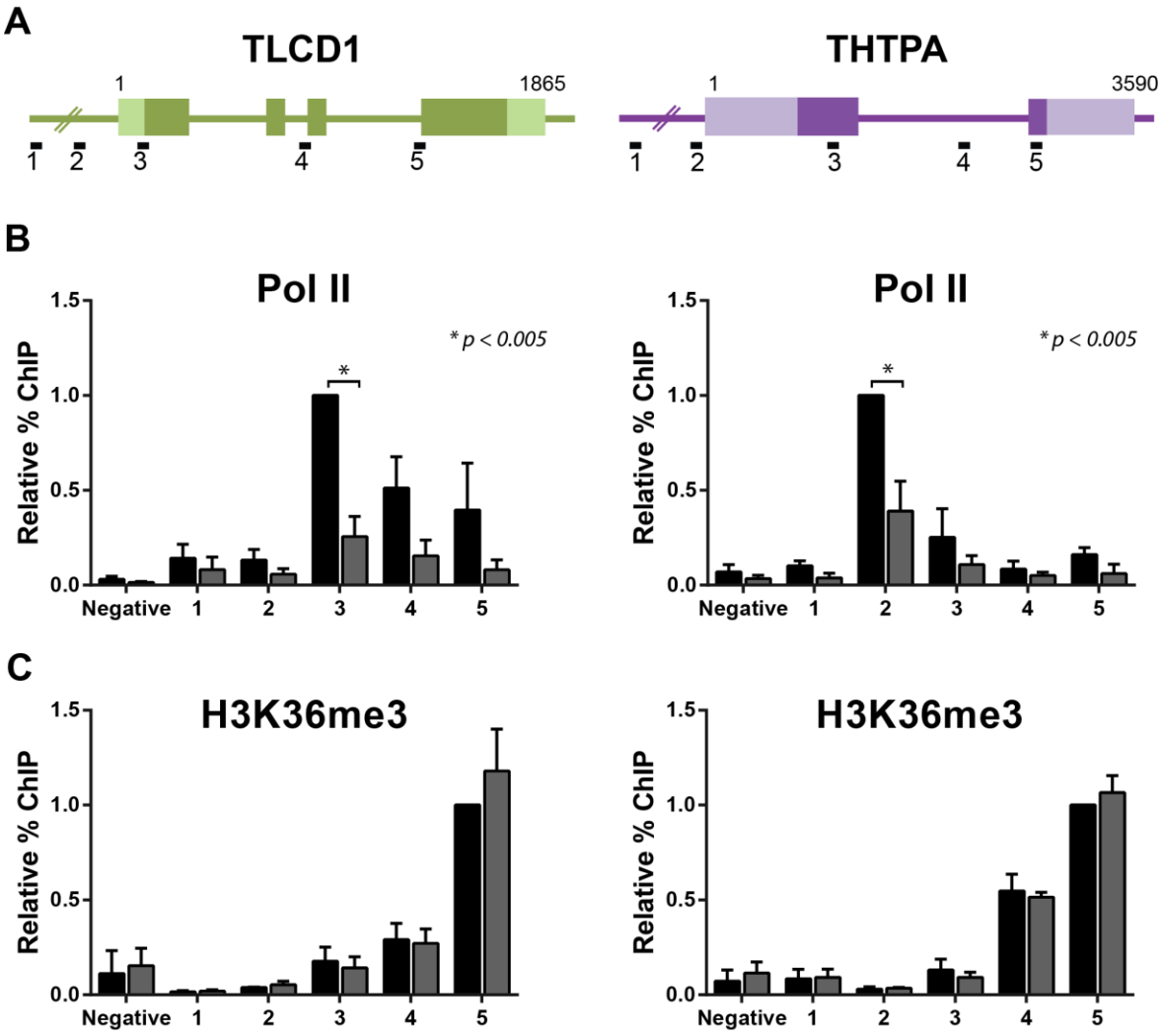

Figure 17. REF/Aly knockdown results in decreased pol II recruitment. (A) Diagram of TLCD1 and THTPA loci and amplicons used for ChIP experiments. (B) Relative ChIP signals for pol II are shown in the presence (black bars) or absence (grey bars) of REF/Aly. ChIP signal is normalized to the siControl signal at the amplicon closest to the TSS (TLCD1 #3, THTPA #2). The negative amplicon is located on chromosome 17 in a region that is at least 20 kb away from an annotated gene. (C) Relative ChIP signals for H3K36me3 in the presence or absence of REF/Aly as in part (B), except that ChIP signal is normalized to the siControl signal at the 3′-most amplicon (TLCD1 #5, THTPA #5). Error bars represent standard deviation from the mean (n=3). P values were calculated using two-tailed, unpaired Student's t test.

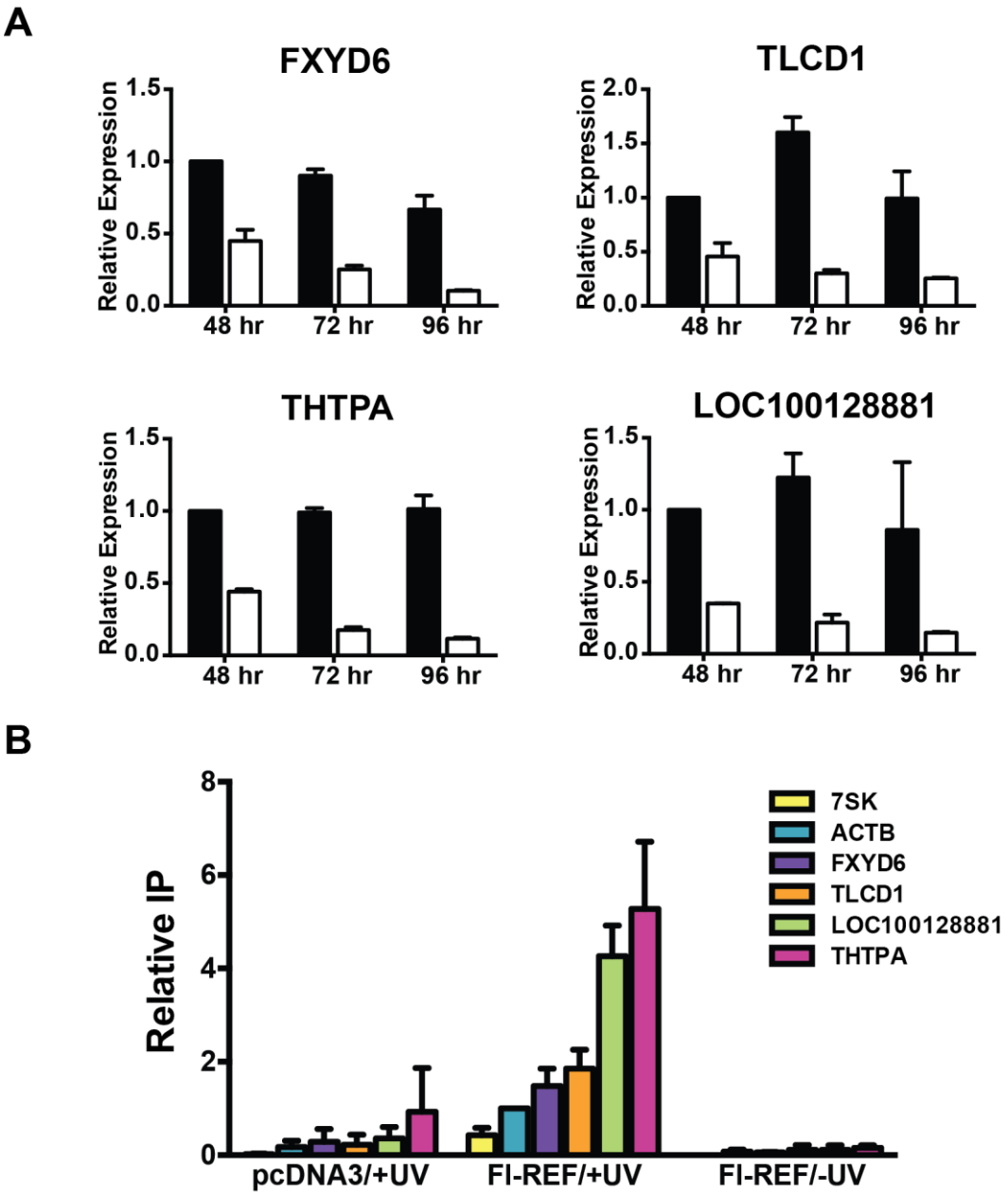

Figure 18. Candidate RNAs levels decrease quickly upon REF/Aly depletion and bind REF/Aly. (A) Relative expression of candidate transcripts were assayed by RT-qPCR analysis after a 48, 72, or 96-hr knock down of REF/Aly. Expression is represented relative to siControl sample after a 48-hr knock down. Error bars represent standard deviation from the mean (*n*=3). (B) Results from UV crosslinking RT-qPCR experiments. Cells were transfected with Fl-REF or a vector (pcDNA3) control and treated with UV as indicated. The bars represent the mean percent immunoprecipitation of RNA relative to ACTB, and error bars represent standard deviation from the mean (*n*=3).

REF/Aly preferentially binds to candidate transcripts.

Because REF/Aly is an RNA-binding protein, we hypothesized that REF/Aly binds directly to our candidate RNAs. To test this, we employed UV irradiation cross-linking followed by RNA immunoprecipitation (148). 293A-TOA cells were transfected with an expression construct for murine REF2-I protein that is N-terminally Flag-tagged (Fl-REF). Murine REF2-I is 74% identical to human REF/Aly, with the greatest variability in the N and C-variable regions, which are shorter in mouse than human (149). Twenty-four hr after transfection, cells were exposed to UV light to covalently crosslink protein with RNA. Following crosslinking, cells were lysed under stringent conditions and Fl-REF was immunoprecipitated using an anti-Flag antibody. Co-immunoprecipitated RNA was then detected by RT-qPCR. As seen in Figure 18B, all four candidate RNAs were immunoprecipitated more efficiently than a 7SK control RNA. Importantly, when cells were not exposed to UV irradiation, no immunoprecipitation of any RNAs was observed. This indicates that REF/Aly interaction with RNA occurs in cells and is not a result of protein re-assortment after cell lysis (116). Because UV irradiation only cross-links proteins that are in direct contact with RNA (27), the UV-dependent immunoprecipitation of candidate RNAs with Fl-REF indicates that REF/Aly directly binds to the candidate transcripts.

REF/Aly effects on gene transcription share both general and specific properties

REF/Aly has been ascribed both general and gene specific roles in RNA processing. We employed the same pol II ChIP assays to examine the housekeeping genes β-Actin (ACTB) and GAPDH, neither of which was identified as a DEG in all three samples in the

RNA-Seq analysis. ChIP assays showed an ~2-fold decrease in pol II density at the 5´ ends of both the ACTB and GAPDH genes (Figure 19A), suggesting that REF/Aly is important for transcription of additional genes not identified in the RNA-Seq. These data are consistent with the idea that REF/Aly acts as a general transcription factor or that cells are globally shutting down transcription in response to REF/Aly depletion. To assess global mRNA production, we depleted REF/Aly and examined the generation of newly made bulk poly(A) RNA using a labeling strategy similar to the 4SU experiments described above (51). Following 48, 72, or 96 hr knockdown, cells were incubated for 2 hr with an alkyne-modified uridine analog, 5-Ethynyl Uridine (EU). Like 4SU, EU is incorporated into nascent RNA during the pulse and can be conjugated with biotin to capture the RNAs generated during the 2-hr pulse (Figure 16A). Following streptavidin selection, RNA was treated with RNase T1, a G-specific endonuclease, to degrade all RNAs while leaving the poly(A) tails intact. Poly(A) RNA was then detected by northern blot. As seen in Figure 19B, bulk poly(A) RNA levels did not change as a result of REF/Aly knockdown, whether REF/Aly was depleted for 48, 72, or 96 hours. We therefore conclude that depleting REF/Aly does not lead to a global decrease in mRNA transcription. However, the transcription of some genes that were not identified in the RNA-Seq analysis may also be REF/Aly-dependent. When taken together, our data suggest that REF/Aly contributes to the efficient transcription of a wide spectrum of genes, but it is not absolutely required for global transcription.

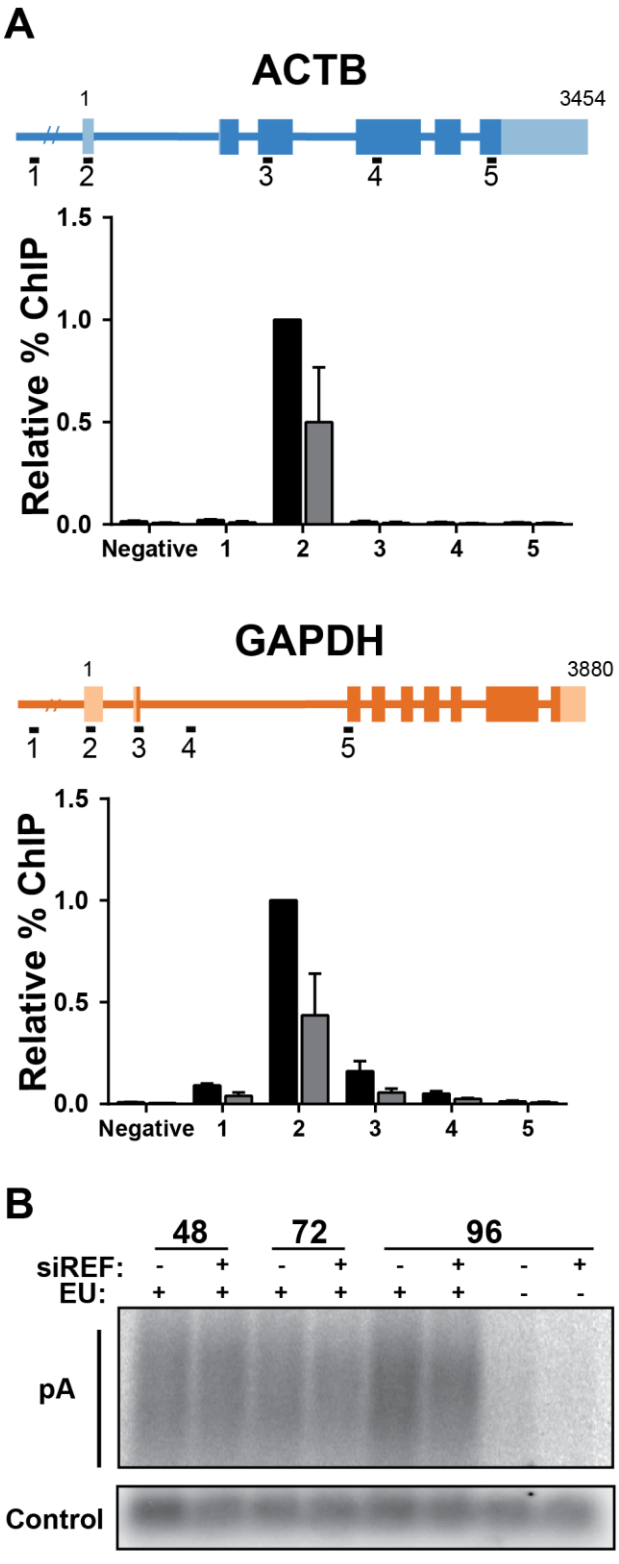

Figure 19. REF/Aly effects have both general and specific properties. (A) Pol II ChIP analysis of β-Actin (ACTB) or GAPDH loci after a 96-hr knockdown. Percent IP is plotted relative to signal in the siControl sample closest to the TSS (ACTB #2, GAPDH #2). Error bars represent standard deviation from mean (*n*=3). (B) Bulk poly(A) tail analysis of RNAs labeled for 2 hr with EU following transfection of siControl or siREF for 48, 72, or 96 hr. An exogenous biotinylated oligonucleotide was added to each sample to control for RNA recovery and gel loading.

Discussion

Much work has been done demonstrating the importance of coupling between nuclear events in gene expression. The data presented here suggest that the RNA export factor, REF/Aly, may be an important component linking transcription, RNA processing, and RNA export. While considerable work has examined the activity of REF/Aly and its homologs in mRNA export, REF/Aly is also capable of stabilizing a viral nuclear RNA and has been implicated in transcriptional control. Our RNA-Seq analysis suggests that a considerable number of transcripts are affected by REF/Aly upstream of export (Figure 14). We chose four candidates and confirmed that depletion of REF/Aly results in decreased steady-state levels (Figure 15). Moreover, stability analysis, metabolic labeling, and ChIP assays demonstrates that the transcription of these candidates is impaired upon REF/Aly depletion (Figure 16 and 17). Furthermore, REF/Aly binds directly to candidate transcripts (Figure 18), supporting a direct role for REF/Aly in their biogenesis. Together, these data suggest that REF/Aly promotes transcription of a subset of genes and may indicate that REF/Aly plays a functional role in linking splicing and export with transcription.

Our work shows that depletion of REF/Aly leads to decreased transcription of particular transcripts, but we cannot conclude from our current data whether that role is direct or indirect. REF/Aly plays important roles in the cell, particularly in facilitating export of mature mRNAs into the cytoplasm. In our studies, we have depleted REF/Aly, potentially disrupting important cellular processes and causing non-specific decreases in gene expression. Thus, we must allow for the possibility that changes in steady-state RNA levels, and particularly changes in transcription, are a result of indirect effects. However, several

pieces of data argue against that idea. After a 48-hr knockdown of REF/Aly, a significant decrease in candidate steady state RNA levels was observed (Figure 18A), even though REF/Aly is only partially depleted at this time point. Importantly, no changes in cell viability or morphology were observed at this time (Figure 14A, data not shown), suggesting that changes in gene expression at these earlier time points are not caused by indirect effects such as cell toxicity. Additionally, REF/Aly preferentially binds to candidate transcript RNAs when compared to a control RNA. Thus, REF/Aly binding to these transcripts may provide a direct link between REF/Aly, the bound mRNA, and the transcription machinery.

So how might REF/Aly lead to increased transcription efficiency? The TREX complex, including REF/Aly, is deposited on a transcript coincident with splicing of the 5´-most intron, a process that occurs co-transcriptionally. Thus, when REF/Aly is bound to a nascent RNA, it is located in close proximity to the chromatin and transcription complexes. The close vicinity of REF/Aly to transcription complexes may provide it with an opportunity to influence transcription. Similarly, Damgaard et al. established that mutation of the 5′ splice site reduces steady-state RNA levels by impairing transcription (33). Furthermore, they show that the 5′ splice site enhances transcription by increasing recruitment of preinitiation complexes (PICs) to gene promoters. Interestingly, a 5′ splice site, even in the absence of splicing, is capable of stimulating transcription, so it seems that nascent transcripts can recruit RNA-binding factors, U1 snRNP in this case, where they jointly serve to process RNA and to stimulate transcription. It is possible that REF/Aly similarly promotes recruitment or activity of transcription factors after its binding to the nascent transcript. Additionally, work by Neugebauer and colleagues (7) demonstrated that ChIP peaks of the

activating histone mark, H3K4me3, coincide with the 5´ splice sites of first introns. Both strong H3K4me3 peaks and H3K4me3 positioning at 5´ splices site depends on splicing. The authors also showed that splicing enhances pol II density. It is therefore tempting to speculate that splicing of the first intron, and the resulting deposition of REF/Aly in the TREX complex specifically to the 5´-most exon, is linked to this phenomenon. However, detailed mechanistic studies need to be performed to test this hypothesis.

Depending on the species and cell type, REF/Aly has been proposed to act as a general mRNA export factor or as a gene-specific factor. We cannot make definitive conclusions regarding the generality of REF/Aly in transcription from our data. Our RNA-Seq data led to the identification of only a subset of genes, suggesting a specific effect, but it is important to point out that normalization schemes used for RNA-Seq experiments can be biased against factors that have general effects (100). Consistent with the idea that REF/Aly plays a broader role in transcription, both GAPDH and ACTB genes showed diminished pol II recruitment (Figure 19A), even though neither transcript was identified in our RNA-Seq analysis. However, we also show that bulk levels of newly synthesized mRNAs are not diminished after REF/Aly depletion, showing that bulk poly(A) RNA production occurs at similar global levels when REF/Aly is knocked down (Figure 19B). Whether or not REF/Aly acts on a specific subset of genes, or plays a more global role in transcription remains to be more definitively characterized.

REF/Aly has been implicated in promoting mRNA export, transcription, and nuclear RNA stability; however the contributions of REF/Aly to each of these activities to the cell remain unknown. Our studies specifically targeted REF/Aly's roles in stability and/or

transcription, so we cannot make any judgments regarding whether export is the primary role of REF/Aly. Interestingly, our RNA-Seq data showed that virtually all of the DEGs identified in the cytoplasmic fractions were down-regulated, strongly supporting a role for REF/Aly in nuclear mRNA export (Figure 14B). Consistent with this idea, a significant fraction of the DEGs in the nuclear fraction were up-regulated (Figure 14B), so it seemed reasonable that these transcripts accumulate in the nucleus upon export block. Surprisingly, however, among the transcripts that were down-regulated in the cytoplasm, only ~10% were also identified as being up-regulated in the nucleus (data not shown). While this suggests that the differential expression of mRNAs identified in the RNA-Seq is not due to export block, our analysis was not optimized to identify targets of REF/Aly mediated mRNA export and further bioinformatic analyses must be performed to directly test this idea. In addition, we were surprised that none of our candidates appeared to have altered stability upon REF/Aly knockdown (88, 89, 148). Of course, until we assay candidates in addition to the four examined here, we cannot rule out that other candidates were destabilized in the absence of REF/Aly. Therefore, our data do not demonstrate a role for REF/Aly in nuclear stability of endogenous transcripts, but this should not be interpreted to mean that no such activity is present.

Until recently, cross-talk between RNA processing events has been thought to take place in a forward manner. That is, transcription machinery positively influences factors involved in pre-mRNA processing. However, a growing body of evidence suggests that enhancement of gene expression occurs in a bidirectional fashion with RNA processing factors positively regulating transcription efficiency. Our work suggests that REF/Aly

influences this bidirectional coupling but additional studies are required to determine the mechanisms of REF/Aly-mediated enhancement of pol II recruitment.

CHAPTER SIX

Conclusions and Future Directions

Conclusions

The work presented here has focused on elucidating additional roles in gene expression for the RNA export protein, REF/Aly. Our data suggests two additional functions for REF/Aly in RNA stability and transcription, independent of its function in mRNA export. Previous work from our lab demonstrated that PAN RNA is stabilized by the viral factor, ORF57. The work presented here demonstrates that ORF57-mediated PAN RNA stabilization is at least in part due to REF/Aly, and that REF/Aly is both necessary and sufficient to confer increased stability to the transcript. Subsequently, we were interested in determining if REF/Aly affected RNA biogenesis of endogenous transcripts, upstream of export. Using an RNA-Seq approach, we found that REF/Aly affects gene expression at the level of transcription. Depletion of REF/Aly results in a decrease of pol II density at the gene promoter, supporting a role for REF/Aly in polymerase recruitment to the gene. Although our work has focused on one RNA processing factor, our data suggests added layers of coupling between steps in RNA biogenesis and supports a multifunctional role for RNA processing proteins.

REF/Aly stabilizes a nuclear transcript

Previous work identified ORF57 as an important protein in many steps of viral gene expression. Two pertinent functions of ORF57 are in viral mRNA export and RNA stability.

A role for ORF57 in RNA export was first proposed because it directly binds the export protein REF/Aly, and subsequently ORF57 was found to recruit all members of the TREX complex (10, 107). Additionally, ORF57 increases the stability of viral transcripts. Particularly, ORF57 binds the *nuclear* PAN RNA through an ORF57-responsive element (ORE) and protects the RNA from nuclear degradation (136, 140). Based on these observations, several important questions arise. Are TREX proteins recruited to the ORF57: PAN RNA complex, or are ORF57 interactions with TREX and PAN RNA independent? Does ORF57 itself protect the transcript, or does it function to recruit other stabilization factors?

The data presented in chapter four seeks to answer some of those questions. We show that ORF57-mediated stabilization of PAN RNA is at least in part a result of REF/Aly activity. First, using UV crosslinking assays demonstrate that REF/Aly is recruited to PAN RNA in an ORF57-dependent fashion and that REF/Aly binds directly to the RNA. REF/Aly binding to PAN RNA exhibits a 5′ bias, reminiscent of REF/Aly interactions with exported RNAs. Additionally, REF/Aly is necessary and sufficient to stabilize PAN RNA. Abrogation of the REF/Aly:ORF57 interaction abolishes ORF57's stabilization function. Using tethering assays we determined that REF/Aly is sufficient to increase PAN RNA stability, and increased stability is not due to PAN RNA being exported to the cytoplasm and thus protected from nuclear decay factors. Taken together, these data support the hypothesis that ORF57-mediated stabilization of PAN RNA is a result of REF/Aly recruitment and that REF/Aly confers stability to the transcript.

How the RNA is stabilized remains elusive. However, it appears that REF/Aly mediated stabilization of PAN RNA might involve protection of the poly(A) tail from nuclear decay machinery. Tethering of REF/Aly to PAN RNA resulted in an increase in poly(A) tail length, supporting the idea that REF/Aly protects the poly(A) tail from deadenylation and/or promotes readenylation of the transcript to counterbalance nuclear deadenylation, which was previously shown to precede PAN RNA destruction (28). Stability may be conferred by REF/Aly alone, or binding of REF/Aly to the RNA may recruit additional proteins, such as other TREX components to the RNA to protect it from degradation.

Does REF/Aly participate in RNA quality control?

Our data has interesting implications for REF/Aly's role in nuclear RNA surveillance mechanisms. When an RNA is misprocessed, unfolded, or forms an aberrant mRNP, it is recognized by RNA surveillance mechanisms, also known as RNA quality control pathways. These pathways then degrade the unsuitable RNAs so that they cannot be exported and translated into defective proteins. While yeast Yra1 has previously been implicated in RNA surveillance, no work has demonstrated a link between human REF/Aly and RNA QC. RNA surveillance proteins are thought to recognize mRNPs at the nuclear pore and allow the mRNP to travel through the nuclear pore. However, Yra1 is not a shuttling protein and must be removed before export. Indeed, Iglesias et al. showed that Yra1 is ubiquitinated which results in removal of Yra1 from the mRNP, making the mRNP competent for export (69). These studies suggest that REF/Aly bound to a transcript is a signal for an mRNP that is in

the process of maturing and protects it from nuclear degradation, indicative of a more indirect role for REF/Aly in RNA QC. In this scenario, it is advantageous for an intronless or poorly spliced transcript to interact with REF/Aly so that it is protected from nuclear decay because these transcripts tend to have a longer nuclear dwell time.

Work performed in these studies supports the hypothesis that REF/Aly actively protects PAN RNA from nuclear decay. First, PAN RNA is stabilized by REF/Aly either through recruitment via ORF57 or through direct tethering of REF/Aly to the RNA. FISH analysis reveals that even though REF/Aly is tethered to PAN RNA, it does not get exported, demonstrating that stabilization of the transcript occurs in the nucleus. Second, tethering of REF/Aly to PAN RNA results in a modest increase in poly(A) tail length. Indeed, previous studies in yeast have proposed models where transcripts that are hyperadenylated get retained at the site of transcription and are more stable (135).

The mechanism of REF/Aly protection to PAN RNA remains unresolved; however several non-mutually exclusive mechanisms are possible. From our studies it is obvious that REF/Aly plays an active role in protecting the transcript from degradation. Interestingly, Yra1 interacts with the poly(A) binding protein, Nab2 in yeast, supporting a model where the 5′ and 3′ ends of the RNA interact with one another. Perhaps the poly(A) tail is protected from nuclear decay by circularization of the transcript through interactions of REF/Aly and poly(A) binding proteins. Based on our observations that REF/Aly tethering to PAN RNA results in an increase in poly(A) tail length and previous work showing that hyperadenylated transcripts are retained at the site of transcription (135), perhaps REF/Aly association with the RNA helps to sequester the mRNP at a specific location in the nucleus where it is

protected from degradation. Indeed, our data support a model where REF/Aly plays a direct role in nuclear RNA surveillance, and not a bystander role as a checkpoint for export.

A role for REF/Aly in KSHV

Our studies took advantage of the virus KSHV and several of its factors to unveil a new role for the export adaptor, REF/Aly. Not only do these studies present interesting data for the function of REF/Aly in RNA stability and quality control, but they also imply an added role for REF/Aly in KSHV RNA metabolism. The function of PAN RNA remains poorly characterized, however it accumulates to extremely high levels during viral infection, inferring an important role for PAN RNA in viral infection. Accumulation of PAN RNA is the result of contributions from multiple steps in RNA biogenesis. The PAN RNA promoter is extremely robust during lytic infection, resulting in high levels of transcription that are driven by the ORF50 protein (162). As mentioned previously, PAN RNA also contains a *cis*-acting stability element, termed the ENE that helps to stabilize the RNA through protection of the poly(A) tail (29, 30, 117). Additionally, ORF57 functions to stabilize PAN RNA (136), and we now demonstrate that REF/Aly is an important cofactor for ORF57-mediated stabilization. Because multiple mechanisms exist to contribute to PAN RNA's expression, it seems reasonable that its function is at least partially dependent on its abundance, which is partially due to REF/Aly.

Depletion of REF/Aly significantly alters gene expression

In the studies presented in chapter five, we analyzed gene expression levels in the presence or absence of REF/Aly by RNA-Seq. RNA-Seq analysis identified a large number of transcripts whose expression was altered in the absence of REF/Aly. We chose to fractionate our samples into nuclear, cytoplasmic, and total fractions in order to look at changes in expression in each compartment. While we focused on transcripts that were down regulated in the absence of REF/Aly in all fractions, we also noticed a significant number of transcripts that were up regulated in at least one fraction in the absence of REF/Aly. Transcripts whose expression is up regulated in the nuclear fraction and down regulated in the cytoplasmic fraction are thought to be transcripts that are retained in the nucleus due to an export block induced by REF/Aly depletion. Because REF/Aly's export function is well characterized, we chose to analyze transcripts that might be affected by REF/Aly early in their biogenesis, particularly at the level of RNA stability and transcription. We hypothesized that transcripts affected by REF/Aly early on would exhibit decreased expression in both the nuclear and cytoplasmic fractions, resulting in decreased expression in the total sample. We confirmed that a handful of our RNA-Seq hits were down regulated in each sample after knockdown and fractionation. To test that REF/Aly was important for their stability we performed transcription inhibition experiments and measured remaining RNA over time. No significant changes in decay rates were observed when REF/Aly was depleted. However, analysis of additional candidates from the RNA-Seq data is necessary to draw a firm conclusion. We also performed transcription pulse experiments for two hours and examined transcript levels after the pulse. Interestingly, we found that in the absence of

REF/Aly, transcript levels are lower, even after a short pulse. Differences in transcript levels during the pulse might indicate that REF/Aly protects transcripts from rapid decay; however, our decay data does not support that hypothesis. Alternatively, changes in expression might be due to changes in synthesis of the RNA in the absence of REF/Aly. Based on these observations, we chose to further explore the possibility that REF/Aly may be involved in transcription.

REF/Aly affects transcription

The data presented in chapter five supports a role for REF/Aly in polymerase recruitment to genes. ChIP assays revealed that levels of pol II on the transcript were reduced when REF/Aly was depleted. Interestingly, changes in pol II density were also observed for genes that were not identified in the RNA-Seq, particularly β-actin and GAPDH. This suggests that depletion of REF/Aly may cause global changes in transcription. Conversely, when a two hour transcription pulse was performed and bulk polyadenylated RNA was analyzed, no significant differences were seen with or without REF/Aly, supporting a role for REF/Aly on a specific subset of genes. However, further exploration is needed to determine if REF/Aly acts as a general or a specific transcription factor.

Bidirectional feedback between transcription and RNA processing is becoming increasingly apparent, and REF/Aly may be acting in the same manner. One reasonable hypothesis is that loading of REF/Aly onto transcripts results in a positive feedback to transcription. For our analysis we used the 8WG16 antibody that recognizes

hypophosphorylated pol II at the gene promoter. Therefore, changes in pol II density that are observed suggest that pol II recruitment is decreased in the absence of REF/Aly. Preliminary studies using an antibody that recognizes elongating polymerase suggest that elongating polymerase density may be decreased in the gene body, at least on TLCD1 (unpublished observations).

Previous reports of REF/Aly function have implicated its involvement in transcription. REF/Aly interacts with and enhances the DNA binding activity of the transcription factors AML-1 and LEF-1, and overexpression of REF/Aly led to an increase in the activation of the TCRα enhancer complex (14). REF/Aly was also shown to promote dimerization of basic region-leucine zipper (bZIP) transcription factors that increase association of bZIP containing proteins and DNA (160). Finally, REF/Aly also associates with Iws1, which interacts with the transcription elongation factor Spt6 (166). Together with these observations, our data suggest a link between REF/Aly and transcription.

REF/Aly is a multifunctional protein

The work presented here demonstrates that REF/Aly is a multifunctional protein that is important for both RNA stability and transcription in addition to its function in RNA export. As seen in Figure 20, REF/Aly may be involved in linking transcription efficiency with splicing and protecting maturing RNPs from nuclear degradation. REF/Aly and the TREX complex are recruited co-transcriptionally to RNA in a splicing dependent manner, placing REF/Aly and TREX in close proximity to transcription complexes. Work from several labs has demonstrated a link between splicing and transcription. For example,

patterning of the activating histone mark, H3K36me3 is influenced by pre-mRNA splicing (83). Additionally, 5´ splice sites appear to be important determinants of transcription. Mutating the 5′ splice site impairs transcription (33) and the activating histone mark, H3K4me3 coincides with the 5´ splice sites of the first introns (7). Because of these links between splicing and transcription, when taken with our data, it is reasonable to hypothesize that REF/Aly acts to link RNA export and transcription in a bidirectional manner.

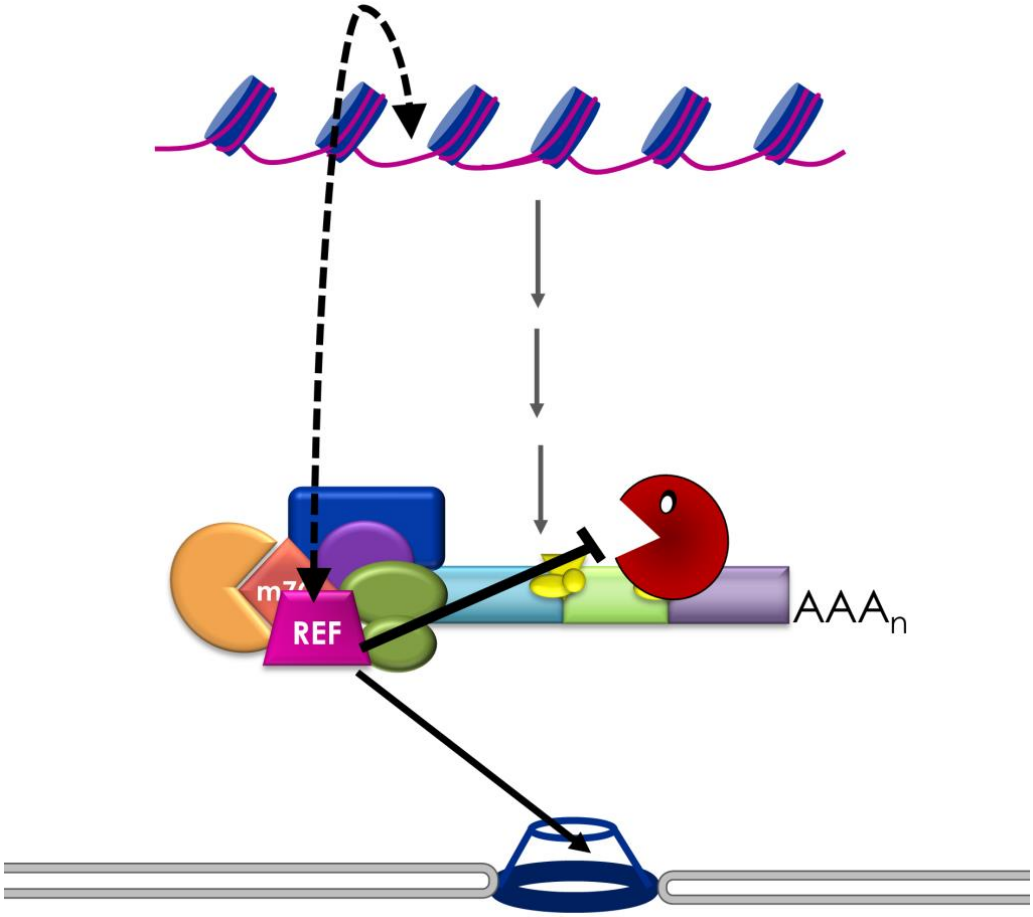

Figure 20. REF/Aly influences multiple steps in RNA biogenesis. REF/Aly plays a well-established role in RNA export, and our data suggests roles for REF/Aly in RNA stability and transcription, demonstrating that REF/Aly is important for multiple steps of RNA biogenesis.

Future Directions

While our work has shown that REF/Aly functions in additional steps in RNA biogenesis aside from export, much work remains to be done to determine how REF/Aly is involved mechanistically in these processes. A greater understanding of REF/Aly's contribution to nuclear stability and transcription is critical to fully grasp all steps in RNA biogenesis. Additionally, further studies determining the importance of REF/Aly in the context of KSHV infection and PAN RNA are needed to better comprehend the biology of the virus and how it uses host machinery for its own production.

We have shown that REF/Aly stabilizes a viral transcript, PAN RNA, but is this function limited only to PAN RNA, or is REF/Aly responsible for stabilizing other transcripts, viral or cellular? Using structural bioinformatics analysis, Tycowski et al. recently identified ENE like elements in six additional viruses, five DNA viruses and one RNA virus. All ENE-like elements in the dsDNA viruses were found in noncoding regions of the genome, likely indicating that they are located within long noncoding (lnc) RNAs. Two PAN RNA homologs were identified in the gammaherpesviruses, Rhesus rhadinovirus (RRV) and Equine herpesvirus 2 (EHV2). Importantly, the RRV ENE was demonstrated to be essential for accumulation of RRV PAN RNA, highly reminiscent of KSHV PAN RNA (157). It will therefore be interesting to determine if REF/Aly also exhibits a stabilizing function on these RNAs since they exhibit similar properties to PAN RNA. Whether or not an ORF57-like protein is used to recruit REF/Aly to PAN RNAs or if REF/Aly is recruited in a unique fashion will also be an interesting area of study.

Transcripts that were identified in our RNA-Seq analysis do not seem to be affected at the level of stability in the absence of REF/Aly. While this data does not support a role for REF/Aly in endogenous RNA stability, a limited number of candidates were tested, and we cannot rule out the possibility that REF/Aly acts on a particular subset of RNAs that were not identified in our RNA-Seq and that were not a focus of our studies. REF/Aly's stability function may act solely on a particular group of noncoding or nuclear RNAs. If REF/Aly functions only in stabilizing nuclear RNAs, we would not have identified this in our RNA-Seq data analysis. Candidates were chosen based on significant decreases in cytoplasmic (and nuclear) expression in the absence of REF/Aly. Strictly nuclear RNAs however would not have been identified because their expression level in the cytoplasmic fraction would remain absent and thus unchanged. Therefore, it will be interesting to determine if REF/Aly acts on other nuclear retained RNAs, similar to its mode of action with PAN RNA by further analyzing the RNA-Seq data and specifically looking for nuclear RNAs.

Our studies also identified REF/Aly as an important co-factor for ORF57 mediated stabilization of PAN RNA, and other recent studies suggest that the REF/Aly:ORF57 interaction is important for the stabilization of several other viral transcripts (92). Interestingly however, recent studies indicate that abrogation of the REF/Aly:ORF57 interaction does not affect viral titer. UIF, an export adaptor with redundant function to REF/Aly, is upregulated upon REF/Aly depletion [unpublished observations,(55)], and has been shown to interact with ORF57 (72). Indeed, depletion of REF/Aly and UIF is needed to inhibit ORF57-mediated viral RNA export. While these studies suggest that UIF may be redundant to REF/Aly in export functions, no work has been done examining UIF's ability to

stabilize RNA. In order to understand the importance of export adaptors in KSHV lytic replication it is imperative to deplete both REF/Aly and UIF and examine viral RNA decay rates.

Exactly how REF/Aly confers increased stability to a transcript is also an area that needs further exploration. As mentioned previously, one way that REF/Aly might promote stability is through protection of the poly(A) tail from nuclear decay factors. It will be important to determine if human REF/Aly also interacts with poly(A) binding proteins in a similar fashion to yeast Yra1, and if this interaction confers protection to the transcript from decay. Interactions between proteins associated at both ends of the RNA suggest a circularization of the RNA which might serve to protect the RNA. This has implications not only for viral transcripts that are stabilized by REF/Aly, but also for cellular transcripts. Circularization of a transcript throughout the maturation process might protect the RNA from nuclear decay pathways by sequestering the poly(A) tail. Identifying other proteins that interact with REF/Aly on the same RNA molecule will give insight into the overall architecture of the transcript, and will yield information into REF/Aly's role in stabilization.

RNA-Seq data revealed that expression of a large group of transcripts are affected when REF/Aly is depleted. Our studies focused on transcripts that were down regulated in the absence of REF/Aly and we determined that changes in expression were occurring at the level of transcription. We identified changes in pol II density on genes that were identified in the RNA-Seq analysis and some genes that were not, although REF/Aly does not appear to globally decrease polyadenylated transcripts. Global assays such as ChIP-Seq when REF/Aly is depleted will be helpful in distinguishing if REF/Aly acts on a particular subset

of genes or if it exhibits a more global effect and will provide more information about REF/Aly's role in RNA biogenesis.

How REF/Aly contributes to transcription and pol II recruitment to a gene will also be an important area to explore. Further studies are needed to understand how REF/Aly is contributing to transcription and RNA pol II recruitment to a gene. REF/Aly is well documented to bind RNA, however data analyzing REF/Aly interactions with DNA is lacking. It will also be interesting to determine if REF/Aly interaction with DNA increases transcription, or if REF/Aly association with RNA is sufficient to regulate transcription. One way to approach these questions is through the use of tethering assays. Using the MS2 binding system that was described earlier coupled with ChIP assays will give insight into the necessity for REF/Aly interaction with RNA for transcriptional effects.

Finally, although we have demonstrated an importance for REF/Aly in nuclear RNA stability and transcription, it will also be interesting to examine additional TREX complex members. Other TREX proteins have been implicated in coupling between processes, so it is not unreasonable to hypothesize that additional TREX factors might play multiple roles in the nucleus aside from facilitating export. Alternatively, REF/Aly may possess a unique function compared with other TREX proteins and may be the only TREX member that is critical for additional steps in RNA biogenesis.

In conclusion, we have identified two additional functions of REF/Aly aside from its well-studied function in RNA export. First, using viral factors we determined that REF/Aly stabilizes the viral transcript, PAN RNA independent of export. Additionally, we found that REF/Aly is recruited to PAN RNA by the multifunctional viral protein, ORF57 to promote

stabilization of the transcript. Using an RNA-Seq approach coupled to REF/Aly depletion, we identified a subset of transcripts whose expression was altered in the absence of REF/Aly and determined that changes in expression are a result of decreased recruitment of pol II to the target genes. Taken together, our data support a role for REF/Aly in multiple functions including RNA export, RNA stability, and transcription.

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