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.


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Abbreviations: ALREX, alternative mRNA nuclear export; JIG, β-globin; CALR, calreticulin; EJC, exon junction complex; EMSA, electro mobility shift assay; ER, endoplasmic reticulum; FISH, fluorescence in situ hybridization; ftz, fushi tarazu; GFP, green fluorescent protein; H1B, histone 1B; INS or Ins, insulin; MHC, major histocompatibility complex; mRNP, messenger ribonucleoparticles; PPR, prion protein; PTH, parathyroid hormone; SG, stress granule; SSCR, signal sequence coding region; ZFD, zinc finger domain; ZFR, zinc finger repeat

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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 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 [4,3].

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 translation in the cytoplasm.
Results

RanBP2 Promotes the Translation of Secretory mRNAs

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 2Ile-MHC-ftz and 2Ile-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 α-tubulin.

We found that cells expressing MHC-ftz mRNA produced about twice as much ftz protein when compared to those expressing 2Ile-MHC-ftz mRNA (Figure 1C). We obtained the same result when we compared INS-ftz to 2Ile-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 (e-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 translational products, for both MHC-ftz and 2Ile-MHC-ftz, were glycosylated indicating that both were efficiently translocated 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 SSCR-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% (±6%, p<0.00001) less protein was produced from 2Ile-MHC-ftz than from MHC-ftz mRNA. Similar results were obtained for INS-ftz and 2Ile-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 1C and 1D) 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...
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. Confirining 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 enucleated 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 βz 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 quantitated 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 (EMSAs), 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 SCCR 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

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**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></td>
<td>Avg ±</td>
<td>Avg ±</td>
</tr>
<tr>
<td>INS-ftz</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>2Ile-INS-ftz</td>
<td>0.63 ± 0.07</td>
<td>1.01 ± 0.03</td>
</tr>
<tr>
<td>PTI-ftz</td>
<td>0.10 ± 0.06</td>
<td>1.01 ± 0.04</td>
</tr>
<tr>
<td>PRP-ftz</td>
<td>0.01 ± 0.00</td>
<td>0.90 ± 0.45</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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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 SSCR, as in Figure 3A, but in the presence of cold competitor RNA. Unlabeled Ins SSCR was a more effective competitor than either the 7A-Ins mutant or the βG RNA fragment (Figure 3C). From these experiments we concluded that one or more factors in HeLa nuclear extracts specifically associate with ALREX-promoting elements. Our data indicate that the addition of adenosines decreases, but does not abolish, the ability of HeLa factors to interact with ALREX-promoting elements. This result is consistent with the observation that adenine substitutions only partially inhibit ALREX (Figure 1 G and 1H) [6,7].

To isolate these interacting proteins from HeLa nuclear extracts we incubated these with biotinylated RNA fragments that were immobilized on streptavidin-conjugated magnetic beads. Bound proteins were eluted by treating the beads with RNase A (Figure 3D, lanes 1–4). Since RNA-binding factors can protect the biotinylated transcripts from digestion, we then treated the beads with SDS at high temperature to denature any remaining proteins (Figure 3D, lanes 5–8). Several Ins SSCR-interacting factors were detected by silver stain. Importantly, these proteins were less abundant when the purification was performed with the 7A-Ins mutant RNA, and almost completely absent with βG RNA. Note that these proteins also associated specifically with the Ins SSCR under less stringent conditions, but were partially obscured by non-specific RNA-binding proteins (Figure S2). Since the nonspecific proteins bound equally well to beads with either the Ins SSCR, 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 Importinα 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 SSCR is either very weak or indirect. We also detected a large number of SUMO peptides in the trichloroacetic acid-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 SSCR pulldown (Figure 3F). In contrast, Nup62 and Nup153 failed to associate...
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 employ 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
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 compared this to the number of zinc fingers encoded by that organism’s RanBP2 gene. As a control we computed the average length of the longest tract of adenine-less sequence in a stretch of 99 nucleotides selected randomly within each ORF. In addition we also analyzed genes that code for signal sequences from Dictyostelium discoideum and Saccharomyces cerevisiae, two organisms that do not contain any known RanBP2 orthologs. Overall, we observed a general correlation between the number of RanBP2 zinc fingers and the length of adenine-less tracts in SSCRs (Figure 5A and 5B; Table S2). Critically, SSCRs from D. discoideum and S.

Figure 4. The RanBP2 zinc fingers directly interact with ALREX-elements. (A) Various His-tagged (TPR, tricopeptide repeat region; RBR1/2, ran binding domain-containing regions 1/2; C-Term, carboxy-terminal domain) or GST-tagged (E3, E3 SUMO ligase domain) fragments of the RanBP2 gene were expressed in bacteria and purified. The proteins were separated by SDS-PAGE and stained with Coomassie blue. In cases where multiple bands appear, proteins that are detected on anti-His-tag immunoblots are denoted by asterisks. (B) Domain structure of RanBP2. Note that this protein contains four type 1 Ran binding domains (RanBD1), eight ZFRs, an E3 ligase domain, a cis-trans prolyl-isomerase domain, and several FG repeats. (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.

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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 (RanBP2Δ6ZFR) 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-RanBP2Δ6ZFR, had no effect (Figure 6J). Note that even in the shRNA3-treated cells, those that express

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**Figure 5. Adenine depletion in SSCRs correlates with the number of RanBP2 zinc fingers across metazoans.** (A–B) For each organism, the ORFs from at least 500 genes encoding secreted proteins were analyzed (for individual values see Table S2). The average length of the longest adenine-less tract within the first 99 nucleotides or a random stretch of 99 nucleotides was analyzed. (A) For each organism, the average adenine-less tract lengths (y-axis) were plotted against the number of ZFRs (x-axis) in the RanBP2 gene. (B) The ratio of adenine-less tract lengths in the first 99 nucleotides versus a random stretch of 99 nucleotides (y-axis) was plotted against the number of ZFRs in the RanBP2 (x-axis) within each species. A trend line and the corresponding coefficient of determination (R²) was also included. Note that for one metazoan species, P. humanus (human head louse), there is neither adenine depletion nor ZFRs within RanBP2. Also note that non-metazoans (D. discoidium and S. cerevisiae) lack both the SSCR-specific adenine depletion and the RanBP2 gene. (C) A phylogenetic tree depicting the likely loss of both adenine depletion and RanBP2 zinc fingers in the lineage that gave rise to P. humanus. doi:10.1371/journal.pbio.1001545.g005
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 < 0.025; **, p < 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-RanBP2Δ6ZFR 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

Discussion

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 ZF Rs 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 Rs does not appear to correlate with adenine depletion across genomes (unpublished data). In particular, the P. humanus 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 thus also impact the translation of supplementary subclasses of mRNA. Furthermore, other factors that have been implicated in both nuclear export and translation, such as Dhp5 and Gie1, 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 and

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 Trapα (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 cells were incubated in media containing [35S]-methionine/cysteine for 20 min. Cells were lysed and BiP and α-tubulin were immunoprecipitated with specific antibodies. The precipitates were then separated by SDS-PAGE and visualized by autoradiography. Band intensities were measured by densitometry, then normalized to control cells and the average and standard error between three independent experiments were plotted (D). (E) U2OS cells were treated with various lentiviruses, then 4 d post-infection cells 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 mRNPs 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× 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-Iso 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× 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× 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 microcapsillary 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 1,832–2,553), 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

[32P]-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× binding buffer (1× binding buffer: 150 mM KAacetate, 5 mM MgAacetate, 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 phosphimager (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 α-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 (MMWs) 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 unprocessed 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 α-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.

**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 the standard error of protein (E, G) or mRNA (F, H) levels between three 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 α-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 between five 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 shRNAs or control shRNA. Three days post-infection cells were co-transfected with plasmids containing MHC-ftz and either GFP-RanBP2 (A) or GFP-RanBP2 Δ6ZFR (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.

**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 was 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.

**References**


