Identification of Novel Stress Granule Components That Are Involved in Nuclear Transport

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Abstract

Background: Importin-α1 belongs to a subfamily of nuclear transport adaptors and participates in diverse cellular functions. Best understood for its role in protein transport, importin-α1 also contributes to other biological processes. For instance, arsenite treatment causes importin-α1 to associate with cytoplasmic stress granules (SGs) in mammalian cells. These stress-induced compartments contain translationally arrested mRNAs, small ribosomal subunits and numerous proteins involved in mRNA transport and metabolism. At present, it is not known whether members of all three importin-α subfamilies locate to SGs in response to stress.

Results: Here, we demonstrate that the oxidant diethyl maleate (DEM), arsenite and heat shock, promote the formation of cytoplasmic SGs that contain nuclear transport factors. Specifically, importin-α1, α4 and α5, which belong to distinct subfamilies, and importin-β1 were targeted by all of these stressors to cytoplasmic SGs, but not to P-bodies. Importin-α family members have been implicated in transcriptional regulation, which prompted us to analyze their ability to interact with poly(A)-RNA in growing cells. Our studies show that importin-α1, but not α4, α5, importin-β1 or CAS, associated with poly(A)-RNA under nonstress conditions. Notably, this interaction was significantly reduced when cells were treated with DEM. Additional studies suggest that importin-α1 is likely connected to poly(A)-RNA through an indirect interaction, as the adaptor did not bind homopolymer RNA specifically in vitro.

Significance: Our studies establish that members of three importin-α subfamilies are bona fide SG components under different stress conditions. Furthermore, importin-α1 is unique in its ability to interact with poly(A)-RNA in a stress-dependent fashion, and in vitro experiments indicate that this association is indirect. Collectively, our data emphasize that nuclear transport factors participate in a growing number of cellular activities that are modulated by stress.
members, the importin-α family is importin-α1 (karyopherin-α2, RPN2); this adaptor belongs to the α2 subfamily and shuttles between the nucleus and cytoplasm under normal growth conditions. At steady-state, the protein localizes predominantly to the cytoplasm and nuclear envelope [33], but stress alters this distribution. For instance, heat shock accumulates importin-α1 in nuclei [34,35] through the increase in nucleoplasmic retention and the reduction of nuclear exit [34]. Additionally, and possibly unique, biological functions for importin-α1 have emerged recently. For example, importin-α1 is implicated in the transcriptional regulation of hydrogen peroxide treated cells [36] and the proliferation of human breast cancer cells [37]; importin-α1 has also been detected in SGs [38]. Unlike importin-α1, the subcellular distribution and biological functions of other importin-α proteins are much less characterized. Among the poorly understood family members is importin-α4 (hSRP1γ), which is part of the α3 subfamily. The importin-α4 coding region was initially isolated from a HeLa cDNA library, and the protein is highly abundant in brain and skeletal muscle cells [39]. On the other hand, importin-α5 (α1 subfamily) regulates proliferation in HeLa cells [40], binds to the transcription regulator STAT1 and interacts with a variety of virus proteins [41,42].

While the importin-α family encompasses seven highly related members, the importin-β family is far more complex. Among the more than 20 importin-β proteins in mammals, some are specialized for nuclear export or import, whereas others promote transport in both directions [43,44,45]. Many importin-β carriers bind the NLS directly (reviewed in [46]); however, importin-β1 also associates with cargo through the adaptor importin-α. This adaptor function and their nuclear export by CAS are features shared by all three importin-α subfamilies. Like importin-α1, CAS plays a role as transcriptional regulator and is thus linked to RNA metabolism [36,47]. Interestingly, several members of the importin-β family have been detected in SGs or PBs. Whereas transportin-1 located to both SGs and PBs upon arsenite treatment, importin-β1 associated with SGs, and importin-13 with PBs [11]. On the basis of their presence in SGs, it was speculated that nuclear transport factors participate in SG assembly [48].

Although there is a growing body of information on importin-α and β isoforms, the full spectrum of their biological function is far from being understood. This includes the response to environmental changes and prompted us to examine the effect of different stressors on importin-α1, α4, α5 as well as importin-β1 and CAS. With our current contribution, we provide several lines of evidence that connect nuclear transport factors to cytoplasmic SGs and RNA metabolism. In particular, we defined the oxidant-induced changes as they relate to transport factor localization and RNA-association. Our work demonstrates that not only importin-α1, but also importin-α4, α5 and importin-β1, but not CAS, locate to SGs under different stress conditions. By contrast, none of the transport factors was detected in PBs under normal or stress conditions. Moreover, we identified importin-α1 as a novel protein that associates with poly(A)-RNA in vivo in a stress-controlled fashion. This distinguishes importin-α1 from other transport adaptors, importin-β1 and CAS. Further characterization of the importin-α1/poly(A)-RNA interaction with in vitro homopolymer binding assays indicate that the protein does not bind RNA directly. Taken together, our results provide new links between the nucleocytoplasmic transport machinery, RNA metabolism and the stress response.

**Materials and Methods**

**Cell Culture and Stress Exposure**

HeLa S3 cells were grown in Dulbecco’s modified eagle medium (DMEM) containing antibiotics and 8% fetal bovine serum. Cultures were maintained in a 37°C incubator with 5% CO₂. To induce the formation of SGs, established conditions were used; oxidative stress was generated with 0.5 mM sodium arsenite for 30 min [49] and controls were incubated with water. Alternatively, HeLa cells were treated with 2 mM diethyl maleate (DEM, [33]) or ethanol (control) for 4 hours. Heat shock was performed for 1.5 hours at 45.5°C; a 1.5 hour heat exposure was selected because it was a reliable condition for SG induction.

**Immunofluorescent Staining**

Cells were grown to 70% confluency on poly-lysine coated cover slips. After treatment, immunofluorescent staining was performed essentially as published [50]. The following antibodies and dilutions were used: importin-α1 (1:400; Santa Cruz, sc-6917), importin-α4 (1:2,000; gift from Dr. K. Weis), importin-α5 (1:500; Zymed), importin-β1 (1:1,000; sc-11376); CAS (1:1,000; sc-1708), HuR (1:2,000 sc-5261; 1:1,000, Millipore 07-1735), G3BP1 (1:1,000, BD Biosciences; 1:2,000; kindly provided by Dr. I. Gallouzi), Dcp1 (1:200, sc-100706; 1:800, gift from Dr. I. Gallouzi). In brief, cells were fixed with 3.7% formaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS and blocked in PBS/2 mg/ml bovine serum albumin/0.05% Tween-20 (PBS/BSA/Tween). Alternatively, blocking and antibody incubations were carried out with PBS/0.05% Tween/5% fetal bovine serum. Samples were incubated overnight with primary antibodies diluted in blocking solution and washed several times. FITC- and Cy3-labeled secondary antibodies (diluted 1:500; Jackson ImmunoResearch) were added for 2 hours. Samples were washed, nuclei stained with 1 μg/ml 4’, 6-diamidino-2-phenylindole (DAPI) and cover slips mounted. Images were acquired with a Zeiss LSM510 confocal microscope in the multi-track mode, using appropriate filter settings to minimize cross-talk between the channels. Image processing was performed in Adobe Photoshop CS4. To monitor non-specific staining, pre-immune serum and isotype controls were tested under identical conditions (Fig. S1). In these experiments, little or no background staining was observed.

**Quantification of SG Localization**

The association of importin-α family members or importin-β1 with SGs was evaluated using the SG marker HuR as a reference. For each transporter three independent experiments were performed for every stressor. SGs were identified based on HuR and the presence of individual nuclear transporter was determined. To this end, 30 SG-positive cells were randomly selected, and all of their SGs were scored. Data for importin-α1, α4, α5 or importin-β1 are depicted in the results section.

**Oligo-(dT) Binding Assay**

HeLa cells were grown to 70% confluency and treated with ethanol (control) or DEM. Cell extracts were prepared in binding
Figure 1. Different forms of oxidative stress target importin-α family members to SGs. HeLa cells were exposed to DEM or arsenite. Importin-α1, α4 and α5 as well as the SG marker HuR were located by indirect immunofluorescence. Nuclei were stained with DAPI; size bar is 20 μm. Co-localization of HuR and importin-α family members is shown for the selected regions (white square in overlay panel) at a magnification of 500 x. doi:10.1371/journal.pone.0068356.g001
Table 1. Oxidants DEM and arsenite promote the association of importin-α family members with SGs. Experiments shown in Fig. 1 were performed with the SG marker G3BP1. Nuclei were detected with DAPI; size bar is 20 µm. Co-localization of G3BP1 and members of the importin-α family can be seen in the selected regions (white square in overlay panel) at a magnification of 500 ×.

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Figure 2. Oxidants DEM and arsenite promote the association of importin-α family members with SGs. Experiments shown in Fig. 1 were performed with the SG marker G3BP1. Nuclei were detected with DAPI; size bar is 20 µm. Co-localization of G3BP1 and members of the importin-α family can be seen in the selected regions (white square in overlay panel) at a magnification of 500 ×.

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### A

| Sample      | DAPI | importin-α | HuR | overlay |
|-------------|------|-------------|-----|---------|
| Imp-α1, control | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α1, heat shock | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α4, control | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α4, heat shock | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α5, control | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α5, heat shock | ![Image] | ![Image] | ![Image] | ![Image] |

### B

| Sample      | DAPI | importin-α | G3BP1 | overlay |
|-------------|------|-------------|-------|---------|
| Imp-α1, control | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α1, heat shock | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α4, control | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α4, heat shock | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α5, control | ![Image] | ![Image] | ![Image] | ![Image] |
| Imp-α5, heat shock | ![Image] | ![Image] | ![Image] | ![Image] |
**Figure 3. Heat shock induces the association of importin-α1, α4 and α5 with SGs.** Following a 1.5-hour heat shock, the distribution of importin-α proteins and SG marker HuR (A) or G3BP1 (B) was determined. DAPI was used to stain DNA; size bar is 20 μm. The area demarcated by the white square shows a 500× magnified view of SGs.

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| DAPI | transporter | HuR | overlay |
|------|-------------|-----|---------|
| Imp-β1, EtOH | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| Imp-β1, DEM | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| CAS, EtOH | ![Image](image7) | ![Image](image8) | ![Image](image9) |
| CAS, DEM | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| Imp-β1, water | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| Imp-β1, arsenite | ![Image](image16) | ![Image](image17) | ![Image](image18) |
| CAS, water | ![Image](image19) | ![Image](image20) | ![Image](image21) |
| CAS, arsenite | ![Image](image22) | ![Image](image23) | ![Image](image24) |

**Figure 4. Importin-β1, but not CAS, binds to SGs in response to oxidative stress.** HeLa cells treated with DEM or arsenite were stained with antibodies against the carrier importin-β1 or CAS. Co-staining was performed with the SG marker HuR; nuclei were detected with DAPI. Size bar is 20 μm; SG-containing areas (white squares) are magnified 500×.

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buffer (100 mM KCl, 25 mM Tris-HCl, pH 7.4, 0.1% Triton-X, 10 mM EDTA, 10 mM vanadyl adenosine complex), containing a protease inhibitor cocktail [7,51]. Samples were passed through a 26.5 gauge needle 4 times and incubated with DNase at 37°C for 15 min. After addition of DTT (5 mM final concentration) samples were centrifuged 1 min at 12,000 rpm (microfuge). Supernatants (starting material) were incubated with 300 µl Oligo-(dT)-cellulose (BioShop, equilibrated in binding buffer) for 30 min at room temperature. The resin was collected by centrifugation and washed twice in binding buffer. Bound proteins were eluted in gel sample buffer and analyzed by Western blotting.

**Figure 5. Oxidant-induced SGs contain importin-β1, but not CAS.** The selective association of importin-β1 with SGs under oxidative stress conditions was examined with the SG marker G3BP1. DAPI was used to identify nuclei; size bar is 20 µm; SG-containing regions (white squares) are shown at 500× magnification. doi:10.1371/journal.pone.0068356.g005

**Generation of Homopolymers for in vitro RNA Binding**

Poly(A), poly(U), poly(C) or poly(G) homopolymers were coupled to BrCN-activated sepharose (Santa Cruz Biotechnology) following standard procedures. To monitor unspecific binding to
the resin, the same procedure was applied to BrCN-activated sepharose, but without addition of a homopolymer.

**In vitro RNA Homopolymer Binding Assay**

His6-tagged importin-β1 or poly(A)-binding protein (plasmid encoding poly(A)-binding protein was kindly provided by Dr. N. Sonenberg) were synthesized in *E. coli* and purified with Ni-NTA agarose (Qiagen). Purified proteins were dialyzed against 20 mM Tris HCl, pH 7.4, 150 mM NaCl and 2.5 mM MgCl₂ and employed for *in vitro* RNA homopolymer binding, essentially as described [52,53]. To reduce nonspecific binding, homopolymers were pre-incubated overnight with 500 µl binding buffer (BB; 20 mM Tris HCl, pH 7.4, 150 mM NaCl, 2.5 mM MgCl₂, 0.5% NP-40, 1 mM vanadyl adenosine complex, protease inhibitors) containing 200 µg BSA/ml. In control experiments, homopolymers were digested with micrococcal nuclease (1,200 Kunitz units per digest) for 1 h at 37°C and then blocked overnight with BSA as described above. Blocked resin (poly(A), poly(U), poly(C), poly(G)-

![Figure 6. Heat shock targets importin-β1, but not CAS, to SGs.](image-url)

In heat-shocked HeLa cells, importin-β1, CAS and HuR (A) or G3BP1 (B) were detected by indirect immunofluorescence. DNA was stained with DAPI; size bar is 20 µm. Magnified views (500 X) of SG-containing regions are depicted for stressed cells.

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sepharose or non-conjugated sepharose) was incubated for 10 min at room temperature with 5 mg purified protein and 100 mg BSA in 500 l BB. Unbound material was removed by centrifugation (microfuge, 1 min, 13,000 rpm), and resins were washed once with BB containing 2 mg/ml heparin (10 min, room temperature) and once with BB/heparin/1 M NaCl (10 min, room temperature). Bound protein was eluted with twofold concentrated gel sample buffer by incubation at 95°C for 15 min and the resin was removed by centrifugation (5 min, 13,000 rpm, microfuge). Eluted and unbound material was analyzed side-by-side by Western blotting with antibodies against importin-a1 or the His6-tag.

Western Blotting

Western blotting and ECL with HRP-conjugated secondary antibodies followed standard procedures [33]. Primary antibodies were used at the dilutions described for immunostaining. None of the proteins were detected by pre-immune or isotype control antibodies (Fig. S2). Poly(A)-binding protein was visualized with antibodies against the His-tag (Affinity Bioreagents, PA1-983A, diluted 1:500).

Statistics

For Western blot analyses results were normalized to controls; three independent experiments were carried out for each treatment. Significant differences were identified by Student’s t-test, all data are shown as averages±SEM.

Results

DEM-induced Oxidative Stress Targets Importin-a1 to Stress Granules

We and others have shown previously that stress interferes with nuclear import, in part by retaining importin-a1 in the nucleus [33,34,35,54]. More recently, Fujimura et al. [38] demonstrated that sodium arsenite, an inducer of oxidative stress, triggers the association of importin-a1 with cytoplasmic SGs. Since the composition of SGs depends on the stressor, it was not known whether other oxidants also cause the translocation of importin-a1 to SGs. We therefore determined how DEM, a compound that generates oxidative stress by depleting intracellular glutathione [55], affects the distribution of importin-a1 in the cytoplasm. Fig. 1

Figure 7. Quantification of the SG association for importin-a1, a4, a5 and importin-b1. HeLa cells were incubated with DEM, arsenite or heat shock and stained with antibodies against importin-a1, a4, a5, or importin-b1. SGs were identified with the marker protein HuR, and 30 SG-containing cells were evaluated for every treatment. Each individual SG was scored for the presence of importin-a1, a4, a5 or importin-b1. A single data point represents the average of three independent experiments±SEM. The tables depict the number of SGs that were positive for the importin analyzed/number of SGs identified with HuR; results are shown for each individual experiment. Note that more than 90% of SGs were positive for the examined members of the importin-a family or importin-b1.

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Transport factors importin-α1, a4, a5, importin-β1 and CAS do not associate with PBs under normal or stress conditions. Transport factors and the PB marker Dcp1 were detected in HeLa cells treated with DEM as described for Fig. 1. Nuclei were stained with DAPI; size bar is 20 μm. Selected PB-containing regions are shown at a magnification of 500×. doi:10.1371/journal.pone.0068356.g008
demonstrates that in response to DEM treatment importin-\(\alpha\)1 not only accumulates in the nucleus, but also associates with SGs, as it co-localizes with the SG marker protein HuR (Fig. 1). In independent experiments, the same results were also observed when the SG nucleating protein G3BP1 was used as a reference (Fig. 2).

**Importin-\(\alpha\)4 and \(\alpha\)5 are Novel Constituents of SGs**

Importin-\(\alpha\)1 belongs to the \(\alpha\)2 subfamily, and little is known about the stress-dependent changes of other subfamilies. We therefore extended our studies to importin-\(\alpha\)4 (\(\text{KPNA3}\)) and \(\alpha\)5 (\(\text{KPNA1}\)), which are representatives of subfamilies \(\alpha\)3 and \(\alpha\)1, respectively. To test whether importin-\(\alpha\)4 or \(\alpha\)5 is present in DEM-induced SGs, we located these proteins by immunocytochemistry, with two different SG markers, HuR (Fig. 1) and G3BP1 (Fig. 2). A comparison of the steady-state distribution of the different family members revealed that in unstressed cells importin-\(\alpha\)1, \(\alpha\)4 and \(\alpha\)5 were predominantly cytoplasmic and concentrated at the nuclear envelope (Fig. 1, 2, 3). However, upon incubation with DEM, importin-\(\alpha\)4 and \(\alpha\)5 accumulated in nuclei and SGs; these results were consistently obtained, with either HuR or G3BP1 as SG marker (Fig. 1, 2). Taken together, DEM treatment is a reliable method to promote the formation of SGs; such DEM-induced SGs contain members of the three importin-\(\alpha\) subfamilies.

**Different Stressors Target Importin-\(\alpha\) Family Members to SGs**

Although SGs assemble when translation is arrested, their composition may vary, as it is dictated by the type of stress. To address this point for importin-\(\alpha\) isoforms, we monitored the impact of arsenite (Fig. 1, 2) and heat shock (Fig. 3A, B), following protocols that are routinely used to induce SGs [38,56]. Like DEM, these treatments led to the formation of SGs, although the granules were smaller in size for arsenite and present in fewer cells.
for heat shock. Despite these differences, importin-α1, α4 and α5 relocated to SGs, which we observed when HuR or G3BP1 was used as a SG marker. Thus, we established that diverse forms of stress lead to the recruitment of different importin-α family members to SGs.

**Importin-β1, but not CAS, Associates with SGs under Various Stress Conditions**

Nucleocytoplasmic transport mediated by importin-β requires that the adapter interacts with importin-β1 during import to move cargo into the nucleus. On the other hand, CAS serves as the nuclear exporter for importin-α. Thus, importin-β1 and CAS, both members of the importin-β family, interact directly with different importin-α isoforms. Importin-β1 had been detected in arsenite-induced SGs [11]; however, to the best of our knowledge the impact of other stressors has not been determined. Moreover, it is not known whether CAS associates with SGs under stress conditions. Data depicted in Fig. 4, 5, and 6 reveal that importin-β1 is present in SGs following DEM, arsenite or heat treatment. As described for importin-α above, DEM produced a marked relocation of importin-β1 to SGs (Fig. 4), and the carrier was also detected in heat-induced SGs (Fig. 6). By contrast, none of the stressors caused CAS to concentrate in SGs; these results were independent of the SG marker, as they were obtained with HuR or G3BP1 as a reference. Collectively, our experiments demonstrate that the stressors which recruit importin-α proteins to SGs also promote the association of importin-β1, but not CAS, with this compartment.

**Importin-α1, α4, α5 and Importin-β1 are bona fide SG Components**

To better define the association of nuclear transport factors with SGs, cells were stressed with DEM, arsenite or heat and SGs were demarcated with the marker protein HuR. SGs were then assessed for the presence of nuclear transport factors in three independent experiments for each stressor. As shown in Fig. 7, importin-α1, α4, α5 and importin-β1 were present on average in more than 90% of SGs, while importin-α3 did not significantly accumulate in SGs that were induced by arsenite or heat shock. In control experiments, HuR, a protein known to bind poly(A)-RNA [7], was efficiently pulled down with the resin (Fig. 9). Interestingly, the interaction between importin-α1 and poly(A)-RNA was strongly affected by oxidative stress. Although this adaptor bound poly(A)-RNA in control cells, the association was reduced significantly in DEM-treated cells. On the other hand, the binding of HuR was only slightly diminished by DEM. Taken together, our studies show, for the first time, that the nuclear transport factor importin-α1, but not other transport components analyzed here, associates with poly(A)-RNA. Notably, this interaction is sensitive to stress.

**In vitro Binding of Importin-α1 to RNA Homopolymers**

Data described in Fig. 9 show that importin-α1 co-purified with poly(A)-RNA isolated from growing cells. One possible mechanism underlying this association is the direct binding of importin-α1 to RNA. A commonly used assay to detect such direct interactions relies on immobilized RNA homopolymers and proteins purified from *E. coli*. For example, this method was used to pull down purified poly(A)-binding protein in *vivo* [37]. Therefore, we included the interaction between poly(A)-binding protein and poly(A)-sepharose as a positive control that validated the assay (Fig. 10). Several negative controls monitored the non-specific interactions of importin-α1. First, each homopolymer-sepharose was pre-treated with micrococcal nuclease to reduce the number of binding sites provided by RNA. Second, purified importin-α1 was incubated with non-conjugated resin, i.e. resin that was not coupled to a homopolymer. For each sample, aliquots of the unbound (F1) and bound protein (B) were probed by Western blotting with antibodies against importin-α1. With this assay, little or no difference of importin-α1 binding was detected when reactions were treated with or without nuclease (Fig. 10). This indicates that importin-α1 is unlikely to bind homopolymers directly. By contrast, purified poly(A)-binding protein associated efficiently with immobilized poly(A), and this interaction was diminished when the poly(A)-sepharose was pre-treated with micrococcal nuclease. Taken together, importin-α1 purified from *E. coli* exhibited no or only weak interactions with homopolymers in *vivo*.

**Discussion**

Importin-α plays an essential role in nucleocytoplasmic transport by serving as an adaptor between the nCQLS and importin-β1. One of the immediate responses to stress is nuclear transport inhibition. We have shown previously that heat shock and DEM interfere with nuclear import and export by affecting multiple transport factors [33,34,54]. Besides altering nuclear transport, stress also leads to translational arrest which is accompanied by the formation of SGs that contain RNA-binding proteins and poly(A)-RNA.

Our current work demonstrates that the oxidant DEM induces the formation of SGs that contain importin-α1. Furthermore, we show for the first time that importin-α4 and α5, members of the α3 and α1 subfamilies, are targeted to SGs as well. The SG composition is stress-specific [3,58]; yet, importin-α4 and α5 also accumulated in SGs that were induced by arsenite or heat shock. Thus, several importin-α proteins of distinct subfamilies are
recruited to SGs under different stress conditions, and importin-α, α4 and α5 can be regarded as bona fide SG constituents. Importantly, their association with SGs is specific, because they do not concentrate in PBs under normal or stress conditions (summarized in Table 1). This property is shared by importin-β1, but not CAS, both of which are carrier proteins that interact directly with importin-α family members.

Aside from its accumulation in SGs under different stress conditions, importin-α1 associated with poly(A)-RNA in vivo, and DEM significantly reduced this interaction. Interestingly, the RNA association of importin-α1 was a distinguishing feature of this isoform, which was not shared by any of the other transport adapters analyzed here. Our binding assays suggest that importin-α1 synthesized in E. coli did not efficiently associate with RNA homopolymers in vitro. There are several potential explanations for this observation. First, it is possible importin-α1 does not contact RNA directly, and a linker protein is necessary to connect importin-α1 to poly(A)-RNA in growing cells. Second, posttranslational modifications of importin-α1 may be necessary for its binding to RNA, and these modifications are absent when importin-α1 is purified from E. coli. Third, importin-α1 recognizes specific RNA sequences that are not provided by homopolymers. Future studies will have to distinguish between these possibilities to determine the precise mechanisms that promote the interaction between importin-α1 and poly(A)-RNA in vivo.

Independent of the nature of its RNA-binding, the coincidence of importin-α1 release from poly(A)-RNA and SG association could suggest that the stress-induced dissociation of RNA-importin-α1 complexes is linked to SG recruitment. In support of this recruitment, SG components could provide binding sites for importin-α1; HuR is a potential candidate for this interaction, as it is an established binding partner of importin-α1 under nonstress conditions [59].

In an alternative model, nuclear transport factors may promote SG assembly by moving individual constituents to SGs. According to this idea, members of the importin-α family and importin-β1 will deliver material to the growing granule [48], a hypothesis compatible with the fact that importin-α1 knockdown reduces SG size [38]. Hence, nuclear transport factors may not only deliver macromolecules across nuclear membranes, but also to specialized cytoplasmic compartments, as they are exemplified by SGs. This model is further supported by the observation that transportin-1 participates in the movement of material between SGs and PBs, while importin-8 is involved in export from PBs [11,60].

Collectively, our experiments provide novel insights into the biological roles of several members of the importin-α family, especially the multifunctional protein importin-α1. The newly identified ability of importin-α1 to associate with poly(A)-RNA is particularly interesting, because it is controlled by stress. Moreover, we identified additional members of the importin-α family, which belong to different subfamilies, as novel SG constituents. While these factors have an established role in nuclear protein transport or gene expression regulation [36,47], our results suggest additional isoform-specific functions that are related to RNA metabolism and the stress response.

Supporting Information

Figure S1 Specificity of anti-importin-α antibodies for immunolocalization. Primary antibodies against members of the importin-α family and isotype-specific IgG controls (for importin-α1 and α5) or pre-immuniserum (control for importin-α4) were tested under the same conditions. Staining was evaluated for ethanol and DEM-treated cells as described for Fig. 1. All samples were co-stained with antibodies against HuR, and nuclei were detected with DAPI. Size bar is 20 μm. (TIF)

Figure S2 Western blot analysis determines the specificity of antibodies against nuclear transport factors and HuR. Crude HeLa cell extracts were tested with antibodies against nuclear transport factors, HuR or control antibodies as indicated. Negative control antibodies, either isotype-specific IgG or pre-immuniserum (PS), were used at the same concentration as primary antibodies. For each antigen, the same filter was probed with primary and control antibodies, with identical exposure times during ECL. (TIF)

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Author Contributions

Conceived and designed the experiments: HM, US. Performed the experiments: HM, ES, DK, US. Analyzed the data: HM, US. Wrote the paper: HM, US.

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