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A role for transportin in deposition of TTP to cytoplasmic RNA granules and mRNA decay

Wei-Lun Chang[1,2] and Woan-Yuh Tarn[2,*]

[1]Graduate Institute of Life Sciences, National Defense Medical Center and [2]Institute of Biomedical Sciences, Academia Sinica, 128 Academy Road Section 2, Nankang, Taipei 11529, Taiwan

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ABSTRACT

Importin-β family members, which shuttle between the nucleus and the cytoplasm, are essential for nucleocytoplasmic transport of macromolecules. We attempted to explore whether importin-β family proteins change their cellular localization in response to environmental change. In this report, we show that transportin (TRN) was minimally detected in cytoplasmic processing bodies (P-bodies) under normal cell conditions but largely translocated to stress granules (SGs) in stressed cells. Fluorescence recovery after photobleaching analysis indicated that TRN moves rapidly in and out of cytoplasmic granules. Depletion of TRN greatly enhanced P-body formation but did not affect the number or size of SGs, suggesting that TRN or its cargo(es) participates in cellular function of P-bodies. Accordingly, TRN associated with tristetraprolin (TTP) and its AU-rich element (ARE)-containing mRNA substrates. Depletion of TRN increased the number of P-bodies and stabilized ARE-containing mRNAs, as observed with knockdown of the 5′–3′ exonuclease Xrn1. Moreover, depletion of TRN retained TTP in P-bodies and meanwhile reduced the fraction of mobile TTP to SGs. Therefore, our data together suggest that TRN plays a role in trafficking of TTP between the cytoplasmic granules and whereby modulates the stability of ARE-containing mRNAs.

INTRODUCTION

Importin-β family members function as receptors for the nucleocytoplasmic transport of macromolecules through the nuclear pore complex (1,2), where importin-β proteins also interact directly with nucleoporins. The prototypical importin-β1 protein forms a heterodimer with importin-α, which acts as an adaptor to bind transport cargoes. Nevertheless, each importin-β member can recognize specific nuclear localization signals of respective cargoes and mediate their nuclear transport. For example, transportin (TRN) recognizes the glycine-rich domain of heterogeneous nuclear ribonuclear protein (hnRNP) A1, and transportin-SR (TRN-SR) interacts specifically with the phosphorylated arginine/serine dipeptide-rich motif of precursor mRNA splicing factor SR proteins (3–5). After import into the nucleus, importin-β–cargo complexes are dissociated by the action of GTP-bound Ran. In contrast, importin-β-like nuclear export receptors associate concomitantly with their cargo and RanGTP during transport. During mitosis, importin-β1 acts as an effector of Ran to negatively regulate the assembly of mitotic spindles and centrosomes as well as the nuclear membrane and pores (6). Similarly, the nuclear export receptor CRM1 also functions with RanGTP to recruit factors to kinetochores for microtubule nucleation and chromosome segregation (7,8). The possibility that other importin-β family members play roles in cellular processes apart from nucleocytoplasmic transport remains to be investigated.

In eukaryotic cells, regulation of gene expression can be determined by subcellular localization of *trans*-acting factors. Cellular signaling pathways that reprogram gene expression often induce post-translational modification of these regulatory factors, thereby altering their affinity or accessibility to the nuclear transport machinery (9). However, it is also possible that cellular signaling pathways affect transporters directly. For example, heat shock stress induces nuclear retention of importin-α and thereby inhibits the conventional nuclear import pathway involving importin-α/β1 (10). The cell cycle may modulate the interaction between importins α and β, thereby affecting transport efficiency (11). However, whether cellular signals can regulate nucleocytoplasmic transport in general by modifying the transport machinery and how regulatory specificity is achieved remain unclear.

In this study, we observed that TRN localized to cytoplasmic RNA-containing granules including processing bodies (P-bodies) and stress granules (SGs). These granules exist in most cell types and play roles in mRNA metabolism (12). SG formation is induced by

*To whom correspondence should be addressed. Tel: +8862 2652 3052; Fax: +8862 2782 9142; Email: wtarn@ibms.sinica.edu.tw

environmental stress. Factors deposited into SGs include the small ribosomal subunit, translation initiation factors, and a number of RNA binding proteins. Therefore, translationally stalled transcripts that are associated with 48S translation–preinitiation complexes act as essential factors in SGs (13). However, P-bodies lack ribosomal proteins as well as the majority of initiation factors but contain mRNA degradation factors (12,13). Thus, P-bodies primarily serve as sites of mRNA decay (14). Except for granule-specific components, P-bodies and SGs share a number of factors involved in mRNA metabolism, and such factors may be exchanged between these granules (13). Nevertheless, the precise nature of why certain proteins exist in both P-bodies and SGs remains to be investigated.

In higher eukaryotes, several pathways regulate mRNA decay, and each pathway involves a specific set of factors. Indeed, most of these factors localize to P-bodies (12,13). The decay of short-lived transcripts is often mediated by AU-rich elements (AREs) within their 3′-untranslated region (UTR). AREs exist in a diverse repertoire of mRNAs, particularly including those encoding oncoproteins, growth factors and cytokines (15). The stability and translation of ARE-containing mRNAs are controlled by ARE-binding proteins (16), among which tritetraprolin (TTP) and BRF, present in both P-bodies and SGs, activate ARE-mRNA decay. TTP and BRF may escort ARE-containing mRNAs to SGs under stress conditions, where mRNAs can be further sorted for translation or transferred to P-bodies for degradation (13). Moreover, mRNAs in P-bodies can also return to polysomes for reinitiation of protein synthesis (13), but the mechanism by which mRNAs move reciprocally between polysomes and P-bodies for the control of translation and degradation has not been fully elucidated.

In this study, we initially characterized the subcellular localization of nuclear transport receptors under cell stress conditions and observed that TRN localized to P-bodies and SGs. Accordingly, TRN interacted with TTP, a component of both types of granules. Therefore, we examined the role of TRN in TTP-mediated mRNA decay and dynamic distribution of TTP in cytoplasmic granules.

MATERIALS AND METHODS

Plasmid construction

The GST-TRN and GFP-TRN expression vectors were constructed by subcloning the human TRN open reading frame (ORF) from PVP16-HA-TRN (Bryan R. Cullen, USA) to pGEX-2T (GE Healthcare) and pEGFP-C1 (Clontech), respectively. The GFP-TIA1 expression vector was constructed by subcloning the TIA1 ORF from pMT2-HA-TIA1 (Nancy Kedersha, USA) to pEGFP-C1. The DNA fragments encoding CRM1 (Iain W. Mattaj, Germany), importin-13 (KIAA0724; Takahiro Nagase, Japan), Ran (Hela cDNA library) and RanQ69L mutant (Iain W. Mattaj, Germany) were obtained by PCR and inserted in-frame with the HA-epitope coding sequence in the pCEP4 vector (Invitrogen). To construct the FLAG-tagged exportin-5 expression vector, the exportin-5 ORF cDNA (Bryan R. Cullen, USA) was amplified by PCR and cloned into pCDNA3.1 (Invitrogen) in-frame with the FLAG coding sequence. The TTP ORF was amplified by RT–PCR from a HeLa cell cDNA library (Clontech) and cloned into pCDNA3.1 and pEGFP-C3 in-frame with the FLAG-tag and GFP, respectively. The cDNA encoding the N-terminal 251 residues of Dcp1a (Jens Lykke-Andersen, USA) was amplified by PCR and cloned into pET-29b (Novagen) and pGEX-2T to generate plasmids expressing His-Dcp1aN and GST-Dcp1aN, respectively. The plasmids encoding GFP-mCPEB1 and His-RanQ69L were generous gifts of Yi-Shuian Huang (Taiwan) and Iain W. Mattaj (Germany). Plasmids expressing HA-tagged TRN-SR, PABP1 and HuR have been described (5,17). All plasmid constructs used in this study were verified by sequencing.

For construction of pTRE-β-globulin-TNF-UTR, the DNA fragment containing the β-globin gene containing the TNF-α 3′-UTR was amplified by PCR from the c-fos-TNF-α 3′-UTR plasmid (Jiahuai Han, USA) and subsequently cloned into the pTRE vector (Clontech). The AREs from the TNF-α 3′-UTR were deleted by PCR with appropriate primers to generate pTRE-β-globulin-TNF-ΔARE. The probes used for RNase protection assays were nucleotides 307 to 1 of the GFP coding region and nucleotides 390 to 214 of the rabbit β-globin gene (accession number V00882); each corresponding DNA fragment was amplified by PCR and subcloned into pBluescript® II KS (+) (Stratagene).

Cell culture and transfection

Human HeLa cells and HEK293 cells were cultivated and transfected using Lipofectamine 2000 (Invitrogen) or calcium phosphate as described (17). For stress induction, cells were treated with 0.5 mM sodium arsenite (Sigma) for 1 h. siRNAs used in knockdown experiments were purchased from Invitrogen (si-TRN and si-XRN1) and Dharmacon (si-TRAP150 and si-Lamin A/C); their sense-strand sequences are as follows: si-TRN, 5′-acaggaaucaacuuaggaagaugcc-3′; si-Xrn1, 5′-gggaucugg aaagaugcaauacuuu-3′; TRAP150, 5′-gguauaagcuccgagau gauu-3′; si-Lamin A/C, 5′-gguggugacgaucugggcu-3′.

Preparation of recombinant proteins and antibodies

Recombinant GST, GST-TRN, GST-Dcp1aN and His-Dcp1aN were overexpressed in *Escherichia coli* strain BLR (Novagen) by induction with 0.8 mM isopropyl-1-thio-β-D-galactopyranoside (Protech). GST-fusion proteins and His-tagged proteins were purified from *E. coli* lysate using glutathione-Sepharose 4B (GE Healthcare) and nickel-agarose (Novagen), respectively, according to the manufacturers' instructions. Purified proteins were dialyzed against buffer D containing 20 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol. Recombinant His-Dcp1a was used as antigen to immunize rabbits, and GST-Dcp1aN was used to purify antibodies against Dcp1a from antisera as described (5). Recombinant His-RanQ69L was purified and loaded with GTP

(Sigma) according to the procedures described earlier (5).

Indirect immunofluorescence

HeLa cells were plated onto coverslips. Transfection was performed at 70% cell confluency. For knockdown experiments, cells were transfected with siRNAs 2 days before analysis or further transfection with pcDNA3.1-TTP-FLAG. The primary antibodies used for immunofluorescence included monoclonal anti-TRN1 (Sigma), anti-importin-β1 (Affinity BioReagents), anti-PABP1 (Sigma), anti-FLAG (Sigma) and anti-HA (gift of Soo-Chen Cheng, Taiwan), and polyclonal anti-Dcp1a, anti-eIF4A (Abcam), anti-HA (Covance), anti-myc (Upstate Biotechnology), anti-EDC4 (Abcam) and anti-FLAG (Sigma). Secondary antibodies were fluorescein- or rhodamine-conjugated anti-mouse or anti-rabbit IgG (Cappel Laboratories). Cells were fixed with 3% formaldehyde for 30 min followed by permeabilization with 0.5% Triton X-100 for 10 min. For immunofluorescence using anti-importin-β, fixation and permeabilization of the cells were performed in 4% paraformaldehyde for 10 min and in 0.1% Triton X-100 for 10 min, respectively. Cells were incubated with appropriately diluted antibodies and subsequently washed with phosphate-buffered saline as described (18). Nuclei were counterstained with Hoechst 33258. The specimens were observed using confocal microscopy (Bio-Rad Radiance 2100 and Zeiss LSM 510 META).

To evaluate the number of FLAG-tagged TTP-expressing cells that colocalized with endogenous PABP1 and Dcp1a, we randomly selected 25 areas, which totally contained more than 100 cells, in individual transfections. Averages were obtained from three independent experiments.

Fluorescence recovery after photobleaching

HeLa cells were transfected with GFP-TRN, GFP-TIA1, or GFP-CPEB1. At 24 h post-transfection, cells were treated with 0.5 mM arsenite for 30 min. To assess the dynamics of TTP in the absence of TRN, HeLa cells were transfected with si-TRN or mock-transfected for 2 days, followed by transfection with the vector encoding GFP-TTP. Cells were treated with 1 μM FCCP (Sigma) in glucose-free medium (Life Technologies) for 1 h before analysis by Fluorescence recovery after photobleaching (FRAP). FRAP experiments were performed on an LSM 510 META confocal microscope with a 100× oil immersion objective using the 488-nm line of a 25-mW argon laser. An area of 3.5×2.4 μm containing a selected cytoplasmic granule was bleached by scanning at the maximum laser power for 100 iterations. Fluorescence recovery was measured every 0.8 s for 40 times, followed by every 5 s for more than 40 times. The raw data were subtracted for background and corrected for the loss of fluorescence signal over time, and subsequently normalized with respect to the intensity of the minimum (value = 0) and the prebleaching intensities (value = 1). The resulting relative intensity was plotted, and the mobile fraction was determined as the span of the recovery curve. The halftime of fluorescence recovery ($t_{1/2}$) was determined with LSM software.

Immunoprecipitation

HEK293 cells were transfected with pcDNA3.1-TTP-FLAG for 2 days. Cells were incubated in RSB100 (10 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, and 100 mM NaCl) containing 40 μg/ml digitonin (Calbiochem), 1 mM β-glycerophosphate, 1 mM KF and a mixture of protease inhibitors (Roche). Cells were incubated on ice for 10 min before passage through 26 gauge needles for lysis, and centrifuged at 4000*g* for 15 min. Supernatants were subjected to immunoprecipitation using α-FLAG M2 agarose (Sigma). After washing with RSB100 containing 0.1% NP-40, the beads was treated with 20 mg of RNase A (Sigma) at 4°C for 1 h. Bound proteins were recovered for immunoblotting using anti-TRN and anti-FLAG (Sigma).

Immunoprecipitation and RT–PCR

HeLa cells were transfected with pTRE-TNF-UTR or pTRE-TNF-ΔARE. At 24 h post-transfection, the cells were lysed in RSB100 as described earlier, followed by incubation with α-TRN-conjugated protein A Sepharose (Amersham) for 2 h. Precipitates were treated with proteinase K. Subsequently, RNA was recovered and treated with RQ1 DNase (Promega). Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen) and a TNF-3′-UTR-specific primer 5′-gattacagtcacggctccc-3′. The primer above and a second primer, 5′-ccagattcttccctgagg-3′ were used for the following PCR.

GST pull-down

Recombinant GST and GST-TRN (2 μg each) were incubated with *in vitro* translated ^{35}S-labeled TTP or HuR in a 100-μl mixture at 30°C for 30 min. For competition, 2 μg of RanQ69L-GTP was added. The mixture was then incubated with 12 μl of glutathione-Sepharose 4B in NET-2 buffer containing 0.1% NP-40 at 4°C for 2 h. The beads were subsequently washed with NET-2 buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% NP-40, and, if necessary, followed by RNase A (2 μg/μl) treatment at 37°C for 30 min. Bound proteins were resolved by SDS–PAGE and analyzed by autoradiography.

RNA decay assay

HeLa tet-off cells (gift of S.-Y. Shieh, Taiwan) were transfected with siRNAs for 2 days, followed by further transfection with the pcDNA3.1-GFP control vector and the pTRE-TNF-UTR or pTRE-TNF-ΔARE reporter. After 24 h, cells were replated and cultured in DMEM supplemented with 10% Tet system approved fetal bovine serum (BD Biosciences). After another 24 h, 10 μg/ml doxycycline (Sigma) was added to stop transcription of the reporter. RNase protection assays were performed essentially as described (18). For each reaction, 20 μg of RNA and 2×10^5 cpm of the probe

were used. The resulting products were resolved on 6% denaturing polyacrylamide-urea gels.

RESULTS

Nuclear transport receptors relocate intracellularly during cellular stress

We initially explored whether cell stress-induced signaling affects the subcellular localization of nuclear transport receptors. We first examined several importin-β family members under different cell stress conditions. In the absence of stress, TRN distributed throughout HeLa cells, with a higher concentration in the nucleus, and also localized somewhat to cytoplasmic granules (Figure 1A). When cells were treated with arsenite, TRN primarily accumulated in cytoplasmic granules concomitantly with a decrease in nuclear abundance (Figure 1A). Heat shock or treatment of cells with the mitochondrial oxidative phosphorylation inhibitor FCCP also induced TRN localization to cytoplasmic granules (Supplementary Figure S1). In unstressed cells, importin-β1 was primarily located in the nucleus and the nuclear envelope (Figure 1B). After arsenite treatment, importin-β1 was not only detectable at the nuclear periphery but also localized to cytoplasmic granules (Figure 1B), although its granule localization was less visible than TRN. Because a transiently expressed green florescent protein (GFP)-TRN fusion protein localized similarly to endogenous TRN (data not shown; also see below),

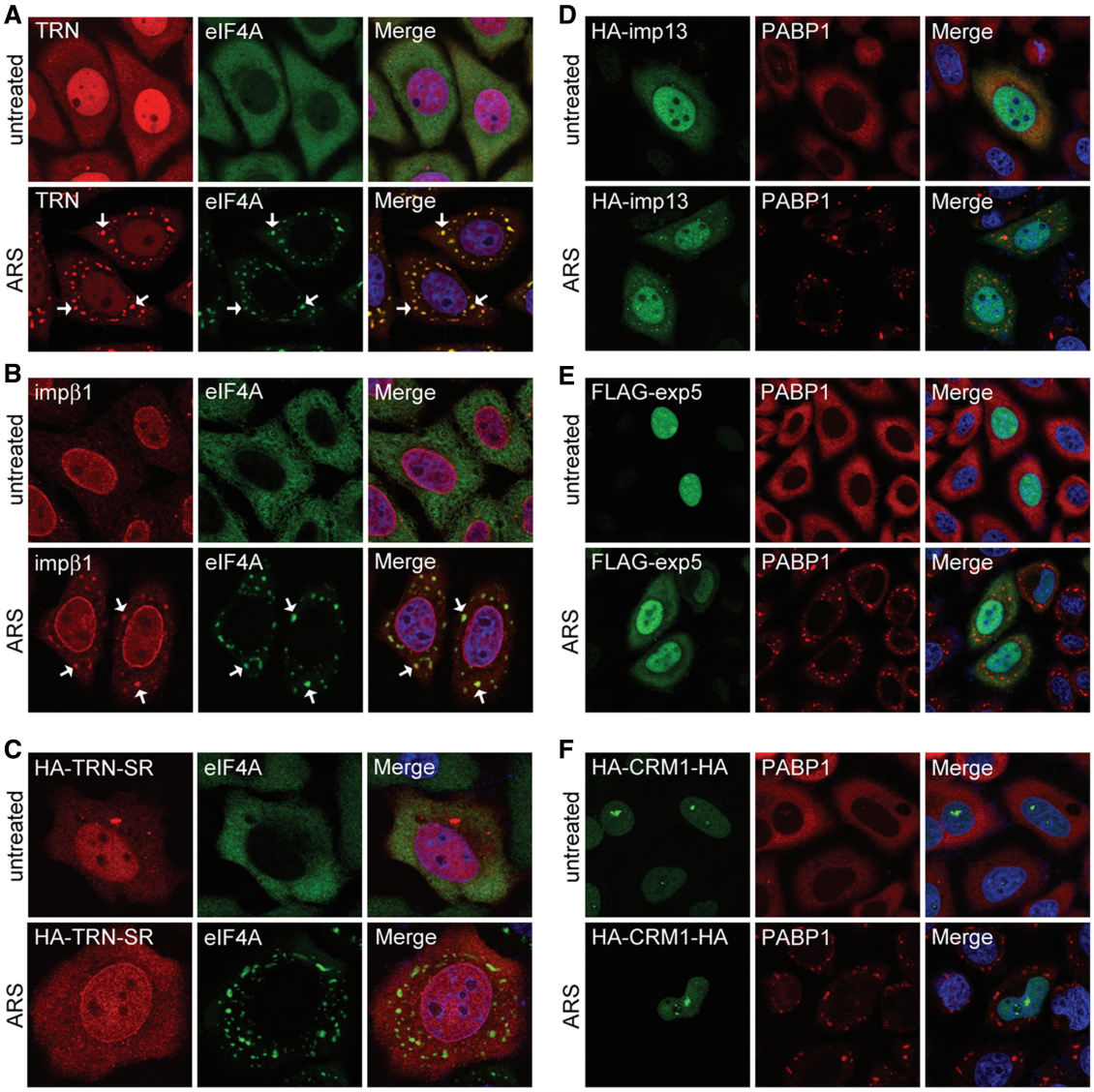

Figure 1. Subcellular localization of importin-β family transporters in arsenite-treated cells. Double immunofluorescence was performed using a monoclonal antibody against TRN (**A**) or importin-β1 (**B**) as well as polyclonal anti-eIF4A in mock-treated and arsenite-treated (ARS) HeLa cells. For each of (**C**–**F**), an analogous immunofluorescence experiment was performed in HeLa cells that transiently expressed the indicated epitope-tagged importin-β family member using a polyclonal antibody against HA, FLAG or eIF4A and monoclonal anti-PABP or anti-HA. In A and B, arrows indicate colocalization of importin-β proteins with endogenous SG marker proteins.

we examined the localization of other importin-β family proteins using epitope-tagged constructs. TRN-SR and exportin-13 distributed throughout the whole cell, albeit with a higher concentration in the nucleus (Figure 1C and D). When cells were treated with arsenite, the cytoplasmic signal of TRN-SR slightly increased whereas importin-13 could be minimally detected in cytoplasmic granules (Figure 1C and D). Finally, two nuclear export receptors, exportin-5 and CRM1, showed a predominant nuclear localization pattern in unstressed cells and the former could modestly accumulate in the cytoplasm after arsenite treatment (Figure 1E and F).

TRN localizes to both SGs and P-bodies during cellular stress

We next examined whether the cytoplasmic granules detected by TRN and importin-β1 immunostaining also contained SG or P-body components. After arsenite treatment, TRN colocalized with the endogenous SG maker protein eIF4A (19,20) (Figure 1A) as well as overexpressed SG factors, TIA1, HuR and PABP1 (Figure 2A), indicating that TRN was recruited to SGs in response to cell stress. Moreover, a part of the TRN foci, although much less intense, colocalized with or was juxtaposed to foci of the endogenous P-body-specific factors Dcp1a and Edc4 (21,22) in both unstressed and arsenite-treated cells (Figure 2B). Overexpression of Myc-tagged Dcp1a substantially recruited TRN to P-bodies (Figure 2C). Therefore, TRN might be a component of both SGs and P-bodies. Among other importin-β proteins examined, arsenite stress only induced importin-β1 and importin-13 to colocalize with eIF4A and Dcp1a in granules, respectively (Figures 1B and 2D, and Table 1). Therefore, TRN appeared to have a unique cellular distribution pattern in response to cellular stress, i.e. localization in both P-bodies and SGs (Table 1).

TRN shuttles rapidly in and out of cytoplasmic granules

Our earlier data suggested that TRN may continuously move in and out of the cytoplasmic granules. Therefore, we performed FRAP analysis by using GFP-TRN. Like endogenous TRN, transiently expressed GFP-TRN accumulated in cytoplasmic foci, and such accumulation was greatly enhanced by arsenite treatment (data not shown). FRAP of a selected GFP-TRN granule was monitored over a 200-s period; during FRAP, GFP-TRN fluorescence recovered to ~80% of the prebleaching level (data not shown). The halftime ($t_{1/2}$) of recovery for GFP-TRN granules was ~8.5 s at ambient temperature under arsenite-stressed conditions (Figure 3, bottom). In comparison, GFP-TIA-1 had a slightly faster recovery ($t_{1/2}$ = ~4.7 s), whereas the recovery of GFP-CPEB1 was much slower ($t_{1/2}$ = ~27 s) (Figure 3, bottom). This observation demonstrated that GFP-TRN may continuously exchange between the cytoplasmic pool and granules rather than merely form aggregates.

Depletion of TRN enhances P-body formation

Our observation of TRN localization in SGs and P-bodies prompted us to examine whether TRN functions in these granules. First, we attempted to examine whether depletion of TRN affected the generation of SGs and P-bodies. Transient transfection of a small interfering RNA (siRNA) targeting TRN reduced its protein level by ~80% in HeLa cells (Figure 4A, immunoblotting), which was also confirmed by immunofluorescence (Figure 4A, immunofluorescence). Using anti-Dcp1a, we quantified the number of microscopically detectable P-bodies in HeLa cells transfected with various siRNAs. Most of the mock-, TRAP150 siRNA-, or lamin A/C siRNA-transfected cells contained less than five P-bodies (Figure 4A, bar graph). As earlier reported (22,23), the number of P-bodies increased in cells depleted of the 5′–3′ exonuclease Xrn1. TRN knockdown greatly enhanced P-body formation, with the number reaching >10 per cell (Figure 4A, bar graph). A similar result was also observed by using antibody against another P-body marker Edc4 (Supplementary Figure S3). Meanwhile, we examined whether TRN depletion affected stress-induced SG formation. Using anti-PABP, there was no significant difference between mock-treated and TRN-depleted cells in the number or the size of arsenite-induced SGs (Figure 4B). Therefore, TRN might play a role in the function or biogenesis of P-bodies but appeared not to be critical for arsenite stress-induced SG assembly.

TRN interacts and colocalizes with TTP

Dynamic localization of TRN in P-bodies and SGs suggested that TRN may participate in mRNA metabolic activities associated with these two types of granules. Because TTP localizes to both P-bodies and SGs (24), similar to that observed with TRN, we therefore examined whether it could be a cargo of TRN. Using anti-FLAG in HEK293 cells, we observed that transiently expressed FLAG-tagged TTP coprecipitated with endogenous TRN and that arsenite treatment enhanced this interaction somewhat (Figure 5A, lanes 5–8). Moreover, a pull-down experiment showed that recombinant GST-TRN indeed interacted with *in vitro* translated TTP even in the presence of RNase (Figure 5B, lanes 3 and 4). Therefore, TRN might directly interact with TTP. We also examined whether these two proteins indeed colocalize in cytoplasmic granules; for this purpose we transiently expressed FLAG-TTP because endogenous TTP is of low abundance in HeLa cells. FLAG-TTP formed cytoplasmic foci in ~50% of transfected HeLa cells and appeared to colocalize with endogenous TRN (Figure 5C). Therefore, we hypothesized that TTP may be an interacting partner of TRN in cytoplasmic granules.

The association of nuclear import receptors with their cargoes can be disrupted by GTP-bound Ran (2). Therefore, we examined whether RanQ69L, a mutant Ran defective in GTP-hydrolysis, could interfere with the interaction of TTP with TRN and their cellular localization. Figure 5D shows that recombinant RanQ69L effectively disrupted TRN binding to its previously known cargo HuR but had only modest effect

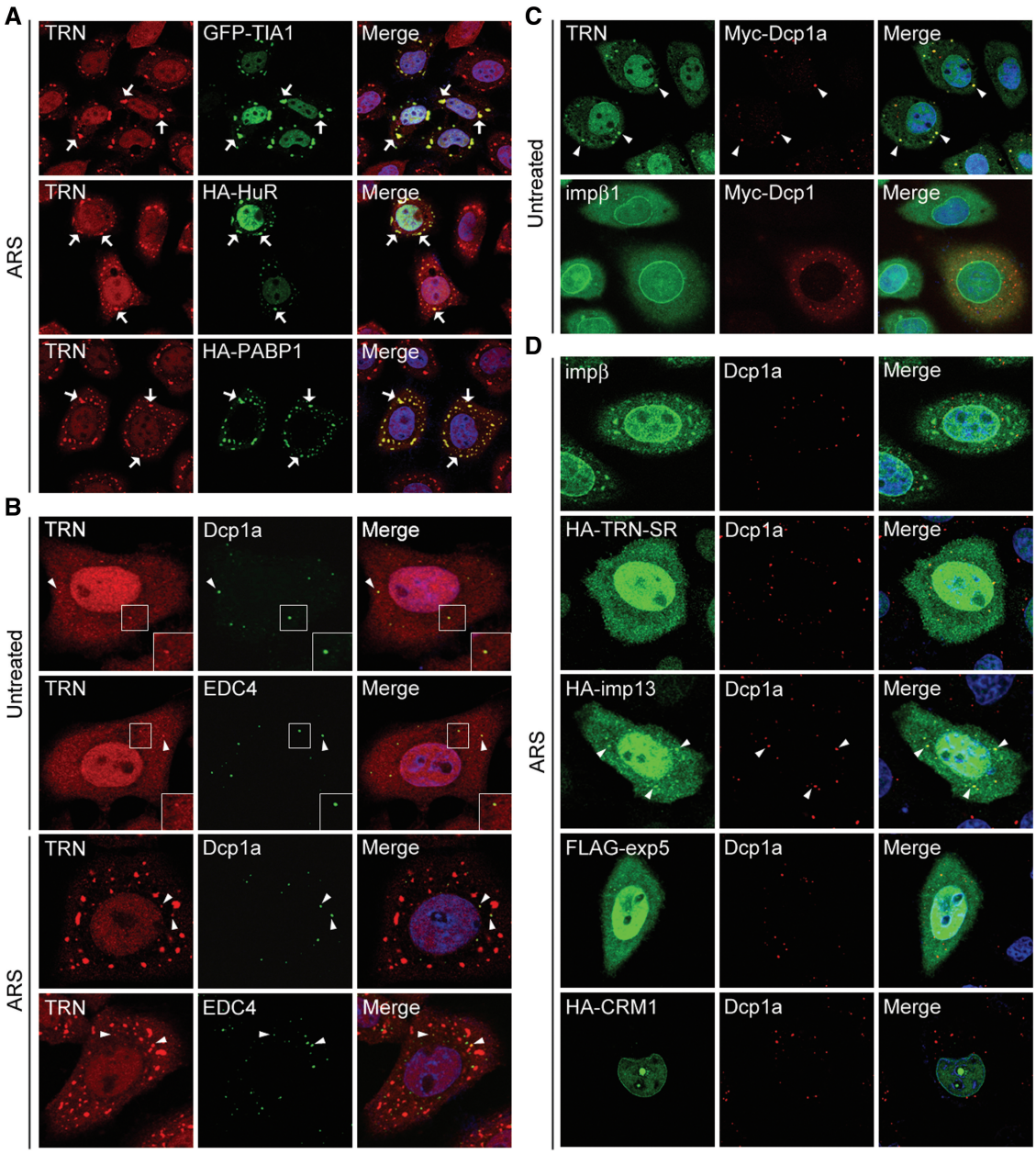

Figure 2. Subcellular colocalization of TRN with SG and P-body markers. (**A**) HeLa cells that transiently expressed GFP-TIA1, or HA-tagged HuR or PABP1 were treated with arsenite (ARS) and subjected to immunofluorescence using anti-TRN. GFP-TIA1 fluorescence was directly detected under a microscope whereas HA-tagged HuR and PABP1 were detected by using anti-HA. Merged signals indicated by arrows represent SGs. (**B**) Double immunofluorescence was performed to detect endogenous TRN and Dcp1a or EDC4 under normal and arsenite-stressed conditions using monoclonal anti-TRN and polyclonal anti-Dcp1a or anti-Edc4. Insets represent enlargements of indicated regions. (**C**) The myc-tagged Dcp1a was transiently overexpressed in HeLa cells. Double immunofluorescence was performed to detect endogenous TRN or importin-β1 and myc-Dcp1a. (**D**) Immunofluorescence was used to detect endogenous importin-β1 or the indicated transiently expressed nuclear transporter as well as endogenous Dcp1a in arsenite-stressed HeLa cells. Epitope-specific antibodies were used to detect tagged proteins in C and D. Merged signals indicated by arrowheads represent P-bodies in B–D.

on TTP (top panel). Moreover, overexpression of RanQ69L neither redistributed FLAG-TTP between the subcellular compartments nor had any significant effect on its localization in cytoplasmic granules (Figure 5D, immunofluorescence). A similar result was also observed with endogenous TRN (data not shown). Therefore, we apparently concluded that Ran perhaps has no critical role in TTP localization in cytoplasmic granules.

TRN interacts with TTP-associated mRNPs and participates in ARE-mediated mRNA decay

TTP binds AREs within certain labile mRNAs and induces their degradation, and TTP also is involved in nucleating P-body formation (25). Therefore, we examined whether TRN associates with ARE-containing mRNAs via its interaction with TTP. The 3′-UTR of the

reporter used was derived from the mouse TNFα gene that harbors AREs. The reporter mRNA was transiently expressed in HeLa cells. Immunoprecipitation of endogenous TRN followed by RT–PCR analysis using primers specific to the reporter showed that TRN indeed bound to the ARE-containing reporter RNA but not considerably to a control RNA lacking AREs (Figure 6A). Moreover, under a similar condition, co-expressed FLAG-TTP was coprecipitaed with TRN, suggesting that both TRN and TTP are in ARE-mRNPs (Supplementary Figure S4).

Association of TRN with ARE-mRNA-containing ribonucleoproteins raised the possibility that TRN participates in metabolic pathways of ARE-mRNAs. To test this, we transfected the ARE-mRNA reporter, that is controlled by a tetracycline-responsive promoter, as well as a control vector expressing GFP 2 days after introducing TRN siRNA into cells. We measured ARE-mRNA level after addition of tetracycline analog doxycycline, which turned off transcription of the reporter. The siRNA-mediated depletion of TRN

Table 1. Localization of importin-β family members in P-bodies and SGs under normal and arsenite-stressed conditions

Nuclear transport receptor	P-body localization		SG localization	
	Normal	Arsenite	Normal	Arsenite
TRN	+	+	–	+
Importin-β1	–	–	–	+
TRN-SR	–	–	–	–
Importin-13	–	+	–	–
Exportin-5	–	–	–	–
CRM1	–	–	–	–

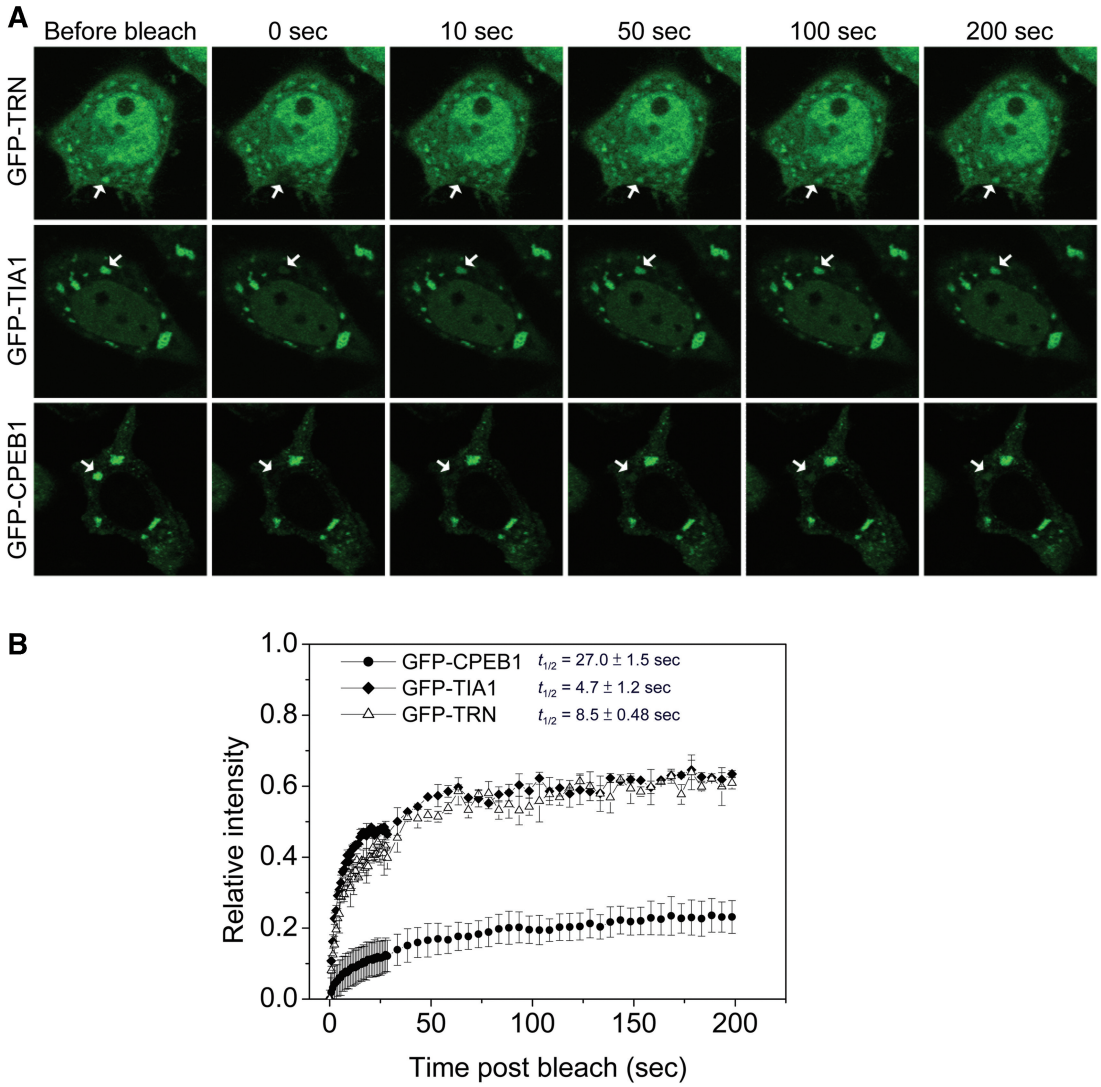

Figure 3. FRAP analysis of granule-localized proteins in arsenite-treated cells. FRAP was performed in HeLa cells that transiently expressed GFP-TRN, GFP-TIA1 or GFP-CPEB1. FRAP was performed following arsenite treatment. Fluorescence signal of selected granules was irradiated with the 488-nm laser, and its recovery was recorded for 200 s. (**A**) Selected images of GFP-fusion protein-expressing cells before bleaching and at the indicated intervals post-bleaching (from 0 to 200 s) are shown. Arrows indicate the targeted SGs. (**B**) The fluorescence intensity of GFP-fusion proteins after photobleaching was normalized to that before bleaching as described in 'Materials and Methods' section; relative intensity of each protein was averaged from at least three independent experiments ($n = 5$ for each experiment). The $t_{1/2}$ value is also indicated.

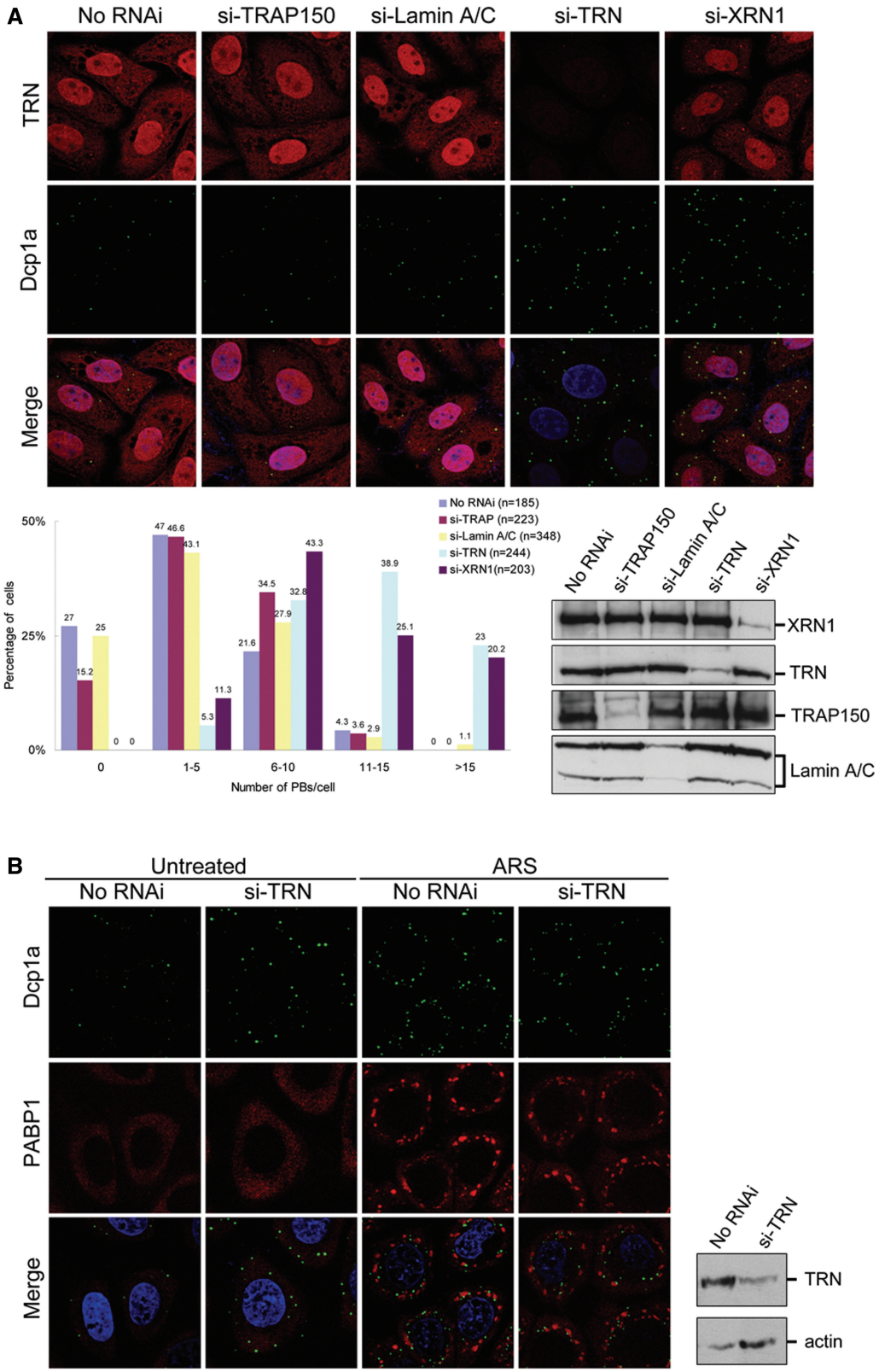

Figure 4. Effect of TRN knockdown on cytoplasmic granules. (**A**) HeLa cells were mock-transfected or transfected with siRNA against TRAP150, lamin A/C, TRN or Xrn1. Representative images of double immunostaining using anti-TRN and anti-Dcp1a. Bar graph: percentage of P-bodies in mock-transfected or siRNA-transfected cells (*n*, the number of cells used for quantification). Proteins in total cell lysates were subjected to immunoblotting using antibodies against the indicated proteins. (**B**) As in A, TRN was depleted by siRNA in HeLa cells. Cells were mock-treated or treated with arsenite followed by double immunostaining with anti-Dcp1a and anti-PABP. Merge indicates superimposed images. Immunoblotting of TRN and β-actin was performed.

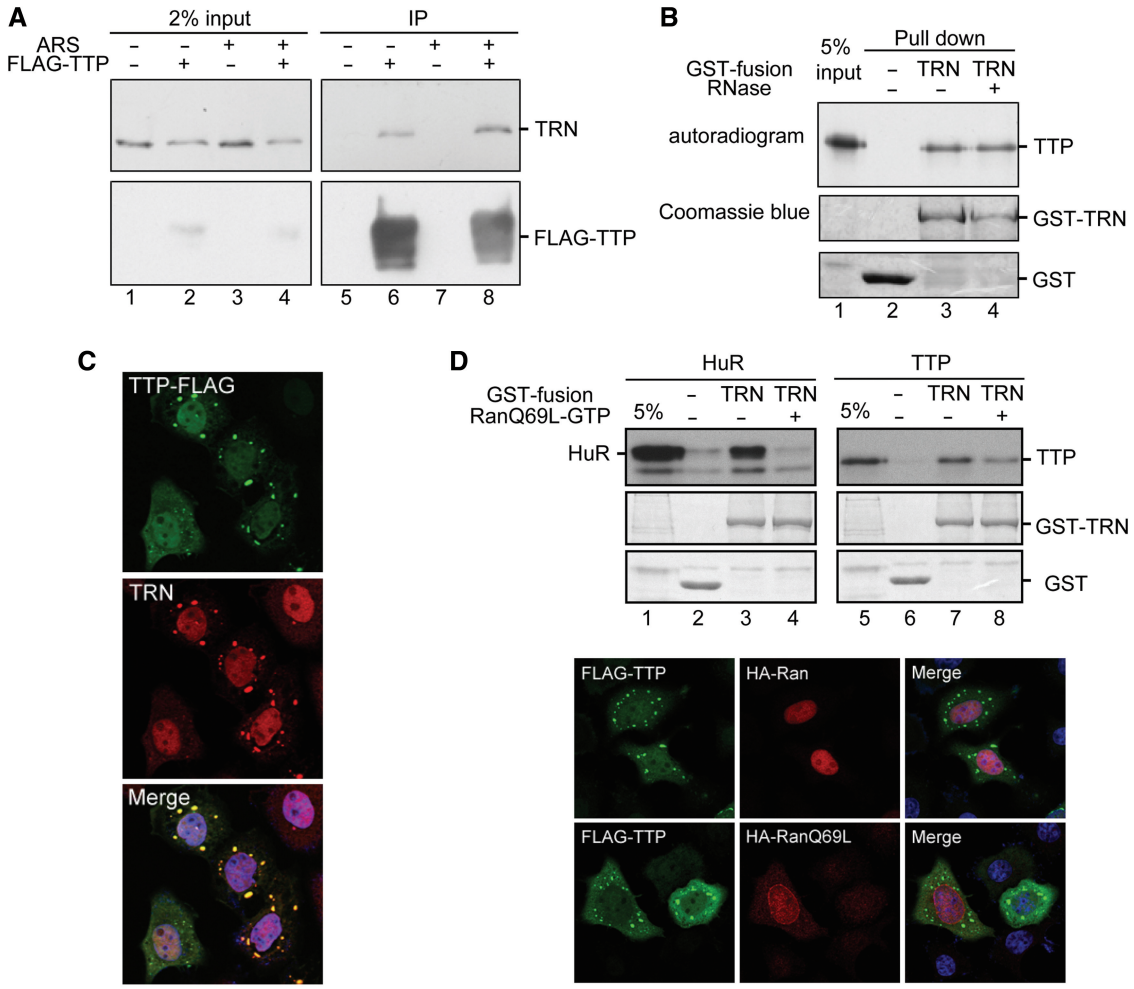

Figure 5. The interaction between TRN and TTP. HEK293 cells were transiently transfected with the FLAG-TTP expression vector or empty vector. (**A**) Cells were treated with arsenite or mock-treated for 1 h before harvest. The cell lysates were subjected to immunoprecipitation with anti-FLAG in the presence of RNase A. The precipitates were analyzed by immunoblotting with anti-TRN and anti-FLAG. (**B**) The *in vitro* translation reaction containing ^{35}S-labeled TTP was incubated with GST or GST-TRN followed by chromatography on glutathione-Sepharose. Bound proteins were fractionated on SDS–PAGE and detected by autoradiography. (**C**) HeLa cells that transiently expressed FLAG-TTP were double immunostained with anti-FLAG and anti-TRN. (**D**) Upper panel: ^{35}S-labeled HuR and TTP proteins were each subjected to the pull-down assay as in B. Recombinant RanQ69L was loaded with GTP and subsequently added into the pull-down reaction (lanes 4 and 8). Bottom panel: HeLa cells that transiently expressed FLAG-TTP and HA-Ran or HA-RanQ69L were double immunostained with anti-FLAG and anti-HA.

prolonged the half-life of the ARE-containing reporter mRNA (Figure 6B), as observed with Xrn1 knockdown (data not shown). In contrast, the stability of the ARE-lacking reporter RNA had no significant difference between the mock- and TRN-depleted cells (Figure 6B). Taken together, our data showed that TRN depletion not only increased P-body abundance (Figure 4) but also stabilized ARE-mRNAs, a scenario similar to that of Xrn1 knockdown (22,23). Therefore, TRN may play a role in ARE-mediated mRNA stability control in P-bodies, perhaps through its interaction with TTP.

Depletion of TRN modulates TTP localization in P-bodies and SGs

The above data suggested that TRN interacts and colocalizes with TTP and participates in TTP-mediated mRNA stability control. Next, we investigated whether TRN depletion would affect the subcellular localization of TTP, particularly in P-bodies and SGs. Because TRN depletion might impair nuclear translocation of its cargos, we measured the abundance of FLAG-TTP protein and two known TRN cargos, hnRNP A1 and DDX3, in subcellular fractions. Our data showed that an efficient depletion of TRN did not significantly increase the cytoplasmic level of the three examined proteins (Supplementary Figure S2). However, under this condition, we observed that colocalization of FLAG-TTP with endogenous Dcp1a in cytoplasmic granules was substantially enhanced (Figure 7A). This result coincided with our above observation that TRN depletion induced P-body assembly (Figure 4A). Moreover, we noted that transient expression of FLAG-TTP promoted endogenous PABP1 to form cytoplasmic foci even in the absence of cell stress (Figure 7A). FLAG-TTP indeed colocalized with PABP1 in these granules, consistent

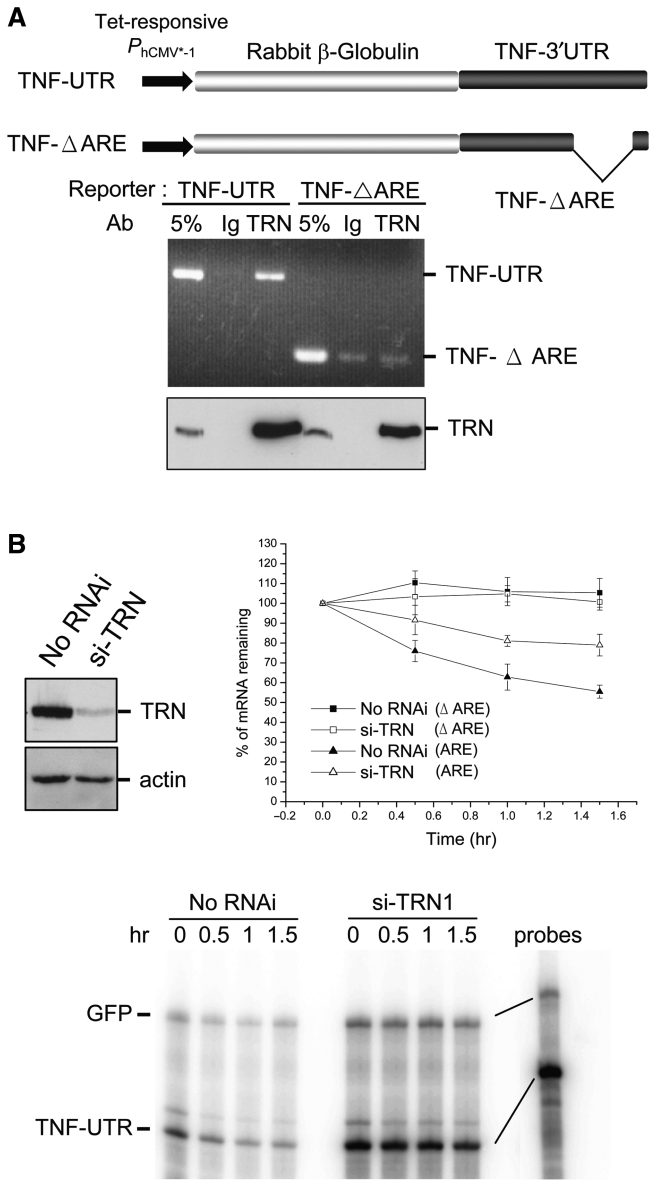

Figure 6. Association of TTP with ARE-containing mRNAs. (**A**) The TNFα ARE-containing β-globin reporter (TNF-UTR) or the control reporter (TNF-ΔARE), as shown in the top schematics, was transfected into HeLa cells. The cell lysates were subjected to immunoprecipitation using anti-TRN. Precipitated RNAs were analyzed by RT–PCR using primers specific to the reporters. (**B**) HeLa cells were mock-transfected or transiently transfected with siRNA against TRN. Depletion of TRN was examined by immunoblotting using anti-TRN. For mRNA stability analysis, the reporters used were the same as those in A, and meanwhile a GFP expression vector was cotransfected as a reference. Cells were harvested at the indicated time points after doxycycline addition. RNase protection was performed using ^{32}P-labeled probes specific to the reporter or the GFP mRNA. The graph shows the level of reporter mRNA remaining at each time point relative to that of the GFP reference. Arbitrary mRNA intensity was determined by phosphoimaging. Average and standard deviation were obtained from three independent experiments. The representative gel shows an experiment using the TNF-UTR reporter.

with the reported observation that TTP is a component of SGs (24,26). Although TRN knockdown did not significantly affect PABP1 or GFP-TIA1-containing SGs (Figure 4B and Supplementary Figure S5, respectively), localization of FLAG-TTP in SGs was largely compromised in TRN-depleted cells (Figure 7A). These results indicated that, in the absence of sufficient TRN, overexpressed TTP was likely retained in P-bodies and failed to localize to SGs. Therefore, TRN may modulate the dynamics of TTP exchange between the cytoplasmic pool and granules.

To confirm that TRN is involved in TTP shuttling between granules and the cytoplasm, we performed FRAP analysis using GFP-TTP. When viewed with direct fluorescence, native GFP-TTP-containing granules were marginally detected. Therefore, we treated cells with FCCP (26) to enhance GFP-TTP granule formation. Under this condition, GFP-TTP largely colocalized with PABP1 (data not shown), indicating that FCCP-induced TTP granules are primarily SGs. We then photobleached GFP-TTP granules and measured the recovery of the fluorescent signal in the absence or presence of TRN. GFP-TTP recovery in bleached granules had a halftime ($t_{1/2}$) of ~9 s, which was not significantly different from that measured in TRN knockdown cells (Figure 7B). However, the mobile fraction of GFP-TTP, as indicated by the normalized intensity of fluorescence recovery after photobleaching, was reduced from ~34% to ~22% (Figure 7B). This result indicated that TRN depletion may reduce the fraction of mobile TTP within each FCCP-induced SGs, and further suggested a role for TRN in trafficking of TTP from the cytoplasm to P-bodies or SGs.

DISCUSSION

Cytoplasmic RNA-containing granules play important roles in mRNA metabolism. P-bodies exist constitutively in the majority of cell types for mRNA degradation whereas SGs are induced in accordance with translation shutoff during cell stress (12,13). Our current results indicate that the importin-β protein TRN exists in P-bodies and that it localizes to SGs during cellular stress. We also found that depletion of TRN results in accumulation of P-bodies and affects the localization and activity of its interacting partner TTP in cytoplasmic granules.

Members of the importin-β family play a primary role in nucleocytoplasmic transport of macromolecules. We assessed whether the subcellular distribution of several importin-β members is altered in response to environmental signal-mediated changes. One study essentially revealed that a minimal fraction of TRN colocalized with Dcp1a and Edc4 in P-bodies, but upon exposure to cellular stress TRN accumulated in SGs; moreover, among other importin-β members, only importin-β1 and importin-13 could be minimally detected in cytoplasmic granules during stress (Figures 1 and 2; Table 1). Previous reports have revealed the possibility that nucleocytoplasmic transport factors may participate in intracellular compartment trafficking. In injured neuronal cells, the importin-α/β1 heterodimer moves along microtubules to mediate retrograde transport of injury signals from the axon periphery to the cell

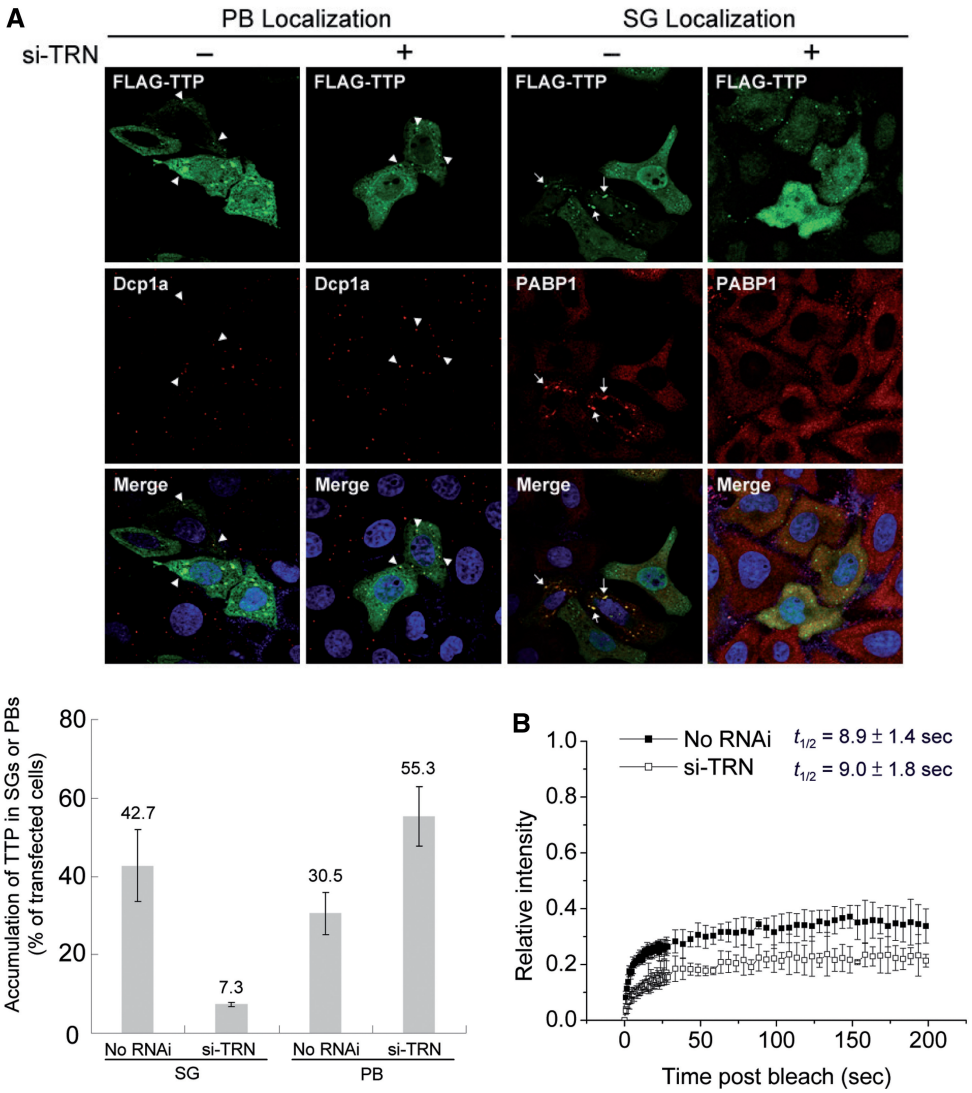

Figure 7. Localization of TTP in cytoplasmic granules in TRN-depleted cells. (**A**) HeLa cells were transiently transfected without or with TRN siRNA and the FLAG-TTP expression vector. Double immunostaining was performed using anti-FLAG and an antibody specific to Dcp1a or PABP1. Arrowheads and arrows indicate merged immunofluorescence of FLAG-TTP with that of Dcp1a and PABP1, respectively. Bar graph: percentage of transfected cells that contained FLAG-TTP-costained SGs or P-bodies. The average was obtained from three independent experiments (~100 transfected cells were counted in each experiment). (**B**) FRAP analysis of GFP-TTP in mock- and TRN-depleted cells. Following photobleaching, the fluorescence recovery of GFP-TTP in granules was recorded for ~200 s. Relative fluorescence intensity of GFP-TTP was measured and calculated as in Figure 3.

body (27). Importin-β1 itself can serve as a potential motor adaptor for movement along microtubules (6). Moreover, CRM1, in conjunction with PHAX, participates in transport of the U3 snoRNP precursor among nuclear bodies (28). Recent reports show that importin-8 and the nuclear RNA export factor NXF7 colocalize with their respective cargos, Argonaute (Ago) proteins and hnRNP A3, in P-bodies (29,30). Importantly, importin-8 recruits Ago2 to P-bodies, which allows efficient microRNA-mediated gene silencing (30). Perhaps, analogous to importin-8, TRN participates in TTP and its target mRNAs trafficking to cytoplasmic granules. Together, our observations suggest that TTP, in association with ARE-containing mRNPs, arrives in P-bodies in a TRN-independent manner, whereas its translocation from P-bodies to cytosol or to SGs requires TRN. In the absence of TRN, ARE-mRNPs fail to exit from P-bodies and are thus stabilized. Notably, cell stress can also induce both NXF7 and importin-8 to SGs, as we found for TRN. Because cytoplasmic granule movement depends on microtubules (31), perhaps TRN also acts as a cofactor or an adaptor of its cargoes to traffic along cytoplasmic microtubules between the granules. Taken together, TRN provides an additional example of how importin-β proteins may function in cytoplasmic trafficking and even granule targeting.

P-bodies primarily serve a site for degradation or storage of translationally stalled mRNAs that are newly released from polysomes (20,32). The integrity of P-bodies

can be profoundly influenced by their intrinsic components and modulated by the mRNA translation status of the cell. Experimental knockdown or overexpression of mRNA degradation factors or effectors shows different effects on P-body assembly. Degradation of mRNAs is primarily initiated by deadenylation, followed by association of the Lsm complex with deadenylated mRNAs (33,34). Assembly or accumulation of P-bodies is impeded when deadenylation factor Pan3 or Lsm1 is knocked down (35,36). Following deadenylation, mRNAs may be degraded from both ends. Downregulation of the decapping factor Dcp1a or the 5′-to-3′ exonuclease Xrn1 results in retention of mRNAs in P-bodies, thereby increasing the number of microscopically visible P-bodies (22,23). However, the 5′-to-3′ degradation factors are essentially absent from P-bodies. Moreover, P-body size can be increased upon depletion of Upf2/3 or by overexpression of SMG7, which are involved in nonsense-mediated mRNA decay (37,38). Knockdown of accessory factors that escort translationally repressed mRNPs to granules also affects P-body assembly. For example, downregulation of 4E-T, which interacts with the mRNA cap-binding protein eIF4E, decreases the number of P-bodies (39). As observed with knockdown of the 5′-to-3′ mRNA degradation factors, we found that depletion of TRN significantly increased the number of P-bodies, and also impaired ARE-containing mRNA decay (Figures 4 and 6). We identified TTP as an interacting partner of TRN in cytoplasmic granules; such an interaction likely allows TRN association with an ARE-containing reporter mRNA (Figure 6). In the absence of TRN, ARE-mRNAs may not be efficiently recruited to the mRNA degradation machinery. However, whether TRN-associated ARE-mRNAs are indeed degraded in P-bodies or merely stored transiently in P-bodies needs to be clarified by future experiments. As a conclusion, our results indicate that TRN, perhaps via its cargo(es), such as TTP, plays a role in an important activity of P-bodies, i.e. mRNA degradation.

Frequent contact between P-bodies and SGs suggests that their biogenesis or function may be coordinated. SG assembly is essentially dependent on P-body formation in both yeast and mammalian cells (40). However, a recent report indicated that the components of newly generated SGs may come from the cytosol rather than pre-existing granules (41). When cell stress is relived, translationally stalled mRNAs may transit from P-bodies to SGs and further return to polysomes for translation (40). Our results showed that TRN knockdown significantly increased the number of P-bodies but had no effect on SGs (Figures 4 and 7). Moreover, in TRN-depleted cells, TTP was retained in P-bodies and failed to localize to SGs (Figure 7). Therefore, TRN may escort TTP in redistribution from P-bodies to SGs under stress conditions. However, whether TTP transits from P-bodies to the cytoplasm or SGs together with its mRNA cargoes and whereby modulates further metabolic activity of these mRNAs remains to be investigated.

From our present study, we conclude that TRN may not only play a role in translocation of TTP between different cytoplasmic granules but also modulate ARE-containing mRNA decay during cell stress.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Gorlich,D. and Kutay,U. (1999) Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.*, **15**, 607–660.
2. Kuersten,S., Ohno,M. and Mattaj,I.W. (2001) Nucleocytoplasmic transport: ran, beta and beyond. *Trends Cell Biol.*, **11**, 497–503.
3. Pollard,V.W., Michael,W.M., Nakielny,S., Siomi,M.C., Wang,F. and Dreyfuss,G. (1996) A novel receptor-mediated nuclear protein import pathway. *Cell*, **86**, 985–994.
4. Kataoka,N., Bachorik,J.L. and Dreyfuss,G. (1999) Transportin-SR, a nuclear import receptor for SR proteins. *J. Cell Biol.*, **145**, 1145–1152.
5. Lai,M.C., Lin,R.I., Huang,S.Y., Tsai,C.W. and Tarn,W.Y. (2000) A human importin-beta family protein, transportin-SR2, interacts with the phosphorylated RS domain of SR proteins. *J. Biol. Chem.*, **275**, 7950–7957.
6. Harel,A. and Forbes,D.J. (2004) Importin beta: conducting a much larger cellular symphony. *Mol. Cell*, **16**, 319–330.
7. Arnaoutov,A., Azuma,Y., Ribbeck,K., Joseph,J., Boyarchuk,Y., Karpova,T., McNally,J. and Dasso,M. (2005) Crm1 is a mitotic effector of Ran-GTP in somatic cells. *Nat. Cell Biol.*, **7**, 626–632.
8. Scott,R.J., Cairo,L.V., Van de Vosse,D.W. and Wozniak,R.W. (2009) The nuclear export factor Xpo1p targets Mad1p to kinetochores in yeast. *J. Cell Biol.*, **184**, 21–29.
9. Jans,D.A., Xiao,C.Y. and Lam,M.H. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays*, **22**, 532–544.
10. Furuta,M., Kose,S., Koike,M., Shimi,T., Hiraoka,Y., Yoneda,Y., Haraguchi,T. and Imamoto,N. (2004) Heat-shock induced nuclear retention and recycling inhibition of importin alpha. *Genes Cells*, **9**, 429–441.
11. Yasuhara,N., Takeda,E., Inoue,H., Kotera,I. and Yoneda,Y. (2004) Importin alpha/beta-mediated nuclear protein import is regulated in a cell cycle-dependent manner. *Exp. Cell Res.*, **297**, 285–293.
12. Anderson,P. and Kedersha,N. (2006) RNA granules. *J. Cell Biol.*, **172**, 803–808.
13. Anderson,P. and Kedersha,N. (2008) Stress granules: the Tao of RNA triage. *Trends Biochem. Sci.*, **33**, 141–150.
14. Fillman,C. and Lykke-Andersen,J. (2005) RNA decapping inside and outside of processing bodies. *Curr. Opin. Cell Biol.*, **17**, 326–331.

15. Chen,C.Y. and Shyu,A.B. (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.*, **20**, 465–470.
16. Barreau,C., Paillard,L. and Osborne,H.B. (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res.*, **33**, 7138–7150.
17. Lai,M.C., Lee,Y.H. and Tarn,W.Y. (2008) The DEAD-box RNA helicase DDX3 associates with export messenger ribonucleoproteins as well as tip-associated protein and participates in translational control. *Mol. Biol. Cell*, **19**, 3847–3858.
18. Lai,M.C., Teh,B.H. and Tarn,W.Y. (1999) A human papillomavirus E2 transcriptional activator. The interactions with cellular splicing factors and potential function in pre-mRNA processing. *J. Biol. Chem.*, **274**, 11832–11841.
19. Mazroui,R., Sukarieh,R., Bordeleau,M.E., Kaufman,R.J., Northcote,P., Tanaka,J., Gallouzi,I. and Pelletier,J. (2006) Inhibition of ribosome recruitment induces stress granule formation independently of eukaryotic initiation factor 2alpha phosphorylation. *Mol. Biol. Cell*, **17**, 4212–4219.
20. Kedersha,N. and Anderson,P. (2007) Mammalian stress granules and processing bodies. *Methods Enzymol.*, **431**, 61–81.
21. Fenger-Gron,M., Fillman,C., Norrild,B. and Lykke-Andersen,J. (2005) Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. *Mol. Cell*, **20**, 905–915.
22. Cougot,N., Babajko,S. and Seraphin,B. (2004) Cytoplasmic foci are sites of mRNA decay in human cells. *J. Cell Biol.*, **165**, 31–40.
23. Sheth,U. and Parker,R. (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science*, **300**, 805–808.
24. Kedersha,N., Stoecklin,G., Ayodele,M., Yacono,P., Lykke-Andersen,J., Fritzler,M.J., Scheuner,D., Kaufman,R.J., Golan,D.E. and Anderson,P. (2005) Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell Biol.*, **169**, 871–884.
25. Franks,T.M. and Lykke-Andersen,J. (2007) TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. *Genes Dev.*, **21**, 719–735.
26. Stoecklin,G., Stubbs,T., Kedersha,N., Wax,S., Rigby,W.F., Blackwell,T.K. and Anderson,P. (2004) MK2-induced tristetraprolin:14–3–3 complexes prevent stress granule association and ARE-mRNA decay. *EMBO J.*, **23**, 1313–1324.
27. Hanz,S., Perlson,E., Willis,D., Zheng,J.Q., Massarwa,R., Huerta,J.J., Koltzenburg,M., Kohler,M., van-Minnen,J., Twiss,J.L. *et al.* (2003) Axoplasmic importins enable retrograde injury signaling in lesioned nerve. *Neuron*, **40**, 1095–1104.
28. Boulon,S., Verheggen,C., Jady,B.E., Girard,C., Pescia,C., Paul,C., Ospina,J.K., Kiss,T., Matera,A.G., Bordonne,R. *et al.* (2004) PHAX and CRM1 are required sequentially to transport U3 snoRNA to nucleoli. *Mol. Cell*, **16**, 777–787.
29. Katahira,J., Miki,T., Takano,K., Maruhashi,M., Uchikawa,M., Tachibana,T. and Yoneda,Y. (2008) Nuclear RNA export factor 7 is localized in processing bodies and neuronal RNA granules through interactions with shuttling hnRNPs. *Nucleic Acids Res.*, **36**, 616–628.
30. Weinmann,L., Hock,J., Ivacevic,T., Ohrt,T., Mutze,J., Schwille,P., Kremmer,E., Benes,V., Urlaub,H. and Meister,G. (2009) Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. *Cell*, **136**, 496–507.
31. Aizer,A., Brody,Y., Ler,L.W., Sonenberg,N., Singer,R.H. and Shav-Tal,Y. (2008) The dynamics of mammalian P body transport, assembly, and disassembly in vivo. *Mol. Biol. Cell*, **19**, 4154–4166.
32. Parker,R. and Sheth,U. (2007) P bodies and the control of mRNA translation and degradation. *Mol. Cell*, **25**, 635–646.
33. Meyer,S., Temme,C. and Wahle,E. (2004) Messenger RNA turnover in eukaryotes: pathways and enzymes. *Crit. Rev. Biochem. Mol. Biol.*, **39**, 197–216.
34. Tharun,S., Muhlrad,D., Chowdhury,A. and Parker,R. (2005) Mutations in the Saccharomyces cerevisiae LSM1 gene that affect mRNA decapping and 3′ end protection. *Genetics*, **170**, 33–46.
35. Stoecklin,G., Mayo,T. and Anderson,P. (2006) ARE-mRNA degradation requires the 5′–3′ decay pathway. *EMBO Rep.*, **7**, 72–77.
36. Zheng,D., Ezzeddine,N., Chen,C.Y., Zhu,W., He,X. and Shyu,A.B. (2008) Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells. *J. Cell Biol.*, **182**, 89–101.
37. Sheth,U. and Parker,R. (2006) Targeting of aberrant mRNAs to cytoplasmic processing bodies. *Cell*, **125**, 1095–1109.
38. Unterholzner,L. and Izaurralde,E. (2004) SMG7 acts as a molecular link between mRNA surveillance and mRNA decay. *Mol. Cell*, **16**, 587–596.
39. Ferraiuolo,M.A., Basak,S., Dostie,J., Murray,E.L., Schoenberg,D.R. and Sonenberg,N. (2005) A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. *J. Cell Biol.*, **170**, 913–924.
40. Buchan,J.R., Muhlrad,D. and Parker,R. (2008) P bodies promote stress granule assembly in Saccharomyces cerevisiae. *J. Cell Biol.*, **183**, 441–455.
41. Mollet,S., Cougot,N., Wilczynska,A., Dautry,F., Kress,M., Bertrand,E. and Weil,D. (2008) Translationally repressed mRNA transiently cycles through stress granules during stress. *Mol. Biol. Cell*, **19**, 4469–4479.