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Accessibility
RanBP2/Nup358 Potentiates the Translation of a Subset of mRNAs Encoding Secretory Proteins

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Abstract

In higher eukaryotes, most mRNAs that encode secreted or membrane-bound proteins contain elements that promote an alternative mRNA nuclear export (ALREX) pathway. Here we report that ALREX-promoting elements also potentiate translation in the presence of upstream nuclear factors. These RNA elements interact directly with, and likely co-evolved with, the zinc finger repeats of RanBP2/Nup358, which is present on the cytoplasmic face of the nuclear pore. Finally we show that RanBP2/Nup358 is not only required for the stimulation of translation by ALREX-promoting elements, but is also required for the efficient global synthesis of proteins targeted to the endoplasmic reticulum (ER) and likely the mitochondria. Thus upon the completion of export, mRNAs containing ALREX-elements likely interact with RanBP2/Nup358, and this step is required for the efficient translation of these mRNAs in the cytoplasm. ALREX-elements thus act as nucleotide platforms to coordinate various steps of post-transcriptional regulation for the majority of mRNAs that encode secreted proteins.

Introduction

In eukaryotic cells, mRNA synthesis and processing occur in the nucleus while the translation of mRNA into protein is restricted to the cytoplasm. Although these various mRNA metabolic steps take place in distinct compartments, these events are biochemically coupled [1,2]. For example, the 5’ cap binding complex and the spliceosome collaborate to deposit the transcription export (TREX) complex on the 5’ end of a newly synthesized transcript [3]. TREX then recruits the TAP/p15 heterodimer, which ultimately allows the mRNA to be exported from the nucleus into the cytoplasm [4,5].

Despite the fact that the vast majority of transcripts contain introns and should therefore use the splicing-dependent export mechanism, we previously demonstrated that most mRNAs that encode secreted proteins contain RNA elements that promote an alternative mRNA nuclear export (ALREX) pathway that is independent of both splicing and a 5’ cap structure [6]. In addition, these ALREX-promoting sequences are found within the signal sequence coding region (SSCR) at the 5’ end of the ORF. SSCRs from vertebrates (and to a lesser extent in invertebrates) tend to contain long stretches of nucleotide sequence that lack adenine. This depletion in adenines is due to the enrichment in both amino acids that are encoded by adenine-poor codons and synonymous codons lacking adenine. Indeed, ALREX activity can be inhibited when nucleotides within the SSCR are silently substituted for adenines, so that the encoded amino acid remains unaltered [6].

Recently, we demonstrated that SSCR-containing genes tend to lack introns in their 5′ UTR (i.e., upstream of the SSCR) [7]. When SSCRs were present in genes that contained 5′ UTR introns, these SSCRs were not as depleted of adenines, and did not promote the export of a reporter mRNA [7]. These results suggested that the 5′ most element in a transcript, be it an intron or SSCR, dictates whether the mRNA is exported by either the splicing or ALREX pathway. This model is supported by the observation that ALREX-promoting elements only potentiate export when present near the 5′ end of a reporter transcript [8].

Interestingly, the incorporation of silent adenine mutations into the ALREX-promoting SSCR not only inhibited nuclear export, but also induced the formation of cytoplasmic stress granules (SGs) into which the mutated mRNAs partially accumulated [6]. Typically, these cellular structures form in response to an accumulation of cytoplasmic transcripts that fail to initiate proper
Author Summary

About one-fifth of the protein-coding genes in the human genome code for secreted and/or membrane-bound proteins. In the nucleus these genes are transcribed into messenger RNAs (mRNAs), which are then exported to the cytoplasm. These mRNAs are then transported to the surface of the endoplasmic reticulum where they are translated into proteins destined for the secretory pathway. Most of these mRNAs contain signal sequence coding regions (SSCRs), which code for short hydrophobic polypeptides that target the newly synthesized proteins for translocation across the endoplasmic reticulum membrane. Previously, we found that many SSCRs also act as RNA elements that promote the efficient nuclear export of mRNAs. Here we present evidence that upon the completion of nuclear export, SCCR-containing mRNAs interact with RanBP2/Nup358, a large protein found on the cytoplasmic face of the nuclear pore. This interaction is mediated by direct binding between the SSCR and zinc finger repeats found within RanBP2/Nup358, and is ultimately required for the efficient translation of SCCR-containing mRNAs into secretory and/or membrane-bound proteins. Our work demonstrates that SSCRs act as nucleotide platforms that recruit various factors to the mRNA throughout its life to regulate distinct events, such as nuclear export and translation.

translation [9,10]. This observation suggested that the ALREX-promoting element might influence not only export, but also other downstream events, such as how the mRNA is localized in the cytoplasm and how efficiently the translation-initiation complex is assembled onto the transcript.

Here we provide evidence that ALREX-promoting elements potentiate translation. Furthermore, our data indicate that these RNA elements associate with the zinc finger repeats (ZFRs) of RanBP2, a large protein found on the cytoplasmic face of the nuclear pore complex. We then demonstrate that the depletion of RanBP2 not only prevents the normal potentiation of translation by ALREX-promoting sequences, but also inhibits the global synthesis of secreted and likely mitochondrial proteins.

Results

ALREX-Promoting SSCRs Enhance Protein Production

Previously, we investigated the function of ALREX-promoting SSCRs in mediating mRNA localization of the fushi tarazu mRNA (ftz), a reporter transcript that has been used to study mRNA splicing [11] and nuclear export [6,12,13]. We also found that ALREX-promoting SSCRs promote the translation of the ftz reporter mRNA into protein (AFP, unpublished observations). However, these initial observations were uninformative as they could not determine whether ALREX-promoting SSCRs contain an RNA element that potentiates protein production in a manner independent of codon effects. Indeed, there is a correlation between [1] the frequency with which a codon is present in the genome (i.e., “codon usage”), [2] the amount of cognate tRNA that is present in the cytoplasm, and [3] the number of copies of that tRNA gene [14–16].

To disrupt features that promote ALREX, such as adenine depletion, while simultaneously controlling for codon usage effects, we altered constructs that had ALREX-promoting SSCRs by mutating leucine codons so that they now encoded isoleucine. While leucine codons are relatively adenine-poor, all of the isoleucine codons contain at least one adenine. Moreover, isoleucine and leucine have almost the same hydrophobicity and the substitution of one for the other is unlikely to alter the strength of the encoded signal sequence [17–19]. We chose to alter two constructs, MHC-ftz and insulin (INS)-ftz, which contain ALREX-promoting SSCRs derived from the mouse major histocompatibility H2kb gene and the human insulin gene, so that each had two leucine-to-isoleucine mutations (creating the 2lle-MHC-ftz and 2lle-INS-ftz constructs; see Figure 1A, 1B, and Table S1). In each construct, the substituted codons had similar usage frequencies as the original ones (Figure 1A and 1B) [20]. We then transfected cells with plasmids containing these various ftz constructs and monitored ftz protein production 18–24 h later. Note that the ftz reporter contains sequences that code for HA and FLAG epitopes, as described previously [6], and can be used to detect the protein by immunoblot. To control for transfection efficiency and gel loading, we co-transfected the cells with plasmids containing the Histone 1B (H1B)-GFP gene, and immunoblotted for green fluorescent protein (GFP) and β-tubulin.

We found that cells expressing MHC-ftz mRNA produced about twice as much ftz protein when compared to those expressing 2lle-MHC-ftz mRNA (Figure 1C). We obtained the same result when we compared INS-ftz to 2lle-INS-ftz (Figure 1D). Strikingly, the total steady state levels (Figure 1E and 1F) and cytoplasmic/nuclear distributions (Figure 1G and 1H) of the two mutant mRNAs were almost identical to their wild-type counterparts. In contrast, no protein could be detected when the ftz construct lacked an SSCR (c-ftz) (Figure 1C), and very little protein was expressed from the parathyroid hormone (PTH)-ftz and prion protein (PRP)-ftz constructs (Figure 1D; Table 1), which contain SSCRs derived from the parathyroid hormone and prion protein genes. Both of these genes have an intron in their 5’ UTR, and as we previously documented, their SSCRs are adenine-rich (Table S1), and lack ALREX activity (Figure 1H; Table 1) [7]. Interestingly, these two mRNAs migrated more slowly, when compared to INS-ftz on a denaturing gel (Figure 1F). This last observation suggests that mRNAs with weak ALREX activity have longer poly(A)-tails and is consistent with reports that poorly exported mRNAs are hyper-polyadenylated [21,22].

The difference in protein levels between cells expressing reporter genes with normal and mutant ALREX-promoting SSCRs could potentially have been caused by alterations in the translocation, processing or steady state level of the resulting proteins. However, all of the detectable translatable products, for both MHC-ftz and 2lle-MHC-ftz, were glycosylated indicating that both were efficiently translated into the endoplasmic reticulum (ER) lumen (Figure S1A). This is not surprising as signal sequences containing either leucine or isoleucine serve equally well to promote translocation [17]. Moreover, since the various protein products differ only in their signal sequence, which is cleaved (Figure S1A–S1C), the various SCCR-containing ftz constructs all produce the exact same final processed polypeptide. In agreement with this, these two protein products had similar half-lives (Figure S1D–S1F). Thus the final difference in protein levels was not due to changes in translocation, processing, or protein stability.

In support of the idea that the mutations inhibit translation, SG formation was up-regulated in cells expressing the mutant forms of ftz as compared to those expressing their wild-type counterparts (Figure 1I–1L). When controlling for transfection efficiency, mRNA distribution and mRNA levels, 54% (±2%, p<0.00001) less protein was produced from 2lle-MHC-ftz than from MHC-ftz mRNA. Similar results were obtained for INS-ftz and 2lle-INS-ftz (Table 1).

In conclusion, these results strongly suggest that ALREX-promoting SSCRs act as an RNA element that enhances protein production from a reporter mRNA. In addition, our data indicate that the incorporation of a few silent adenine mutations has a
much more dramatic impact on translation (Figure 1G and 1H) than on mRNA export (Figure 1G and 1H). This finding is consistent with our previous observations that many silent adenines had to be incorporated into ALREX-promoting SSCRs before we could observe effects on mRNA export [6,7].

ALREX-Promoting SSCRs Stimulate the Production of a Natural Protein

Next we monitored the production of calreticulin (CALR), a protein chaperone that resides in the lumen of the ER [23]. The CALR gene lacks a 5' UTR intron and has an SSCR that is highly depleted of adenines. Within the first 55 nucleotides of the human CALR ORF, there is a tract of 43 nucleotides that lacks adenine, which is above average for the typical human SSCR [6].

To detect the expression of this protein, we inserted a sequence that codes for HA epitope into the 3' end of the ORF. We also created a mutant form of the CALR-HA gene where four leucine codons were substituted for isoleucine codons that had similar usage frequencies (4Ile-CALR-HA; Figure 1M; Table S1). In agreement with our ftz reporter construct results, cells transfected

Figure 1. ALREX-promoting SSCRs promote translation. (A) For each codon in the MHC, 2Ile-MHC SSCRs (x-axis), the frequency with which it appears in the human genome per 1,000 codons ("Freq/1000"; y-axis) was plotted. Note that the two leucine-to-isoleucine mutations are circled. (B) Codon analysis of Ins, 2Ile-Ins SSCRs as in (A). (C–L) U2OS cells were co-transfected with plasmids that contained various versions of the ftz gene, and a second plasmid that either contained (''+'') or lacked (''−'') the H1B-GFP gene. As a control, cells were transfected with a plasmid lacking the ftz gene (''Vector''). The cells were then analyzed 18–24 h post-transfection. (C–D) Cell lysates were separated by SDS-PAGE and were probed with antibodies against the HA epitope, GFP, and α-tubulin. (E–F) RNA was extracted from the cell lysates, separated on a denaturing agarose gel, and analyzed by Northern blotting using [32P]-labeled probes directed against ftz and GFP transcripts. (G–H) The percentage of total mRNA found in the cytoplasm and nucleus as determined by the distribution of ftz mRNA by FISH staining. Each bar represents the average and standard error between three separate experiments (each experiment consisting of the average of at least 30 cells). (I–L) Cells expressing the indicated version of ftz were analyzed for the number of SGs. The number of SGs detected by Tia1 immunostaining, but almost always contained an enrichment in ftz mRNA. For each graph, >160 cells were analyzed. Note that while >15% of 2Ile-INS-ftz expressing cells had SGs, this number dropped to <1% in cells expressing INS-ftz. (M) For the first 17 codons (x-axis) in the wild type and 4Ile mutant CALR gene, the frequency with which it appears in the human genome per 1,000 codons (y-axis). Note that the four leucine-to-isoleucine mutations in 4Ile-CALR are circled. (N–P) U2OS cells were co-transfected with plasmids that contained either version of the CALR gene, and a second plasmid that either contained (''+'') or lacked (''−'') the 4Ile-CALR-HA gene. As a control, cells were transfected with a plasmid lacking the CALR gene (''Vector''). The cells were analyzed 18–24 h post-transfection. (N) Cell lysates were separated by SDS-PAGE and were probed with antibodies against the HA epitope, GFP, and α-tubulin. (O) RNA was extracted from the cell lysates, separated on a denaturing gel and analyzed by Northern blotting analysis using [32P]-labeled probes directed against CALR and GFP RNA. (P) The percentage of total mRNA found in the cytoplasm and nucleus as determined by the distribution of CALR mRNA by FISH staining.

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with plasmids containing the CALR-HA cDNA gene produced more protein than those transfected with 4Ile-CALR-HA (Figure 1N). Since the two protein products differ only in their signal sequences, which are cleaved during processing, we could again rule out that the difference in levels was due to changes in protein stability. Furthermore, we could also rule out that the change in protein production was due to differences in the level (Figure 1O) or cytoplasmic/nuclear distribution (Figure 1P) of CALR-HA mRNA.

From these experiments we conclude that ALREX-promoting SSCRs can potentiate the translation of a natural mRNA. When controlling for transfection efficiency, mRNA distribution and mRNA levels, 57% (±13%, *p*<0.005) less protein was produced from 4Ile-CALR-HA than from CALR-HA mRNA. Confirming our results with the βz reporter construct, we again observed that the disruption of the ALREX-element had a more pronounced effect on translation than on mRNA export.

### ALREX-Promoting Elements Require a Nuclear Factor in order to Stimulate ftz Protein Synthesis

When the translation of MHC-ftz and 2-Ile-MHC-ftz was monitored using an in vitro reticulocyte extract, both mRNAs produced the same amount of protein (Figure S1C). From this result we concluded that there is no inherent difference in the translatability of these two mRNAs and that reticulocyte lysate, which is derived from nucleated cells, may lack factors that are required for the efficient translation of transcripts harboring ALREX-elements. Indeed, ALREX-elements are likely to associate with nuclear factors that promote mRNA export and then accompany the transcript to the cytoplasm where they may help to potentiate translation. To test this idea, we microinjected in vitro synthesized and polyadenylated MHC-ftz mRNA into either the cytoplasm or nucleus of cells, and after various time points, we visualized βz mRNA by fluorescence in situ hybridization (FISH). Cytoplasmic or nuclear microinjection was confirmed by co-injecting fluorescently labeled 70 kDa dextran, which is too large to passively cross the nuclear pores. When injected into the cytoplasm, but not the nucleus, MHC-ftz mRNA accumulated in aggregates that were positive for Tia1, a general marker for SGs (Figure 2A–2B) [24]. In general, SGs are thought to form under a variety of conditions, including the accumulation of mRNA with defective or incompletely assembled translation initiation complexes [25,26]. Since these SGs were also enriched in eIF3B (Figure 2C), a translation initiation factor, it is likely that the mRNAs that were injected into the cytoplasm assembled incomplete translation-initiation complexes, rather than simply aggregating non-specifically. Interestingly, mRNA that was injected into the cytoplasm appeared to be stable, as the FISH signal remained strong for up to 9 h post-injection (unpublished data).

We next investigated whether the injected MHC-ftz mRNAs were translated. Since too few cells are microinjected in a single experiment to allow for biochemical analysis, we instead monitored the expression of protein by immunofluorescence against the HA and FLAG epitopes, which are encoded within the ftz ORF. When MHC-ftz mRNA was microinjected into the cytoplasm, few of those cells produced detectable levels of ftz protein (Figure 2D). In contrast, most cells whose nuclei were injected with the same mRNA expressed protein after about 3 h (Figure 2E). As expected, all expressed protein co-localized with the ER marker Trapz (unpublished data), as previously reported [6]. When we quantified the total intensity of the FLAG and HA immunostain signals 3 h post-injection, there was a 10- to 15-fold increase in protein production when the mRNA was microinjected in the nucleus (Figure 2F). Interestingly, when we monitored this expression over time, cells whose cytoplasm was injected with MHC-ftz mRNAs eventually expressed protein, but only after a 2-3 h delay when compared to those that received MHC-ftz mRNAs in the nucleus (Figure 2G). We speculated that the delay in expression was due to the fact that over time these mRNAs were slowly accumulating a factor that cycled between the nucleus and the cytoplasm. Remarkably, when MHC-ftz mRNA was pre-incubated with HeLa nuclear extract and then injected into the cytoplasm, efficient protein production was restored (Figure 2F–2H). When c-ftz mRNA was injected either into the nucleus or cytoplasm, with or without nuclear extract, no translational product was ever detected (unpublished data). These observations suggest that ALREX-promoting elements potentiate translation by recruiting one or more nuclear factors to the translational start site.

### Identification of Nuclear Proteins That Associate with ALREX-Promoting Elements

Since HeLa nuclear extract appeared to potentiate translation, and would also be enriched in putative nuclear export factors, we wanted to determine whether any proteins in these extracts directly associate with ALREX-promoting elements. Using electrophoretic mobility shift assays (EMSA), we observed the formation of a complex between nuclear extract factors and [32P]-labeled RNA fragments from the Ins and MHC SSCRs (Figure 3A and 3B). Complex formation was detected when we tested a mutant form of the Ins SSCR containing seven silent adenine substitutions (7A-Ins; Figure 3A; see Table S1 for the sequence of this mutant). In contrast, very little complex was formed with an RNA fragment derived from the beginning of the

### Table 1. Analysis of various SSCR-containing ftz constructs.

<table>
<thead>
<tr>
<th>Protein Levels</th>
<th>mRNA Levels</th>
<th>Fraction of the Total mRNA in the Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Avg</strong></td>
<td><strong>±</strong></td>
<td><strong>Avg</strong></td>
</tr>
<tr>
<td>INS-ftz</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2Ile-INS-ftz</td>
<td>0.63</td>
<td>0.07</td>
</tr>
<tr>
<td>PRP-ftz</td>
<td>0.10</td>
<td>0.06</td>
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Each data point consists of the average and standard error of three independent experiments. Protein levels were determined by densitometry analysis of immunoblots, mRNA levels were determined by densitometry analysis of northern blots, and the fraction of the total mRNA was determined as in Figure 1G and 1H. In each experiment, the protein and mRNA levels were normalized to that of INS-ftz.

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Figure 2. ALREX-promoting elements require a nuclear factor in order to stimulate ftz protein synthesis. (A–C) The cytoplasm (A, C) or nuclei (B) of COS-7 cells were microinjected with in vitro synthesized, capped, and polyadenylated MHC-ftz mRNA and FITC-labeled 70 kDa dextran. After 2 h cells were fixed and stained by FISH using probes against ftz mRNA and by immunofluorescence using antibodies directed against Tia1 (A–B) or eIF3B (C). Each row represents a single field of cells. Note that in cytoplasmically injected cells the MHC-ftz mRNA accumulated in cytoplasmic aggregates that are enriched in Tia1 and eIF3B (see overlays in (A and C)). (D–E, H) NIH-3T3 cells were microinjected with FITC-labeled 70 kDa dextran and in vitro synthesized, capped, and polyadenylated MHC-ftz mRNA, which was either untreated (D–E) or pre-incubated with HeLa nuclear extract (H). The cells were incubated for 3 h then fixed and stained by immunofluorescence using antibodies directed against the FLAG epitope. Each row represents a single field of cells that were microinjected into the cytoplasm (D, H) or nucleus (E) as determined by the distribution of 70 kDa dextran. Cell nuclei are stained with DAPI. (F, G) NIH-3T3 cells were microinjected with MHC-ftz mRNA and FITC-labeled 70 kDa dextran, alone or with HeLa nuclear extract (“NE”). Scale bar = 20 μM. (F–G) NIH-3T3 cells were microinjected with MHC-ftz mRNA and FITC-labeled 70 kDa dextran, alone or with HeLa nuclear extract (“NE”). MS. (F–G) NIH-3T3 cells were microinjected with MHC-ftz mRNA and FITC-labeled 70 kDa dextran, alone or with HeLa nuclear extract (“NE”). RNAs, but not to the beads alone, we are somewhat confident that equal amounts of RNA were used in all pulldowns. Bands were excised and identified using mass spectrometry as being RanBP2/Nup358 (henceforth termed RanBP2), Importinβ, RanGAP1, Ran, and Ubc9. When proteins from RNAse A and SDS eluates were precipitated with trichloroacetae and analyzed by mass spectroscopy, we could also detect TAP, RCC1, and Importinβ in the Ins purified fraction, but not the other fractions. Since these proteins could not be readily visualized on a silver-stained gel (Figure 3D), we believe that their binding to the Ins SSR is either very weak or indirect. We also detected a large number of SUMO peptides in the trichloroacetate-precipitated fraction. Interestingly, RanBP2 is a known E3 SUMO ligase, which transfers SUMO from Ubc9 (an E2 SUMO ligase) to substrates such as RanGAP1 [28]. Moreover RanBP2, which is present on the cytoplasmic face of the nuclear pore, forms a stable complex with Ubc9 and sumoylated RanGAP1 [29,30]. RanBP2 also interacts with most of the other identified proteins, including Ran [31,32], TAP [33,34], and Importinβ [35].

To validate our findings, we assessed the association of HeLa extract proteins to the RNA-coated beads by immunoblot. To ensure that the binding was specific, we washed the beads extensively with high salt buffer. Indeed, RanBP2, RanGAP1, and Ran bound exclusively to Ins, but not 7A-Ins, βG RNAs, or to the beads alone (Figure 3E). When the fractions were probed with mAb14, an antibody that recognizes several nucleoporins [36,37], only RanBP2 was detected in the Ins SSR pulldown (Figure 3F). In contrast, Nup62 and Nup153 failed to associate with either the Ins SSR, the 7A-Ins mutant, and βG RNAs, but not to the beads alone. 

β-globin (βG) ORF (Figure 3A and 3B), which does not promote export [27]. To test for specificity, we formed complexes between HeLa nuclear extracts and the Ins SSR, as in Figure 3A, but in the presence of cold competitor RNA. Unlabeled Ins SSR was a more effective competitor than either the 7A-Ins mutant or the βG RNA fragment (Figure 3C). Since the presence of cold competitor RNA strongly inhibited the Ins SSR, the 7A-Ins mutant, and βG RNAs, but not to the beads alone, we are somewhat confident that equal amounts of RNA were used in all pulldowns. Bands were excised and identified using mass spectrometry as being RanBP2/Nup358 (henceforth termed RanBP2), Importinβ, RanGAP1, Ran, and Ubc9. When proteins from RNAse A and SDS eluates were precipitated with trichloroacetate and analyzed by mass spectroscopy, we could also detect TAP, RCC1, and Importinβ in the Ins purified fraction, but not the other fractions. Since these proteins could not be readily visualized on a silver-stained gel (Figure 3D), we believe that their binding to the Ins SSR is either very weak or indirect. We also detected a large number of SUMO peptides in the trichloroacetate-precipitated fraction. Interestingly, RanBP2 is a known E3 SUMO ligase, which transfers SUMO from Ubc9 (an E2 SUMO ligase) to substrates such as RanGAP1 [28]. Moreover RanBP2, which is present on the cytoplasmic face of the nuclear pore, forms a stable complex with Ubc9 and sumoylated RanGAP1 [29,30]. RanBP2 also interacts with most of the other identified proteins, including Ran [31,32], TAP [33,34], and Importinβ [35].

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with the RNA (Figure 3F, lane 1) despite the fact that they are present in this extract (Figure 3F, lane 5).

From these results we conclude that the Ins SSCR interacts with RanBP2 and several RanBP2-associated proteins.

The Zinc Finger Repeats of RanBP2 Interact Directly with the Ins SSCR

Since all of the proteins that associated with the Ins SSCR are known to bind to RanBP2, it is highly probable that the RNA is making contact to one subunit of a large complex. We expressed and purified five His-tagged, and one GST-tagged, fragments of RanBP2. Each fragment corresponded to a different region of the protein (Figure 4A and 4B, tagged proteins that react positively on an immunoblot with anti-His antibodies are denoted by asterisks). We found that the zinc finger domain (ZFD) fragment, which contains all eight of the RanBP2 ZFRs (see Figure 4B), was able to form a complex with [32P]-labeled Ins RNA by EMSA (Figure 4C and 4D). In contrast, none of the other fragments were able to form a complex (Figure 4C and 4D). In addition we did not detect binding between [32P]-labeled Ins SSCR and either purified bacterially expressed RanGAP1 or Importinβ alone, or in the presence of GTP-bound Ran (unpublished data). The ZFD also formed a complex with the MHC SSCR, but not the βG RNA (Figure 4E). Although, the mutant 7A-Ins SSCR associated with purified ZFD (Figure 4E), this RNA showed reduced affinity as compared to wild-type Ins SSCR in competition assays (Figure 4F and 4G). From these results we conclude that RanBP2 has the ability to bind directly to the Ins and MHC SSCRs through its ZFRs.

Ran and the Ins SSCR Interact with the ZFD in a Mutually Exclusive Manner

Interestingly, the eight ZFRs from RanBP2 are highly related to repeats found in Nup153 and ZRanB2. Indeed, the ZRanB2 zinc fingers bind directly to single-stranded RNA in a sequence-specific manner [38]. Although the zinc fingers from RanBP2 and Nup153 had not been previously reported to interact with RNA, they were shown to directly associate with both GDP- and GTP-bound Ran [39]. Interestingly, structural and biochemical analysis indicates that the RanBP2 and Nup153 zinc fingers interact with Ran using the same analogous surface that the ZRanB2 repeats employs to bind RNA [38,39]. This raised the possibility that the Ins SSCR and Ran may compete for binding to the RanBP2 ZFRs. In agreement with this idea, GST-tagged Ran, loaded with either GTP or GDP (Figure 4H), effectively prevented the ZFD protein fragment from associating with the Ins SSCR (Figure 4I, lanes 2 and 3). In contrast, BSA, which does not associate with the Ins SSCR (see Figure 4D), had no effect on complex formation (Figure 4I, lane 1). From these results we conclude that the binding of the Ins SSCR and Ran to ZFD is mutually exclusive. Our results

Figure 3. Identification of ALREX-element associating proteins from HeLa nuclear extract. (A–B) Various [32P]-labeled RNA fragments, either 82 (Ins SSCR, 7A-Ins SSCR, and the 5’ end of the βG ORF) or 86 (MHC SSCR) nucleotides long, were incubated with or without HeLa nuclear extract (‘‘NE’’) and separated on a non-denaturing polyacrylamide gel and visualized by autoradiography. (C) [32P]-labeled Ins SSCR RNA was mixed with increasing amounts of various unlabeled RNA fragments, then incubated with NE. The reactions were separated on a non-denaturing polyacrylamide gel and visualized by autoradiography. (D–F) Streptavidin-coated magnetic beads, bound with either various biotinylated RNAs (each 76 nucleotides long) or without any RNA (‘‘No RNA’’), were used to isolate proteins from HeLa nuclear extract. Proteins were eluted by first treating the beads with RNase A (D, lanes 1–4), followed by denaturation in SDS at 90 °C for 5 min (D, lanes 5–8). (D) Silver stained gel of the eluted proteins. Bands identified by mass spectrometry are indicated on the right. (E–F) Eluted proteins and NE were analyzed by immunoblots using an antibody that recognizes, RanBP2, RanGAP1, Ran, or FG-repeat Nucleoporin proteins (mAb414).

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are consistent with the idea that the ZFD uses a similar surface to bind to both molecules, although we cannot exclude the possibility that Ran-binding may induce a conformational change in the ZFD that ultimately alters a different RNA binding-site.

The Number of RanBP2 Zinc Fingers Correlates with the Average Length of SSCR-Specific Adenine-Less Tracts across Metazoans

Adenine depletion is a hallmark of ALREX-promoting elements [6,7]. We previously capitalized on this fact to estimate the size of ALREX-promoting elements in various organisms by measuring the longest tract of adenine-less sequence within the first 69 nucleotides of ORFs that encode signal sequences [6]. Indeed, the average length of adenine-less sequence per SSCR varied greatly between species [6]. We speculated that this variation between organisms might correlate with the number of zinc fingers present in their respective copy of RanBP2. To test this idea, we expanded our analysis to 36 different metazoans where we calculated the average length of the longest tract of adenine-less sequence within the first 99 nucleotides of the ORFs that encode signal sequences, and then visualized by autoradiography. (C–D) [32P]-labeled Ins SSCR RNA was incubated with purified proteins, separated on a non-denaturing polyacrylamide gel, and then visualized by autoradiography. Note that the ZFD forms a complex with the Ins RNA. (E) Various [32P]-labeled RNA fragments were incubated with the ZFD protein, separated on a non-denaturing polyacrylamide gel, and then visualized by autoradiography. (F) [32P]-labeled Ins SSCR RNA was incubated with or without the ZFD protein fragment in the absence or presence of various unlabeled RNAs. The complexes were separated on a non-denaturing polyacrylamide gel and then visualized by autoradiography. (G) Graph of the amount of [32P]-containing complexes (y-axis) in the presence of various amounts of unlabeled RNAs (x-axis). Each point is the average and standard deviation of two independent experiments. (H) Coomassie stain of bacterially expressed ZFD and GST-tagged Ran before and after loading with GTP or GDP. In order to estimate the levels of Ran and ZFD proteins, various amounts of BSA were also loaded on the gel. (I) ZFD was preincubated with either BSA or Ran loaded with GDP or GTP, then incubated with [32P]-labeled Ins SSCR RNA. The complexes were separated on a non-denaturing polyacrylamide gel and then visualized by autoradiography. doi:10.1371/journal.pbio.1001545.g004
cerevisiae did not contain long adenine-less tracts when compared to control sequences (Figure 5A and 5B). Since RanBP2 has yet to have been documented outside of metazoans, our data suggest that the selective pressure necessary to effectively deplete adenines from SSCRs arose only after the appearance of RanBP2 at the base of the metazoan tree (Figure 5C). Even more interesting is the case of Pediculus humanus (commonly referred to as the human head louse), which did not display any significant increase in length of adenine-less tracts in their SSCRs and whose RanBP2 gene lacked ZFRs.

In contrast, six other invertebrates (including aphids) had both a significant increase in the length of the adenine-less tracts in their SSCRs and versions of RanBP2 that contained ZFRs. Thus, it is likely that the lineage from which P. humanus arose, lost both features relatively recently (i.e., after this lineage had diverged from aphids, Figure 5C).

This analysis indicated that these two features (i.e., zinc fingers within RanBP2 and SSCR-specific adenine depletion) not only correlated, but are either both present or absent in all organisms analyzed thus far. We thus concluded that adenine-less tracts and the zinc fingers of RanBP2 display an evolutionary relationship towards each other. This finding strongly indicates that a major role of the RanBP2 ZFRs is to recognize adenine-less tracts in ALREX-promoting SSCRs.

RanBP2 Is Required for the Potentiation of Translation by ALREX-Promoting SSCRs

We next examined whether RanBP2 was required for the efficient translation of mRNAs containing ALREX-promoting SSCRs. To accomplish this we transiently transfected reporter constructs into U2OS cells treated with lentiviral-delivered shRNAs directed against RanBP2. We found that cells depleted of RanBP2 (Figure 6A) did not efficiently produce MHC-ftz protein when compared to control cells (Figures 6B and 6C). In contrast, the expression of PTH-ftz and H1B-GFP protein was relatively unaffected (Figures 6B and 6C). Interestingly, we also observed a drop in the production of 2ile-MHC-ftz. This result is not surprising when considering that the RanBP2 zinc-finger repeats bind to ALREX-promoting sequences that had adenine mutations, albeit at a lower level than wild-type sequences (see Figure 4F and 4G). The decrease in MHC-ftz and 2ile-MHC-ftz protein synthesis could not be attributable to changes in the total level of mRNAs, which remained unaffected (Figures 6D and 6E), or to changes in the distribution of MHC-ftz mRNA between the cytoplasm and the nucleus (Figure 6F).

This last finding was quite surprising given the fact that RanBP2 was shown to be required for the efficient nuclear export of bulk mRNA in insect and mouse cells [33,40]. In contrast, it had also been reported that RanBP2 depletion had no effect on the distribution of poly(A) mRNA in the human HeLa cell line [41]. In line with this last experiment we found that the cytoplasmic/nuclear ratio of poly(A) mRNA was unaffected by the depletion of RanBP2 in human U2OS cells (Figure 6G). On the other hand, depletion of the two RNA helicases, UAP56 and URH49, by shRNA treatment (Figure 6H) caused a pronounced block in mRNA nuclear export (Figure 6G) as described previously [42]. Thus it is likely that RanBP2 plays at most a minor role in mRNA nuclear export in human cells. This might be due to the presence of RanBP2 paralogs, which appear to be primate specific [43]. Curiously, these paralogs lack the ZFRs and are thus unlikely to participate in ALREX-mediated events.

We next evaluated the production of protein from a more “natural” transcript. As expected, the depletion of RanBP2 also inhibited the production of CALR-HA, but not 4ile-CALR-HA protein without a significant change in mRNA level (Figure S3A–S3C). To confirm these results, we depleted RanBP2 with a second shRNA construct (“RanBP2 shRNA2”; Figure S3D). In these cells we again observed a consistent reduction in the production of MHC-ftz and CALR-HA protein without a significant change in mRNA levels (Figure S3E–S3H). In contrast, the levels of PTH-ftz and H1B-GFP proteins were relatively unaffected. Finally we found that the depletion of RanBP2 using either of the two shRNAs inhibited the synthesis of INS-ftz protein (Figure S3I and S3J).

To ensure that our results were not due to some pleiotropic effect, we depleted RanBP2 with a third lentiviral-delivered shRNA (i.e., shRNA3) that is complementary to a region in the 3′ UTR. Three days after infection we rescued the knockdown cells by transfecting a plasmid that contains a GFP-RanBP2 construct that lacks the endogenous UTRs and thus was resistant to depletion (Figure 6I). In parallel we transfected control plasmids containing either a version of GFP-RanBP2 that lacks the last six zinc fingers (RanBP2D6ZF) or H1B-GFP. With these various GFP vectors we co-transfected a plasmid containing MHC-ftz. Since, only a subset of the MHC-ftz expressing cells also co-expressed the GFP-RanBP2 constructs (Figure S4), we monitored individual cells for ftz protein levels by immunofluorescence against the HA epitope. Since GFP-RanBP2 expression was very low we also immunostained cells with an antibody against GFP. In cells transfected with MHC-ftz alone, RanBP2 depletion by shRNA3 resulted in a decrease in protein expression by approximately 2-fold (Figure 6J, “mock”). Co-expression of GFP-RanBP2 rescued the level of ftz protein, whereas co-expression of GFP-RanBP2D6ZF, had no effect (Figure 6J). Note that even in the shRNA3-treated cells, those that express...
Figure 6. RanBP2 is required for ALREX-mediated translation enhancement. (A) U2OS cells were infected with lentivirus containing shRNA1 directed against RanBP2 or control virus. Four days post-infection, cell lysates were collected, separated by SDS-PAGE on a 6% acrylamide gel, and immunostained for RanBP2 or α-tubulin. (B–E) U2OS cells were infected with lentivirus that delivered shRNA1 against RanBP2 or control virus. Three days post-infection, cells were transfected with plasmids containing various ftz constructs and H1B-GFP. 18–24 h post-transfection the level of protein was analyzed by immunoblot (B, quantification in C) and mRNA by northern blot (D, quantification in E). Each bar represents the average and standard error of six independent experiments. *, \( p \leq 0.025; **, \ p \leq 0.01. (F) Three days post-infection, U2OS cells were transfected with plasmid containing MHC-ftz. 18–24 hr post-transfection the amounts of MHC-ftz mRNA found in the cytoplasm and nucleus was determined by FISH. One bar represents an average of three experiments, each of which consists of 20–30 cells. Error bars represent the standard deviation between the three experiments. (G) U2OS cells were treated with various lentiviruses. Four days post-infection, cells were fixed and stained for poly(A) mRNA by FISH. The average levels of poly(A) mRNA in the cytoplasm or nucleus were plotted. One bar represents an average of three experiments, each of which consists of 20–30 cells. Error bars represent the standard deviation between the three experiments. (H) UAP56 and URH49 were simultaneously depleted in U2OS cells by lentiviral mediated delivery of shRNAs. Four days post-infection, cell lysates collected, separated by SDS-PAGE, and immunostained for these two proteins and for α-tubulin. (I–K) U2OS cells were infected with lentivirus containing shRNA directed against the 3' UTR of RanBP2 (shRNA3) or control virus. (I) Three days post-infection, cells were transfected with plasmids containing GFP-RanBP2, or GFP-RanBP2ΔΔZFR or without plasmid (“Mock”). After allowing expression for 48 h, cell lysates were collected and detected by immunoblot using antibodies against GFP, RanBP2, and α-tubulin. Note that the GFP-RanBP2 constructs do not contain the endogenous UTRs and are resistant to depletion by shRNA3.
RanBP2 Promotes the Translation of Secretory mRNAs

K) Three days post-infection, cells were co-transfected with plasmids containing MHC-ftz and either GFP-RanBP2, GFP-RanBP2Δ6ZFR, or H1B-GFP and allowed to express for 48 h. Cells were immunostained using anti-GFP and anti-HA primary antibodies, and the appropriate fluorescent secondary antibodies. (L) For cells co-expressing GFP and MHC-ftz, the HA-immunofluorescence intensity was tabulated, averaged, and then normalized to the level of expression in mock, control shRNA-treated cells expressing MHC-ftz. Each bar represents the average and standard error between four independent experiments, each of which consists of 30–50 cells. Examples of cells depleted of endogenous RanBP2 with shRNA3 and expressing various GFP-RanBP2 constructs and MHC-ftz are shown in (K). Each row is a single field co-immunostained for GFP and HA. Note that cells expressing GFP-RanBP2 (top row, arrows), express higher levels of MHC-ftz than neighboring cells that express MHC-ftz alone (top row, arrowheads). In contrast, cells expressing GFP-RanBP2Δ6ZFR (bottom row, arrows) express about as much MHC-ftz protein as cells expressing MHC-ftz alone (bottom row, arrowheads). Un-transfected cells are denoted by asterisks. Scale bar = 20 μM. doi:10.1371/journal.pbio.1001545.g006

GFP-RanBP2, express much more ftz mRNA than neighboring cells that express ftz alone (Figure 6K, compare the level of ftz protein in the cells expressing GFP-RanBP2, which are denoted by arrows, to the cell expressing ftz alone, which is indicated by an arrowhead). In contrast, expression of GFP-RanBP2Δ6ZFR had no effect on ftz protein levels (Figure 6K).

We next examined the association of MHC-ftz with polysomes using sucrose gradient centrifugation. Consistent with our analysis of translational product, MHC-ftz mRNA was associated with fewer ribosomes in RanBP2-depleted cells (Figure 7A–7C). In contrast, the distributions of H1B-GFP mRNA (Figure 7A, 7B, and 7D) and ribosomal RNA (Figures 7A, 7B, and S5) in the gradients were relatively unaffected. Since active translation is required for the efficient localization of MHC-ftz mRNA to the ER [44], we next examined the distribution of this mRNA on this organelle. In order to accomplish this we extracted cells with digitonin, a detergent that selectively permeabilizes the plasma membrane and thus removes cytoplasmic mRNAs that are not anchored to the ER [44, 45]. By analyzing the level of MHC-ftz mRNA in unextracted and extracted cells by FISH, we calculated that the amount of ER-bound MHC-ftz mRNA was reduced in cells depleted of RanBP2 (Figure 7E). Finally we assessed the association of MHC-ftz mRNA with SGs by co-staining this transcript with Tia1. RanBP2-depleted cells transfected with plasmids encoding MHC-ftz mRNA had an increase in SG formation when compared to transfected control cells (Figure 7F). As seen previously, the vast majority of these SGs were enriched in MHC-ftz mRNA. To determine how fast these SGs formed, we microinjected plasmids into these cells and monitored the distribution of newly made mRNA levels. 20 min post-injection we halted the further production of mRNA by adding -amanatin and then monitored the distribution of newly synthesized mRNA by FISH. Indeed, SGs appeared in RanBP2-depleted cells that expressed MHC-ftz mRNA after only 2 h post-injection (Figure 7G). Moreover the newly expressed mRNAs accumulated in these SGs. These observations are consistent with the idea that in RanBP2-depleted cells, there is a buildup of MHC-ftz mRNAs with defective translation-initiation complexes that very quickly aggregate into SGs [9].

From these results we conclude that RanBP2 is required for the potentiation of translation by ALREX-promoting SSCRs. Furthermore, in cells depleted of RanBP2 we observed a decrease in the amount of MHC-ftz transcripts that are associated with the ER and polysomes, and a corresponding increase of this mRNA in both monosomes and SGs, suggesting that these cells have a defect in translation-initiation that is specific for ALREX-containing mRNAs.

Discussion

In summary we conclude that RanBP2 is required for the efficient translation of mRNAs that encode secreted and membrane-bound proteins. Our data also suggest that cells depleted of RanBP2 accumulate mRNAs with defective translation initiation complexes, although the overall level of these defective complexes is likely very low and near the critical amount necessary to form SGs. In support of this, we did not detect a significant increase in ribosomal rRNA associated with the monosomal fraction after RanBP2 depletion (Figure 7A and 7B).

In this study, we provide the first evidence that ALREX-promoting SSCRs act as a platform to coordinate both nuclear export and translation by recruiting factors to the 5’ end of the transcript. Our data suggest that once export is completed, this platform directly binds to the ZFRs of RanBP2, a large nucleoporin present on the cytoplasmic face of the nuclear pore. This interaction is required for downstream events, which likely includes the assembly of a competent translation initiation complex (Figure 9). Our data indicate that these events are critical for the proper translation of many mRNAs that encode secretory, and likely mitochondrial proteins. Our results may explain previous studies that reported that the presence of a signal sequence enhances protein synthesis (for example [46]), and that the translation rate for ER-bound transcripts was much greater than those found in the cytoplasm [47].
It is still unclear how ALREX-promoting elements enhance mRNA export through the pore before these mRNAs encounter RanBP2. Our data suggest that it is unlikely that the nuclear transport complex TAP/p15 binds directly to the element, as purified TAP/p15 heterodimers do not associate with either the Ins or MHC SSCRs in vitro (unpublished data). It is possible that other proteins that contain RanBP2-like zinc fingers can bind to the ALREX-promoting elements, as these are known to interact with adenine-poor motifs. For example, each of the two RanBP2-like zinc fingers of ZRanB2 interacts with a GGU triplet [48], and the RanBP2-like zinc finger of TLS recognizes GGUG [48,49]. One obvious candidate ALREX-element binding protein is Nup153, whose zinc fingers share the highest degree of similarity to those of RanBP2. However, in our experiments we did not detect any association of this protein with the Ins SSCR in HeLa nuclear extracts (Figure 3F). In line with these results, the number of Nup153 ZF domains does not appear to correlate with adenine depletion across genomes (unpublished data). In particular, the P. hominis Nup153 ortholog has five zinc fingers, without any SSCR-specific adenine depletion. Identification of other proteins that associate with ALREX-promoting elements will be critical for our understanding of the molecular mechanisms that drive the ALREX pathway.

Since RanBP2 is thought mainly to reside on the cytoplasmic face of the nuclear pore, it is likely that this protein only interacts with mRNAs after they have been exported. This interaction may be required to remodel the messenger ribonucleoparticle (mRNP) complex after it has emerged from the nuclear pore. The idea is supported by recent observations of single mRNA molecules [50]. These studies demonstrated that after their nuclear export, a subset displayed extended dwell times in a region that was 100–150 nm away from the nuclear pore, a region that is occupied mainly by RanBP2 and presumably its paralogs. In addition to its ZFD, RanBP2 may have additional RNA-interacting domains [51], and this also impact the translation of supplementary subclasses of mRNA. Furthermore, other factors that have been implicated in both nuclear export and translation, such as Dhh5 and Gle1, are also thought to be present on the cytoplasmic face of the nuclear pore [52,53]. Thus it is likely that many critical mRNP remodeling events may occur in this region.

Since it is likely that the first translating ribosome would disrupt the association between the SSCR and RanBP2, it is likely that this protein must enhance the translatability of the mRNA before the pioneer round of translation [54]. This is reminiscent of the exon junction complex (EJC), which is known to stimulate the translatability of any associated mRNA and whose binding to the transcript is also displaced by translating ribosomes [55]. The EJC accomplishes this in part by promoting the phosphorylation of translation factors [56]. Recently, the EJC has been shown to associate with certain motifs that are found in the first exon [57],...
which resemble sequences that are enriched in ALREX-promoting SSCRs [7]. Experiments are currently underway to determine the exact contribution of the EJC to the translation of mRNAs containing ALREX-promoting SSCRs.

Although RanBP2 is not known to be associated with any kinases, it does have the ability to sumoylate proteins. Indeed a large proportion of known sumoylated proteins have RNA-binding activity [58–60]. Interestingly, it has been reported that the sumoylation of the cytoplasmic cap-binding protein, eIF4E, potentiates translation initiation [61]. Indeed, RanBP2 and eIF4E appear to cross regulate one another and thus impacting overall gene expression [62]. We have tried to recapitulate the potentiation of translation using in vitro translation systems, such as reticulocyte lysate; however, we could not detect any change between MHC-ftz and 2Ile-MHC-ftz (Figure S1C). This is not surprising as these lysates lack RanBP2 (Figure S6). Unfortunately, adding HeLa nuclear extracts to reticulocyte lysates inhibits translation of all tested mRNAs (unpublished data). Experiments are now underway that aim to dissect the exact mechanism by which RanBP2 potentiates translation by performing a detailed domain analysis of this protein using an in vivo assay.

Finally it is possible that RanBP2 may play some role in delivering mRNAs to their ultimate destinations. Interestingly, in photoreceptor cells of the retina, it has been reported that RanBP2 can accompany vesicles that pinch off from the nuclear envelope to be delivered to the cell periphery, which contains both ER

Figure 8. RanBP2 is required for the efficient translation of secretory proteins. (A–B) U2OS cells were treated with various lentiviruses, then 4 d post-infection cells were incubated in media containing [35S]-methionine/cysteine for 20 min. The cells were lysed and subfractionated by differential detergent treatments and centrifugation steps to produce three fractions: one enriched in nuclear proteins, a second containing ER and mitochondrial proteins, and the remaining cytoplasmic proteins. (A) The ratio of [35S]-incorporation to total protein from each fraction was measured and normalized to control cells. Each bar represents the average and standard error of three independent experiments. Note that radioactive incorporation into the fractions was blocked when cells were pre-treated with the translation inhibitor homoharingtonine (HHT) 15 min prior to [35S]-methionine/cysteine labeling, demonstrating that the fractions do not contain free (i.e., unincorporated) labeled amino acids. (B) Various cell fractions from knockdown and control cells were analyzed by immunoblot using antibodies against either Traps (a resident ER protein), F1 ATPase α (a mitochondrial protein), α-tubulin (a cytoplasmic protein), and lamin A/C (a nuclear protein). (C–D) U2OS cells were treated with various lentiviruses, then 4 d post-infection were either immediately fixed to visualize cytoplasmic and nuclear poly(A) mRNA, or briefly permeabilized with digitonin to remove all cytoplasmic mRNA that was not anchored to the ER, and then fixed. Poly(A) mRNA was visualized by FISH staining with fluorescent oligo-dT probes and the fraction of fluorescence intensity associated with each compartment was calculated for at least 30 cells in a single experiment. Each bar represents the average and standard error between three separate experiments.

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Figure 9. A general model of ALREX-regulated processes. (1) mRNAs containing ALREX-promoting SSCRs are packaged into mRNP and exported by an unknown mechanism. (2) The exported mRNAs transiently interact with RanBP2 at the cytoplasmic face of the nuclear pore. This interaction likely causes some alteration within the mRNP. (3) Ultimately, this interaction promotes the translation of these mRNAs into secretory proteins at the surface of the ER.

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mitochondria [63]. In other studies, RanBP2 has also been reported to interact with kinesins [64,65] and dynein [65]. Further investigations will need to be performed to determine if RanBP2 plays a direct role in delivering secretory mRNAs to the ER.

Materials and Methods

Identification of ALREX-Binding Proteins

100 µl HeLa nuclear extract (7 mg/ml), which was prepared as previously described [66], was pre-cleared with streptavidin-coated magnetic beads (Dynabeads, Invitrogen), mixed with 10 µl denatured E. coli tRNA (20 mg/ml, Sigma), 10 µl salmon sperm DNA (11 mg/ml, Sigma), 5 µl of RNase-free BSA (20 mg/ml, Ambion), 250 µl of 2 x binding buffer (0.1% TritonX-100, 1.2 M NaCl, 10 mM MgCl₂, 2 mM DTT), and incubated with 20 µl of beads that were pre-bound with 10 µg of Ies, 7A-Ino and βG biotinylated RNA (see the methods section in Text S1 for biotinylated RNA synthesis) for 1 h at 4°C with gentle rotation. The beads were then washed five times by incubation with 500 µl of 1 x binding buffer (0.1% TritonX-100, 600 mM NaCl, 5 mM MgCl₂, 1 mM DTT; Figure 3E). The beads were then isolated, treated with 10 µl of RNase solution (0.1% TritonX-100, 100 mM NaCl, 1 mg/ml RNase A, Sigma) for 15 min at room temperature. The supernatant was removed (Figure 3E, lanes 2–5) and the beads were mixed with 20 µl of 2 x Laemmli sample buffer and incubated at 90°C for 5 min and separated by SDS-PAGE on a 4%–20% gradient gel. The gel was either silver stained (SilverQuest, Invitrogen) or transferred to nitrocellulose for immunoblotting (see the methods section in Text S1 for details). All silver-stained protein bands were cut and identified by microcapillary liquid chromatography tandem mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School).

Expression and Purification of RanBP2 Fragments and
Ran

The RanBP2 TPR domain (amino acid residues 1–601), RBR1 (residues 514–1,245), ZFD (residues 1,335–1,829), RBR2 (residues 1,892–2,553), C-Term (residues 2,765–3,130) were amplified from the pBSK-RanBP2 [28] and cloned into pET28a vector (Novagen) using restriction-free cloning with the addition of N-terminal His-tag.

Electromobility Shift Assays

[35S]-labeled RNA was incubated with HeLa cell nuclear extract (0.44 µg/µl final protein concentration), RanBP2 fragments, or BSA (120 ng/µl) in 1.5 x ϕ buffer (1 x ϕ buffer: 150 mM KAcetate, 5 mM MgAcetate, 20 mM HEPES [pH 7.4]) with 10 µg/ml denatured yeast tRNA at room temperature for 15 min. For the competition EMSA experiments, unlabeled RNA was first mixed with tRNA and radiolabeled RNA, then incubated with nuclear extract or recombinant proteins. For the Ran competition assay, 170 ng/µl BSA or GST-Ran was pre-incubated with 50 ng/µl RanBP2 ZFD, then incubated with labeled RNA. RNA-protein complexes were resolved by native PAGE (TBE, 3.5%, 5%, and 10% acrylamide; acrylamide/bisacrylamide ratio of 19:1). Radiolabelled RNA was visualized using a Typhoon phosphomager (GE Healthcare).

Cell Biological Methods

Cell culture, transfection, microinjection, extraction, fixation, FISH, and immunofluorescence were performed as previously described [6,13,67]. For more information see the methods section in Text S1.

Supporting Information

Figure S1 The translational products of the MHC-ftz and 2Ile-MHC-ftz mRNAs are translocated into the ER where they are processed into identical protein products. (A–B) Lysates were collected from U2OS cells expressing MHC-ftz, 2Ile-MHC-ftz, or a version of this protein translated from a mutant frame-shifted MHC-ftz (FS-MHC-ftz). This last construct contains a point insertion before, and a point deletion after the SSCR, thus encoding a cytoplasmic version of the ftz protein (see [6] and Table S1). The lysates were first treated with Endo H, which removes ER-specific N-linked glycosylation, or PNGase, which removes all N-linked glycosylation. The lysates were then denatured and separated by SDS-PAGE, and probed with antibodies against the HA epitope (A–B), and against β-tubulin as a loading control (A). An image of colored molecular weight standards is included in (A). Note that after either Endo H or PNGase treatment, the mobility of all of the MHC-ftz and 2Ile-MHC-ftz proteins increased. This is to be expected as the mature ftz polypeptide has two consensus glycosylation sites. In contrast FS-MHC-ftz protein was unaffected by PNGase treatment. From these data we concluded that MHC-ftz, but not FS-MHC-ftz, is present in the ER lumen where it is glycosylated. Also note that the mobility of deglycosylated MHC-ftz is greater than that of FS-MHC-ftz despite the fact that the two protein products should have similar molecular weights, suggesting that the former is processed by signal peptidase. (C) In vitro transcribed and capped mRNAs were translated in vitro using reticulocyte lysate in the presence of [35S]-methionine/cysteine. Samples were separated by SDS-PAGE, and newly synthesized proteins were detected by autoradiography. An image of colored molecular weight markers (MWMs) was included as a reference. Note that reticulocyte lysate does not contain ER-derived microsomes, thus the resulting ftz protein products are expected to be processed and unmodified. Also note that the molecular weight of these in vitro synthesized proteins (>17 kDa) was greater than the final deglycosylated in vivo translated forms (see PNGase and Endo H treated samples in (A), which are <17 kDa). This observation indicates that in cells, MHC-ftz and 2Ile-MHC-ftz are proteolytically processed, likely by signal peptidase. (D–F) To calculate the half-life of the two protein products, U2OS cells that were transfected with plasmids that contained either the MHC-ftz or 2Ile-MHC-ftz genes (18–24 h post-transfection) were treated with cyclohexamide (CHX) for indicated time points. Cell lysates were collected and separated by SDS-PAGE, then probed with antibodies against the HA epitope and β-tubulin. (D) The amount of ftz protein (y-axis) at each time point (x-axis) was analyzed by densitometry and then normalized to the initial protein level and plotted. Each data point represents the average and standard deviation of three independent experiments. (F) Each bar represents the average and standard error of the half-lives derived from three independent experiments. (TIF)

Figure S2 Identification of ALREX-element associating proteins from HeLa nuclear extract. Streptavidin-coated magnetic beads, bound with various biotinylated RNAs (each 76 nucleotides long) or without any RNA (“No RNA”), were used to isolate ALREX-element associating proteins from NE under low salt conditions (i.e., 200 mM NaCl instead of the 600 mM used in Figure 3E–3G). Beads were treated with RNase A and then proteins were denatured in SDS at 90°C for 5 min. Eluted proteins were separated on a 4%–20% gradient gel and silver stained. ALREX-interacting proteins (RanBP2, Importinβ, Ran-GAP1, and Ran) are indicated. Note that under these conditions, many non-specific proteins are present in the RNA-coated beads.
at similar levels, but not in the uncoated beads. These observations indicate that the RNA-coated beads contained similar amounts of bound oligonucleotides. The position of molecular weight markers in kDa are indicated on the right. (TIF)

Figure S3 RanBP2/Nup358 is required for ALREX-mediated translation enhancement. (A–C) U2OS cells treated with shRNA1 against RanBP2 or control lentiviruses. Three days after infection the cells were transfected with plasmids containing various CALR-HA constructs and H1B-GFP. The level of protein and mRNA was analyzed by densitometry analysis of HA and GFP immunoblots (A–B), and of protein and mRNA was analyzed by densitometry analysis of immunoblots (C). Each bar represents the average level and the standard error between five independent experiments. (D) U2OS cells treated with either shRNA2 against RanBP2 or control virus. Four days post-infection the cell lysates were collected and analyzed by immunoblot for RanBP2 and β-tubulin. (E–J) U2OS cells treated with either shRNA1 or shRNA2 against RanBP2 or control virus, were transfected with various βz (E–F, I–J) or CALR-HA (G–H) constructs along with H1B-GFP. Protein and mRNA levels were then analyzed by densitometry analysis of immunoblots (E, G, I) and northern blots (F, H, J). Each bar represents the average level and the standard error of protein (E, G) or mRNA (F, H) levels between three independent experiments. (TIF)

Figure S4 Level of co-expression of GFP-RanBP2 and MHC-ftz constructs in control and knockdown U2OS cells. Cells were treated with lentiviruses that delivered RanBP2 shRNAs3 or control shRNA. Three days post-infection cells were co-transfected with plasmids containing MHC-ftz and either GFP-RanBP2 (A) or GFP-RanBP2A6ZFR (B). 48 h post-transfection cells were fixed and immunostained for HA and GFP using specific antibodies. Cells expressing detectable levels of either protein alone or together were tabulated. For each experiment, the expression of at least 400 cells was tabulated. (TIF)

Figure S5 Densitometry analysis of ribosome intensities in sucrose gradients. For each polysome gradient, ribosomal rRNA was visualized as in Figure 7A and 7B and the intensity of the 28S rRNA and 18S rRNA bands were calculated by densitometry analysis. The fraction of the ribosomal rRNAs in each sucrose gradient fraction was then averaged between experiments. Each data point represents the average and standard error between three independent experiments. (TIF)

Figure S6 Rabbit reticulocyte lysate does not contain RanBP2/Nup358. Rabbit reticulocyte lysates, and lysates from U2OS cells treated with shRNA1 against RanBP2 or control lentiviruses for 4 d, were separated by SDS-PAGE and immunoblotted for RanBP2 and β-tubulin. (TIF)

Table S1 Constructs used in this study. Nucleotides and encoded amino acid sequences for all ftz and CALR constructs used in this study. Also included are the nucleotides and encoded amino acid sequences of human BiP. Note that all nucleotide and amino acid substitutions in 2Ile-MHC, 2Ile-INS, 7A-INS, and 4Ile-CALR are in bold. (XLS)

Table S2 Analysis of SSCR-specific adenine depletion in metazoans. The mean and standard error of the mean of the longest A-less tract in the first 99 nucleotides in ORFs encoding signal sequences. As a control a random 99 nucleotides from each ORF was analyzed. Also listed are the number of ZFRs in that organism's RanBP2 ortholog, and the total number of ORFs analyzed. (XLS)

Text S1 Additional supplemental materials and methods. (DOC)

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Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: KM HZ AFP. Performed the experiments: KM HZ AA XAC SG AFP. Analyzed the data: KM HZ AA XAC CC FPR AFP. Contributed reagents/materials/analysis tools: FPR AFP. Wrote the paper: KM HZ AFP.

References

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