Trafficking of mRNAs containing ALREX-promoting elements through nuclear speckles

Abdalla Akef,1 Hui Zhang,1 Seiji Masuda1 and Alexander F Palazzo1,*

1Department of Biochemistry; University of Toronto; Toronto, ON Canada; 2Division of Integrated Life Science; Graduate School of Biostudies; Kyoto University; Kyoto, Japan

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Abbreviations: ALREX, alternative mRNA export; βG, beta-Globin; CTE, constitutive transport element; FISH, fluorescence in situ hybridization; fex, fushi tarazu; HHT, homoharringtonine; INS, insulin; MHC, major histocompatibility complex; mRNP, messenger ribonucleoprotein; MSCR, mitochondrial-targeting sequence coding region; ORF, open reading frame; PolII, RNA polymerase II; RT-qPCR, reverse transcription–quantitative polymerase chain reaction; TREX, transcription export; shRNA, small hairpin RNA; SRP, signal recognition particle; SSCR, signal sequence coding region

Introduction

In eukaryotes, mRNAs are synthesized in the nucleus where they are capped, spliced, and polyadenylated. During these maturation steps, the mRNAs are loaded with several proteins to form messenger Ribonucleoprotein (mRNP) particles that are capable of crossing the nuclear pore to reach the cytoplasm where the mRNAs are translated into proteins.1 It is believed that the majority of mRNAs in vertebrate cells are exported from the nucleus in a splicing dependent export pathway.2 This pathway is initiated by the nuclear cap binding complex collaborating to deposit the transcription export (TREX) complex at the 5’end of the mRNA.3,4 TREX is a multiprotein complex that is composed of the THO subcomplex, the RNA helicase UAP56, the adaptor molecules Aly and Chop.5-8 This complex acts to recruit the heterodimeric mRNA export receptor TAP/p15 (TAP is also known as NXF1), which directly binds to the mRNA and ferries it across the nuclear pore to the cytoplasm.9,10

In addition to the canonical splicing-dependent pathway, several unconventional mRNA export mechanisms have been characterized. Previously, we reported that the presence of a signal sequence coding region (SSCR) at the beginning of the open reading frame (ORF) of an mRNA promotes the Alternative mRNA Export (ALREX) pathway in vertebrate cells.13,14 In addition to coding for targeting peptides to the endoplasmic reticulum and the mitochondrion respectively, the SSCR and MSCR act as RNA elements that facilitate the nuclear export of their transcripts independently of splicing or a functional 5’ cap.15 These export-promoting SSCRs and MSCRs tend to be depleted in adenovirus and present within the first exon. ALREX-promoting SSCRs also enhance translation of the mRNA, and this likely requires a rearrangement of mRNP components at the cytoplasmic face of the nuclear pore after the completion of nuclear export.16

Presumably ALREX-elements function in export by regulating the composition of the mRNP within the nucleus. ALREX requires TAP/p1513,14 and ALREX-elements likely recruit this nuclear transport receptor to the mRNP through an adaptor molecule. However, little else is known about how these sequences influence mRNP formation. In addition, it is unclear where in the nucleoplasm the assembly of these mRNPs takes place. One potential subnuclear compartment where mRNP assembly may occur is nuclear speckles. These are large nuclear aggregates that contain active RNA polymerase II (PolII), spliceosomal sequence coding region (MSCR) at the beginning of the open reading frame (ORF) of an mRNA promotes the Alternative mRNA Export (ALREX) pathway in vertebrate cells.13,14 In addition to coding for targeting peptides to the endoplasmic reticulum and the mitochondrion respectively, the SSCR and MSCR act as RNA elements that facilitate the nuclear export of their transcripts independently of splicing or a functional 5’ cap.15 These export-promoting SSCRs and MSCRs tend to be depleted in adenovirus and present within the first exon. ALREX-promoting SSCRs also enhance translation of the mRNA, and this likely requires a rearrangement of mRNP components at the cytoplasmic face of the nuclear pore after the completion of nuclear export.16

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components, and splicing cofactors such as SR proteins.26 In addi-
tion, these structures contain TREX complex components.3,5,17,18
Many mRNAs appear to be recruited to nuclear speckles by the
act of splicing.21,22 In the vicinity of speckles, splicing is com-
pleted and the mRNA acquires components of the exon junction
and likely TREX complex.23,24 Depletion of UAP56, enhances
an intron and was dependent on splicing.19-22 In several of these
structures. Finally, it is likely that TAP/p15 is itself loaded onto spliced
mRNAs containing ALREX-promoting elements and other undetermined features that are
present in certain transcripts. Our data suggests that within
speckles, mRNAs containing ALREX-promoting elements undergo a series of mRNP maturation steps. One nuclear speckle
step likely involves the recruitment of the RNA helicases UAP56
and URH49 to the mRNA. A second downstream step requires
TAP activity. In addition, our data suggests that the poly(A)-tail
is also required for a nuclear speckle-associated event. In sum-
mary, our data supports the model that the initial targeting of
mRNA to nuclear speckles licenses an mRNA to use the ALREX
pathway.

Results
Nuclear speckle association is promoted by sequences within
the reporter transcript and by the MHC SSCR. To understand
the early steps of the ALREX pathway, we monitored the dis-
tribution of newly synthesized mRNA that contains or lacks an
ALREX-promoting SSCR. We first investigated different ver-
sions of the intronless ftz-flarus (ftz) reporter mRNA which either possesses (MHC-fsz-Δ) or lacks (c-fsz-Δ) the SSCR from
the mouse Major Histocompatibility Complex (MHC) H2Kb gene. This SSCR promotes efficient nuclear export by the ALREX
pathway.13

We thus microinjected plasmids that contained either ver-
sion of ftz into the nucleus of human osteosarcoma cells (U2OS)
and after various periods of time we imaged the endogenously
synthesized mRNA by fluorescence in situ hybridization (FISH)
and various subcellular markers by immunofluorescence. Both
MHC-fsz-Δ and c-fsz-Δ mRNAs appeared to associate with foci
within the nucleus that were reminiscent of nuclear speckles,
also known as interchromatin granules.26 This observation, was
at odds with previously published findings that indicated that
the ability for mRNAs to associate with these structures required
an intron and was dependent on splicing.19,20 In several of these
studies, an intron-containing, but not an intronless version, of
the human β-Globin (βG) transcript was targeted to speckles. We thus compared the distribution of mRNA transcribed from
microinjected plasmids containing either MHC-fsz-Δ, c-fsz-Δ,
or βG-Δ (an intronless version of the βG gene), and examined
their association with nuclear speckles over time. We observed
that both MHC-fsz-Δ and c-fsz-Δ colocalized with SC35, a
marker of nuclear speckles,26 however this association was only
apparent after at least 30 min of expression (Fig. 1A), compare the distribution of MHC-fsz-Δ with SC35 at 15 min and 1 h
post-microinjection, arrows indicate nuclear speckles. In con-
trast, intronless transcripts, including those that originate from
naturally intronless genes, do not appear to associate with nuclear
speckles.24,26 In several of these transcripts within or in the vicinity of nuclear speckles.24,26 In contrast of the intronless
MHC-ftz-Δ reporter mRNA which was obtained from a different nucleus and then repeated
the process we inserted the mouse MHC SSCR into the 5’end
of the intronless βG gene (creating MHC-Δ, see Fig. 2A) and
monitored its ability to localize to speckles. As a control we
also examined a version of βG that contains its two natural
introns (βG-i). The insertion of MHC SSCR had only a moder-
ate effect on speckle localization (Fig. 1F, compare MHC-βG-Δ
to βG-Δ). These results suggested that the MHC SSCR promotes
weak speckle association. To determine whether other ALREX-
sensitive mRNAs associate with speckles, we next examined
an intronless version of the human insulin (INS-Δ) mRNA tran-
script. This mRNA is efficiently exported and this activity is sensitive to mutations within its SSCR.13 Indeed, this intronless mRNA was enriched in speckles to a higher degree than all the other
transcripts (Fig. 1F). Furthermore, disruption of the MHC SSCR with 5 silent adenine substitutions (5A-INS-Δ) slightly
decreased, but did not abolish, speckle localization (Fig. 1F),
despite the fact these mutations partially inhibit nuclear export.27
Figure 1. For figure legend, see page 329.
From these observations we conclude that various reporter mRNAs appear to have different abilities to localize to nuclear speckles. We can also conclude that the ability to associate with nuclear speckles is not sufficient for mRNAs to be efficiently exported from the nucleus, as exemplified by c-fos Δi. Furthermore, our data indicates that although ALREX-elements may function to promote the localization of mRNAs to nuclear speckles, robust speckle targeting requires the presence of supplementary features within the transcript.

MHC-fz-Δi traffics through nuclear speckles. We next investigated whether these mRNAs could target to the speckles post-transcriptionally. To test this idea we microinjected plasmids containing MHC-fz-Δi and after 20 min we added α-amanitin to the cells. This treatment completely inhibits transcription of microinjected plasmids within 5 min.52 We then monitored the distribution of MHC-fz-Δi at various time points after transcriptional shut-down. Our analysis indicated that over time, newly synthesized MHC-fz-Δi transcripts increased their degree of localization with SC35 (Fig. 1G), suggesting that they can target to these structures post-transcriptionally.

Although colocalization studies indicate whether a particular mRNA is enriched in speckles, this does not indicate how much of that transcript partitions into these structures. Moreover, monitoring the total level of speckle-associated mRNA over time may provide insights into the kinetics of this process. To examine whether the localization to speckles represented a transient event, we used the SC35 immunofluorescence signal to subdivide nuclei into nuclear speckle regions and non-speckle regions (for details see the methods section) and monitored what fraction of MHC-fz-Δi was present in these zones after α-amanitin treatment. To limit the amount of variation between measurements, each cell was imaged at a specific defined by thresholding the brightest 20% (± 0.5%) of pixels in each nucleus using SC35 immunofluorescence. Generally, the total amount of speckle-associated MHC-fz-Δi mRNA decreased over time (Fig. 1H). This result implied that this mRNA was trafficking out of the nuclear speckles over the time course. Interestingly, when only the nuclear MHC-fz-Δi mRNA levels were assessed, the amount associated with speckles only slightly decreased over the same period (Fig. 1H). Speckle/Nuclear mRNA. From these measurements, we could definitively determine whether any of the mRNAs under study were targeted post-transcriptionally. However this data suggested that the partitioning of mRNA between the non-speckle and speckle regions was close to equilibrium.

To obtain a clearer picture of post-transcriptional mRNA trafficking through nuclear speckles, we microinjected in vitro synthesized, capped, and polyadenylated mRNA into nuclei and measured the partitioning of this mRNA over time. Note that microinjected mRNA is exported at a higher rate than endogenously transcribed transcripts. For example, the half-time of export for microinjected MHC-fz-Δi mRNA is about 15 min, while the figure for the same mRNA that is transcribed endogenously off of plasmids is 40–50 min.53 This also holds true for splicing dependent export (Akef A and Palazzo A, unpublished observations). We found that microinjected MHC-fz-Δi mRNA very rapidly accumulated into nuclear speckles, and this peaked at about 10 min post injection (Fig. 1I). Speckle/Nuclear mRNA. After this point the amount of mRNA in nuclear speckles decreased. This result confirmed that mRNA was likely trafficking through nuclear speckles and that this localization could occur post-transcriptionally.

In summary our data suggests that many intronsless mRNAs traffic through nuclear speckles. In light of the role of nuclear speckles in mRNA metabolism,54 it is likely that this trafficking is linked to mRNP assembly. Nonetheless, we cannot exclude the possibility that only a fraction of the MHC-fz-Δi mRNA transits through speckles. The extent of nuclear export promoted by the MHC SCCR varies between reporter mRNAs. Since MHC-β-G-Δi mRNA
had relatively weak targeting to nuclear speckles, we wondered whether it was efficiently exported. To test this idea we monitored the nuclear export of various versions of βG mRNAs (see Fig. 2A) using a standard microinjection based assay. DNA plasmids that contained these various genes were injected into U2OS nuclei along with a 70 kDa fluorescently labeled dextran, which cannot passively cross the nuclear pore and thus be used as a marker for injected cells. After 20 min, the RNA PolII inhibitor α-amanitin was added to halt further transcription. Cells were left for 2 h to allow the newly synthesized mRNA to be exported. Subsequently, cells were fixed and processed for FISH. Interestingly, the MHC SSCR promoted export of βG, but this was substantially weaker than the export promoted splicing (compare MHC-βG-Δi, βG-Δi, and βG-i, Fig. 2B and C). This result suggested that these two reporter constructs (i.e., ftz and βG) are not equivalent.

Interestingly, c-ftz-Δi mRNA encodes an unstable protein (Palazzo A, unpublished observations), raising the possibility that...
it is eliminated by some quality control mechanism. To ensure that c-ftz-i was not degraded co-translationally, we treated cells with the translation inhibitor homeostatargamine (HHT) for 30 min before injecting plasmids containing MHC-ftz-Δi or c-ftz-Δi. HHT-treatment had no effect on the level of nuclear export of either mRNA (Fig. S2A and B). In order to ensure that translation was inhibited, we took advantage of the fact that the fitz reporter encodes a protein fused to an HA epitope and immuno-stained MHC-ftz-Δi injected cells. As expected, treating cells with HHT greatly diminished the HA immunofluorescence (Fig. S2C).

The lack of efficient export by MHC-βG-Δi could be explained by two possible models: either βG contained anti-ALREX activity, or fitz contained one, or several, features that potentiate ALREX. To test these models, we fused the two genes (creating MHC-ftz-βG-Δi; see Fig. 2A) and measured the export of the resulting mRNA. We found that this transcript was efficiently exported (Fig. 2B and C), indicating that fitz contained some feature that was required for ALREX.

From these experiments we conclude that the MHC-SCR promotes nuclear export of various reporter mRNAs, but that the amount of activity may differ. This variation is unlikely to be related to the translational products of the different transcripts. Instead our results are consistent with the model that certain reporters contain additional features that license an mRNA for ALREX. These features may be related to the propensity of a given transcript to localize to nuclear speckles.

The nuclear export of MHC-ftz-Δi requires UAP56 and URH49. Recent studies have suggested that TREX components are required for spliced mRNAs to exit nuclear speckles.27 Previously, we demonstrated that in HeLa cells the co-depletion of the RNA DExD/H-box helicase UAP56, and its close paralog URH49, only partially inhibited the export of microinjected MHC-ftz-Δi mRNA.28 In contrast the export of c-ftz-i was more drastically affected.27 These previous experiments were hampered by the fact that at the time we did not have access to an antibody specific for URH49 and thus could not determine the efficiency of its knockdown.

In light of our localization findings, we decided to revisit these experiments, but this time monitoring the export of endog-enously transcribed mRNAs. We took advantage of a lentiviral delivery system to transduce U2OS cells with plasmids that contain small hairpin RNA (shRNA) constructs that are complementary to both UAP56 and URH49 mRNAs. We took advantage of a lentiviral small hairpin RNA (shRNA) construct that prevents TAP from translation, and the newly synthesized mRNA was allowed to export for an additional 2 h. We observed that when UAP56 and URH49 were co-depleted, both MHC-ftz-Δi and c-ftz-i mRNAs were fully retained in the nucleus (Fig. 3B, quantitation Fig. 3C). In contrast, depletion of either helicase alone had only slight effects on export, although c-ftz-i appeared to be more sensitive to UAP56 depletion than MHC-ftz-Δi (Fig. 3C). As previously published,30 co-depletion of UAP56 and URH49 also caused a drastic accumulation of poly(A) mRNA in the nucleus (Fig. 3F). In contrast, depletion of either UAP56 or URH49 alone did not cause a change in poly(A) mRNA distribution. Depletion of the THO complex member THOC1 (also known as hHpr1 and p84) or the adaptor Aly had little to no effect on the export of either MHC-ftz-Δi or c-ftz-i mRNA (Fig. 3C–E). Furthermore, depletion of either THOC1 or Aly also had no effect on bulk mRNA export (Fig. 3F).

In light of these results we wanted to revisit our previous experimental results investigating the export of in vitro synthesized mRNAs in HeLa cells depleted of UAP56 and URH49.31 One difference between our previous experiments and our current results is the use of α-amanitin in the latter. Nevertheless, when α-amanatin treatment was omitted from DNA injection experiments, MHC-ftz-Δi mRNA was still retained in the nucleus in U2OS cells depleted of UAP56 and URH49 (Fig. 3G). It is possible that microinjected mRNA, which is exported more rapidly than its in vivo transcribed counterpart, is more efficient at utilizing the low levels of UAP56/URH49 remaining in cells, regardless of the cell line. Indeed, the export of microinjected MHC-ftz-Δi mRNA was only partially inhibited in U2OS cells that were depleted of UAP56 and URH49 (Fig. 3H), confirming what we previously reported in HeLa cells. In contrast, the export of microinjected c-ftz-i mRNA was more sensitive to the deple -tion of these two factors.

From these experiments we conclude that both ALREX and the splicing dependent export pathways are mediated by UAP56 and URH49. Furthermore, our data suggests that ALREX may require lower levels of these two helicases than splicing depen-dent export.

UAP56, URH49, and TAP/p15 are required for MHC-ftz-Δi to exit out of nuclear speckles. In the course of our experiments we observed that the depletion of UAP56 and URH49 caused MHC-ftz-Δi mRNAs to accumulate into large nuclear foci and these colocalized with several nuclear speckle markers such as SC35 and Aly (Fig. 4A and B). In agreement with previous findings,27 c-ftz-i also accumulated in nuclear speckles in UAP56/URH49-depleted cells (Fig. 4C). The speckle localization of MHC-ftz-Δi was much more pronounced in the UAP56/URH49 knockdown cells than in control cells, whether this was calculated by Pearson correlation or by comparing the total amount of mRNA associated with these structures (Fig. 4D and E). Indeed, in the knockdown cells practically every SC35-positive speckle had an enrichment of MHC-ftz-Δi mRNA (Fig. 4D). In contrast, the depletion of these two helicases had only minor effects on the speckle association of βG-Δi mRNA (Fig. 4D and F).

Since TAP/p15 is also thought to associate with mRNA in nuclear speckles,28,29 and its activity is required for ALREX,31,33 we next assessed whether inhibition of this protein also resulted in the accumulation of MHC-ftz-Δi mRNAs in these structures. When plasmids containing the MHC-ftz-Δi gene were co-injected with RNA that contained the constitutive transport element (CTE), a viral sequence that prevents TAP from
TREX-components directly promotes the targeting of mRNA to speckles, we favor the model that under normal conditions these proteins enhance the rate of egress from nuclear speckles.

UAP56 associates with MHC-ftz mRNA. In U2OS cells, UAP56 is distributed throughout the nucleoplasm with a slight enrichment in nuclear speckles (Fig. S3A). However, upon UAP56/URH49 co-depletion, the remaining UAP56 was predominantly associated with nuclear speckles (Fig. S3A). Interestingly this change in distribution was seen across almost all expressing cells. Indeed, CTE co-injection also inhibited its export (Fig. 5D) and enhanced its association with speckles (Fig. 5B and E).

These experiments indicate that mRNAs containing ALREX-elements require both UAP56/URH49 and TAP/p15 for nuclear export. Although it is possible that inhibition of TREX-components directly promotes the targeting of mRNA to speckles, we favor the model that under normal conditions these proteins enhance the rate of egress from nuclear speckles.

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the entire cell population (Fig. 3B). Since this shRNA treatment also promoted the enrichment of MHC-ftz-Δi in speckles, we decided to determine whether UAP56 associates with this mRNA in vivo. Previously, it had been demonstrated that UAP56 is retained in mRNAs in a splicing-dependent manner using an in vitro splicing reaction. We thus immunoprecipitated UAP56 from cells expressing various ftz constructs and analyzed the interacting RNAs by reverse transcription-quantitative PCR (RT-qPCR). In control experiments, we repeated these experiments with a UAP56–Δi immunoprecipitate that contained the UAP56-interacting protein Aly (Fig. 6A), suggesting that the isolated complexes are stable throughout the purification procedure. Indeed, we observed a higher level of MHC-ftz-Δi mRNA enrichment in UAP56 immunoprecipitates in comparison to c-ftz-i (Fig. 6B). To further confirm our results, we compared the enrichment of MHC-ftz-Δi mRNA and the 7SL RNA in UAP56 immunoprecipitates. 7SL is a very abundant non-coding RNA that is part of the Signal Recognition Particle (SRP) and is exported to the cytoplasm independently of the TREX-TAP mRNA export pathway. Indeed, while MHC-ftz-Δi was enriched in the UAP56 immunoprecipitates, 7SL was not (Fig. 6C). From these experiments, we conclude that MHC-ftz-Δi is associated with UAP56 in vivo.

The poly(A)-tail is required for the efflux of MHC-ftz-Δi mRNA from nuclear speckles. In yeast, the UAP56 and TAP homologs, Suh2p, and Mem6p, have been shown to affect the retention of hyperpolyadenylated mRNA in nuclear foci. With this in mind, we decided to investigate whether the poly(A)-tail affected how these mRNAs trafficked through speckles. Previously we found that in vitro synthesized MHC-ftz-Δi that lacked a poly(A)-tail (MHC-ftz-Δi-ΔpolyA) was rapidly degraded when microinjected into cells. In order to stabilize this RNA, we treated in vitro synthesized MHC-ftz-Δi-ΔpolyA with sodium periodate which selectively oxidizes the vicinal diols on the 2’ and 3’ carbon atoms of the RNA 5’-phosphate. This treatment prevents the RNA from being degraded by certain 3’-5’exonucleases. Since the 5’ cap is also a substrate for this reaction, we first reacted uncapped RNA with periodate, purified it, and then performed the capping reaction. Indeed, when compared with the non-oxidized mRNA, periodate oxidized MHC-ftz-Δi-ΔpolyA was stable in microinjected U2OS cells (Fig. 7A). This mRNA was also poorly exported when compared with microinjected in vitro transcribed and polyadenylated MHC-ftz-Δi or c-ftz-i mRNA. Cells were fixed 1 h after microinjection and the mRNA was stained by FISH. Quantification of the cytoplasmic mRNA distribution is shown. Each bar represents the average and standard deviation of two independent experiments, each consisting of 15–30 cells.

Discussion

Here we shed further light on the molecular mechanism of the ALREX pathway. Our work indicates that ALREX-promoting elements are sensitive to certain features of the reporter transcript. Two mRNAs that are highly susceptible to ALREX, ftz-i, and insulins, are trafficked through nuclear speckles, while a third that is less dependent on ALREX, ftz-Δi, shows weak association with these structures. Our results are consistent with the idea that egress from speckles, and nuclear export, requires two RNA helicases, which are normally associated with the TREX complex (UAP56 and URH49), and the heterodimeric nuclear transport receptor, TAP/PS. Our data suggests that at least some ALREX-promoting elements may be more efficient at recruiting UAP56/URH49 than particular introns, thus explaining why in certain circumstances the ALREX pathway is less sensitive to the depletion of UAP56/URH49 than splicing-dependent export. Finally, our data indicates that the poly(A)-tail is required for efficient nuclear speckle egress, and nuclear export, of MHC-ftz-Δi mRNA.

From this data we propose a general model for the trafficking of mRNAs containing ALREX elements (Fig. 8). Our data indicates that mRNAs are first targeted to speckles by several possible routes. Although ALREX-promoting elements and splicing may contribute to this, other features within the transcript likely
are general export factors, it remains unclear how these proteins are recruited to mRNA containing ALREX-promoting elements and this likely requires additional nuclear factors. It also remains formally possible that speckle-targeting features also promote export, and that ALREX-promoting SSCRs may simply enhance RNA-stability, as we have previously documented.13 Our data promote this activity. Within speckles, either ALREX-promoting elements or splicing is sufficient to help assemble the mRNAs into an export-competent mRNP. Our data suggests that this speckle-associated maturation involves several stages. One step is a UAP56/URH49-dependent and involves the recruitment of UAP56, and perhaps URH49, to the transcript. Seeing that these

![Figure 4](image-url)

**Figure 4.** Depletion of UAP56 and URH49 causes an enrichment of MHC-ftz-Δi mRNA but not βG-Δi mRNA in nuclear speckles. (A–C and F) U2OS cells were treated with lentiviruses that either mediate the delivery of shRNAs against UAP56 and URH49 or control plasmids. Three days post-infection, cells were microinjected with plasmid containing MHC-ftz-Δi (A–F), c-ftz-Δi (C) or βG-Δi (F). After allowing the plasmid to be transcribed for 20 min, cells were then fixed, probed for either ftz (A–C) or βG (F) mRNA and immunostained for the nuclear speckle markers SC35 (A, C and F) or Aly (B). Each row represents a single field of view. Overlays of mRNA (red) and SC35 (A, C and F) or Aly (B) (green) are shown in the right panels. Scale bar = 5 μm. (D) The percentage of SC35-positive speckles that colocalize with MHC-ftz-Δi, βG-Δi mRNA or dextran in control cells or cells depleted of UAP56 and URH49. The data was analyzed and plotted as in Figure 1D. (E) The percentage of total cellular and nuclear MHC-ftz-Δi mRNA that is present in nuclear speckles in control cells or cells depleted of UAP56 and URH49 as described in Figure 1H. Each bar represents the average and standard error of the mean of 10 cells.
be attributable to the fact that these transcripts have a low level of speckle association, but that it is so transient that it is not detectable under normal circumstances. It has also been documented that certain spliced mRNAs do not traffic through speckles yet are still exported to the cytoplasm,40 although again it is hard to determine whether these mRNAs have a low level of speckle association that is not normally detectable. Although our data supports the model that targeting to nuclear speckles licenses an mRNA for ALREX, thus explaining why MHC-βG-Δi mRNA is poorly exported, we do not know whether trafficking through speckles is absolutely required for ALREX.

The role of the poly(A)-tail in nuclear export is unclear at the moment. Many studies have implicated poly(A)-binding proteins in mRNA export,41-43 while others have found the converse.44 In budding yeast, components of the TREX-complex have been implicated in the regulation of poly(A)-tail length.35,45 In particular cases, hyperpolyadenylation, and the subsequent trimming of also indicates that there is an additional TAP/p15-dependent step that also occurs within speckles, and likely includes the recruitment of TAP to the mRNA. This step may be coupled with the release of UAP56/URH49,37,38 although it is possible that UAP56/URH49 may stay on the mRNA and accompany it to the cytoplasm.39 It is thus likely that egress of export-competent mRNAs from the speckles requires TAP/p15 recruitment, and this is consistent with recent findings.36 Our data also indicates that one of the speckle-associated steps requires a poly(A)-tail, however its exact role remains unclear.

We have yet to define why certain transcripts, such as fit and insulin, have a high degree of speckle-association while others do not. This may be related to certain features that are over-represented in protein coding genes, such as GC-content.3 It is also clear that many naturally intronless mRNA do not normally associate with speckles,13,27,28 and this may be due to the requirements of different mRNA export pathways, although it could also

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Figure 5. TAP inhibition promotes the accumulation of MHC-fit-Δi and INS-Δi in nuclear speckles. (A) U2OS cells were microinjected with DNA plasmid that codes for MHC-fit-Δi with either in vitro synthesized CTE RNA or control buffer. After allowing the plasmid to be transcribed for 20 min, cells were treated with α-amanitin and incubated for an additional 2 h. Cells were then fixed, probed for fit mRNA and immunostained for the nuclear speckle marker SC35. Each row contains a single field of view with an overlay of the MHC-fit-Δi mRNA (red) and SC35 (green) in the far right panel. Scale bar = 5 μm. (B) The percentage of speckles that demonstrate different levels of colocalization with MHC-fit-Δi, INS-Δi mRNA or dextran in control cells or cells co-injected with CTE RNA as described in (A). The data was analyzed and plotted as in Figure 1D. (C) The percentage of total cellular and nuclear MHC-fit-Δi mRNA present in nuclear speckles in control cells or cells co-injected with CTE RNA as described in Figure 1H. Each bar represents the average and standard error of the mean of 10 cells. (D) U2OS cells were either injected with a DNA plasmid that codes for INS-Δi mRNA alone or co-injected in vitro synthesized CTE RNA. After allowing the plasmid to be transcribed for 20 min, cells were treated with α-amanitin and incubated for an additional 2 h. Cells were then fixed, probed for INS mRNA and nuclear export was quantified. Each bar represents the average of three independent experiments and error bars represent standard error of the mean. (E) The percentage of total cellular and nuclear INS-Δi mRNA that is present in nuclear speckles in control cells or cells co-injected with CTE RNA. The data was analyzed and plotted as in Figure 1H.
the poly(A)-tail, is coupled to UAP56 and TAP-dependent steps and may occur in discrete nuclear foci. It however remains unclear whether the poly(A)-tail length is modulated during this phenomenon has never been observed in mammalian cells, 51

**Materials and Methods**

Plasmid constructs. The MHC-ftz-Δi, c-ftz-Δi, c-ftz-i and INS-Δi constructs in pCDNA3 were described previously. Human β2m introns were amplified from U2OS genomic DNA and inserted into pcDNA3 mammalian expression vector containing βG cDNA by restriction-free cloning 53 using the following primer sequences, forward primer: GTGTTGAGGCTGTGGCAGG CCGTGGTAACAGTACAG and the reverse primer: GACCGAGCA GTTGCGCAGGA GCTTGGGGA GAGATAGTTA CGCGTCCAGG TGGTCGCTGT GT and the reverse primer 3: CTCCCTCAGGA GTCTAAGGTA CCGGCCTGGGT CGTACCCAGA GC. This product was then used in a subsequent PCR reaction to insert the MHCI SSCR into the βG-containing vector by restriction-free cloning. The sequence pre-bound to protein G sepharose. The immunoprecipitates were quantified by RT-qPCR. Each bar represents the average of three independent experiments. Error bars represent standard error of the mean.

**Figure 6.** MHC-ftz-Δi associates with UAP56 in vivo. (A) UAP56 was immunoprecipitated from U2OS lysates using rat anti-UAP56 antibodies pre-bound to protein G sepharose. The immunoprecipitates were analyzed by immunoblotting using rabbit polyclonals against UAP56 and Aly. Rat pre-immune serum was used in the mock immunoprecipitation reaction. (B and C) U2OS cells were transfected with plasmids containing MHC-ftz-Δi or c-ftz-i (B) or c-ftz-i (C). One day after transfection, cells were harvested and the nuclear lysates were collected and immunoprecipitated with rat anti-UAP56 antibodies or rat pre-immune serum. RNA was collected from fractions and converted to cDNA using MHC-specific primers (B) or random hexamers (C). The fold enrichment of mRNAs in anti-UAP56 over pre-immune precipitates was quantified by RT-qPCR. Each bar represents the average of five independent experiments. Error bars represent standard error of the mean.

**Cell lines and antibodies.** Both human osteosarcoma (U2OS) and embryonic kidney 293T (HEK293T) were maintained in high glucose DMEM (Wisent) containing 10% FBS (Wisent) and antibiotics (Sigma).

**Lentiviral mediated shRNA protein depletion.** Human embryonic kidney 293T (HEK293T) cells were transiently transduced with gene specific shRNA pLKO.1 plasmids (Sigma) along with the packaging (Δ8-9) and envelope (VSVG) expression vectors using Lipofectamine 293 DNA In Vitro Transfection Reagent (Life Technologies) following the manufacturer’s protocol. Viruses were harvested 48 h after transduction. Human U2OS cells were transduced with viruses in the presence of 8 μg/mL hexadimethrine bromide. One day after transduction, cells were treated with 2 μg/mL Puromycin every other day. The efficiency of the knock-down was assessed 3 d post transduction for UAP56 and URH49 and 4 d for THOC1 and Aly by immunoblotting. The decrease in protein was measured by densitometry analysis, as described previously. These viruses contained the following plasmids obtained from Sigma: TRCN0000074386 (shRNA targeted to UAP56 with sequence CCGGGATAGGA CATCTCCTCC TACATCTCAGG GATGTAGGAG GAGATGTCTA 3: CTCCTCAGGA GTCTAAGGTTA CCGGCCTGGGT CGTACCCAGA GC).

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Microinjection, FISH, and immunostaining. For microinjection experiments, cells were plated on 22 × 22 mm coverslips (VWR) in 35 mm mammalian tissue culture dishes (Thermo Scientific) for 24 h prior to injection. For DNA microinjections, DNA plasmids were prepared using QIAprep Midi Kits (Qiagen). Microinjections were performed as previously described. Briefly, DNA plasmids or mRNA transcripts were microinjected at 200 ng/μL with 70 kDa Dextran conjugated to Oregon Green (Invitrogen) and Injection Buffer (100 mM KCl, 10 mM HEPES, pH 7.4). For pulse chase experiments, cells were treated with 1 μg/mL α-amanitin (Sigma) 20 min after injection. After incubating the cells for the appropriate time at 37 °C, they were washed twice with Phosphate Buffer Saline (PBS) and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 min. Cells were then permeabilized using 0.1% TritonX-100 in PBS (Thermo Scientific). In HHT experiments, cells were treated with 5 μM HHT (or DMSO as a control) 30 min prior to microinjections. The cells were then maintained in HHT up until they were fixed.

For mRNA staining, cells were washed twice in 1× Sodium Saline Citrate (SSC) buffer supplemented with 60% formamide. Cells were then treated with hybridization buffer (60% formamide, 100 mg/ml dextran sulfate, yeast tRNA, 5 mM VRC, 1× SSC) containing 200 nM Alexa 546-conjugated ssDNA probe (Integrated DNA Technologies) for 24 h. Subsequently, coverslips were washed with 1× SSC supplemented with 60% formamide and the coverslips were mounted on DAPI. The probe oligonucleotide sequences included antioxidation, β-G (CTTCATCCAC GTTCACCTTC GCCCCACAGG GCAGTAACGG CAGACTTCTC CTCAGGAGTC A), or antioxidation, insulin (GGTCCTCTGC CTCCCGGCGG GTCTTGGGTG TGTAGAAGAA GCCTCGTTCC CGCACACACT A).

Figure 7. MHC-ftzΔ mRNA lacking a poly(A)-tail accumulates in nuclear speckles and is poorly exported. (A) In vitro transcribed MHC-ftzΔ mRNA lacking a poly(A)-tail (ΔpA) was either oxidized with periodate or left untreated. These mRNA were microinjected into the nuclei of human U2OS cells, which were then immediately fixed (70 hr) or first incubated for 1 h at 37°C then fixed. Cells were probed for ftz and imaged. The total mRNA fluorescence was quantified for each nuclear-injected cell. Each bar represents the average and standard error of 12–30 cells, all values being normalized to the average fluorescent intensity at 0 h. (B) Nuclei of cells were microinjected with either in vitro transcribed and polyadenylated MHC-ftzΔ mRNA or with periodated oxidized MHC-ftzΔΔpA. After allowing mRNA export to proceed for 1 h, cells were fixed, stained using FISH, and imaged. Scale bar = 20 μm. (C) The fraction of mRNA in the cytoplasm and nucleus was quantified. Each bar represents the average and standard error of three independent experiments, each consisting of 12–30 cells. (D) An example of a nucleus microinjected with in vitro transcribed and oxidized MHC-ftzΔΔpA mRNA and then incubated for 1 h before fixed, probed for fitz mRNA and immunostained for the nuclear speckle marker SC35. An overlay of the mRNA (red) and SC35 (green) is shown in the bottom right panel. Scale bar = 5 μm. (E) The percentage of nuclear speckles that demonstrate different levels of colocalization with MHC-ftzΔ, periodated oxidized MHC-ftzΔΔpA and dextran 1 h after mRNA microinjection. Data was analyzed and plotted as described in Figure 1D. (F) The percentage of total cellular and nuclear MHC-ftzΔ and periodated oxidized MHC-ftzΔΔpA mRNA that is present in nuclear speckles as described in Figure 1H.
The Pearson correlation between microinjected fluorescent dextran and the average and standard error were compiled. Totals from 10 cells were compiled. This analysis was repeated for the brightest SC35-positive speckles per cell and the fluorescence intensity of RNA FISH was computed by NIS analysis software. Then Pearson correlation ratio between RNA FISH and SC35 immunofluorescence was computed by NIS analysis software. Examples are shown in Supplementary Materials. For each experiment, the 10 brightest SC35-positive speckles per cell were analyzed and the analysis was repeated for the other SC35-positive speckles. The Pearson correlation between microinjected fluorescent dextran and SC35 was also assessed to determine background levels of correlation.

To calculate the fraction of mRNA in speckles, thresholds were drawn on the SC35 immunofluorescence channel using NIS. The threshold was set so that 20% (± 5%) of the nuclear area was selected per cell and the fluorescence intensity of RNA in the selected area was calculated. The total integrated mRNA signals in the nucleus and the cell body were also computed.

In vitro $MHC_{ftz}\Delta X-\alpha$ mRNA synthesis, polyadenylation, capping and periodate oxidation. pcDNA3 plasmid containing $MHC_{ftz}\Delta X-\alpha$ was linearized by Xhol digestion and precipitated at $-80^\circ C$ for 1 h with 40 mM Potassium Acetate and 2.5× 100% ethanol. The precipitated DNA was resuspended in RNase free water and used as a template to transcribe mRNAs from T7 RNA polymerase (NEB) by incubation with 10 mM ATP, 10 mM GTP, 10 mM CTP, 10 mM UTP for 4 h at 37 °C. Polyadenylation was performed using E. coli Poly A Polymerase (NEB) following the manufacturer’s protocol. To oxidize the 3’end, 500 pmoles of T7-transcribed RNA previously dissolved in 40 μl of water was added to 360 μl of freshly prepared 5M Sodium-Periodate (Sigma). The 400 μl reaction mixture was rotated for 1 h in the dark at room temperature. The oxidized RNA was purified by Purelink RNA purification kit (Ambion) and eluted in 30 μl of RNase free water. Oxidized and non-oxidized RNA was capped using vaccinia capping enzyme (NEB) following the vaccinac-turer’s protocol and purified by Purelink RNA purification kit (Ambion). RNA was then precipitated at $-80^\circ C$ for 1 h with 40 mM Potassium Acetate and 2.5× 100% ethanol and resuspended in injection buffer.

**CTE mRNA synthesis.** CTE-PCR3.1 plasmid$^3$ was linearized by Xhol digestion and precipitated at $-80^\circ C$ for 1 h with 40 mM KAcetate and 2.5× 100% ethanol. The precipitated DNA was resuspended in RNase free water. mRNA synthesis and capping was performed by T7 RNA polymerase (NEB) and Vaccinia capping enzyme (NEB). RNA was purified by Purelink RNA purification kit (Ambion), then precipitated at $-80^\circ C$ for 1 h with 40 mM Potassium Acetate and 2.5× 100% ethanol and resuspended in injection buffer.

**RNA immunoprecipitation.** Human U2OS cells were transfected with different plasmids using GenJet in vitro DNA Transfection Reagent for U2OS (SignaGen Laboratories). After 18–24 h, cell lysate was incubated for 10–14 h with rat anti-UAP56 antibodies$^5$ prebound to protein G sephrose (Invitrogen). Subsequently, the beads were washed five times. The RNA is eluted from the UAP56 bound beads using SDS and harvested using Trizol (Invitrogen) as previously described.$^56,57$ The RNA samples were treated with DNase (Ambion) to remove DNA plasmid contamination. cDNA was synthesized using SuperScript III (Invitrogen) according to the manufacturer’s protocol and purified by Purelink Green Master Mix (Invitrogen) and the reaction was run on a CFX384 Touch Real Time PCR Detection System (Bio-Rad). The efficiency of the IP reaction was checked by immunoblotting with rabbit anti-UAP56 antibodies (Sigma) and rabbit anti-Aly antibodies.$^3$

**Disclosure of Potential Conflicts of Interest**
No potential conflict of interest was disclosed.

**Supplemental Materials**
Supplemental materials may be found here: www.landesbioscience.com/journals/nucleus/article/26052

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