Mammalian Staufen 1 is recruited to stress granules and impairs their assembly

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Summary
Stress granules are cytoplasmic mRNA-silencing foci that form transiently during the stress response. Stress granules harbor abortive translation initiation complexes and are in dynamic equilibrium with translating polysomes. Mammalian Staufen 1 (Stau1) is a ubiquitous double-stranded RNA-binding protein associated with polysomes. Here, we show that Stau1 is recruited to stress granules upon induction of endoplasmic reticulum or oxidative stress as well in stress granules induced by translation initiation blockers. We found that stress granules lacking Stau1 formed in cells depleted of this molecule, indicating that Stau1 is not an essential component of stress granules. Moreover, Stau1 knockdown facilitated stress granule formation upon stress induction. Conversely, transient transfection of Stau1 impaired stress granule formation upon stress or pharmacological initiation arrest. The inhibitory capacity of Stau1 mapped to the amino-terminal half of the molecule, a region known to bind to polysomes. We found that the fraction of polysomes remaining upon stress induction was enriched in Stau1, and that Stau1 overexpression stabilized polysomes against stress. We propose that Stau1 is involved in recovery from stress by stabilizing polysomes, thus helping stress granule dissolution.

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Key words: ER stress, P bodies, Staufen, Oxidative stress, Silencing foci, Stress granules

Introduction
The cellular response to stress involves a global silencing of ongoing translation. The transient formation of large cytoplasmic foci, known as stress granules, was recently reported to be a hallmark of the stress response. Stress granules harbor abortive translation initiation complexes that accumulate upon inactivation of eIF2α by specific kinases activated by distinct stressors. Thus, stress granules contain polyadenylated mRNA in a complex with poly-A binding protein (PABP), several initiation factors including eIF4E, eIF4G, eIF3, eIF2B and phosphorylated-eIF2α, and small, but not large, ribosomal subunits (Kimball et al., 2003) (reviewed by Anderson and Kedersha, 2008). In addition, stress granules include a growing number of RNA-binding proteins (RBPs), which modulate stability or translation, as well as act on nuclear splicing and post-splicing events (Gallouzi et al., 2000; Baez and Boccaccio, 2005; Wilczynska et al., 2005; Vessey et al., 2006; Guil et al., 2006; Stöhr et al., 2006; Mazroui et al., 2007; Kawahara et al., 2008) (reviewed by Anderson and Kedersha, 2008). Stress granules are also induced by overexpression of translational repressors, by pharmacological inhibition of translation initiation or by inosine-modified RNA (Thomas et al., 2005; Baez and Boccaccio, 2005; Wilczynska et al., 2005; Dang et al., 2006; Mazroui et al., 2006; Scadden, 2007; Kim et al., 2008). The transient assembly of abortive initiation complexes in stress granules is mediated by the self-aggregation of the RBP T-cell intracellular antigen (TIA-1), TIA-1-related (TIAR) and RasGAP-associated endoribonuclease (G3BP), among others, and requires O-glycosylation of ribosomal proteins (Kedersha et al., 1999; Tourriere et al., 2003; Ohn et al., 2008) (reviewed by Anderson and Kedersha, 2008). Stress granule disassembly is mediated by the chaperone activity of stress-induced Hsp70 and by phosphorylation of specific RBPs (Tourriere et al., 2003; Tsai et al., 2008) (reviewed by Anderson and Kedersha, 2008). Stress granules are highly dynamic and are in equilibrium with translating polysomes, because disruption of polysomes promotes stress granule formation, whereas polysome stabilization prevents stress granule assembly (Kedersha et al., 2000; Mollet et al., 2008) (reviewed by Anderson and Kedersha, 2008). The processing bodies, also termed decapping bodies or glycine-tryptophan (GW) bodies, are related mRNA-silencing foci. Processing bodies are constitutively present, and they also depend on the global translational state of the cell (Cougot et al., 2004; Andrei et al., 2005; Ferraiuolo et al., 2005; Wilczynska et al., 2005) (reviewed by Eulalio et al., 2007; Anderson and Kedersha, 2008).

We have previously reported the presence of the double-stranded RBPs Staufen 1 (Stau1) and Staufen 2 (Stau2) in stress granules formed in brain oligodendrocytes exposed to oxidative stress (Thomas et al., 2005). Whether Staufen molecules participate in stress granule structure or physiology has not been addressed. The ubiquitously expressed mammalian Stau1 binds to ribosomal subunits through protein-protein and protein-RNA interactions and is shown to be associated to polysomes in several cell types (Kiebler et al., 1999; Marion et al., 1999; Duchaine et al., 2002; Luo et al., 2002; Thomas et al., 2005; Baez and Boccaccio, 2005; Dugré-Brisson et al., 2005). In addition, Stau1
is considered to be a global mRNA-binding factor, as it was shown to bind to different RNA motifs, including G-quartets, the β-actin
zip code, the Myc 3′UTR; the FMR1 3′UTR, the HIV transactivating response region and the so-called Staufen-binding
motif (Rackham and Brown, 2004; Dugré-Brisson et al., 2005; Kim, Y. K. et al., 2005). Recently, at least 7% of cellular mRNAs
were shown to be present in Stau1 ribonucleoparticles (RNPs) (Furic et al., 2008). Consistently with this broad spectrum of
targets, Staufen was associated with a variety of cytosolic functions. Staufen is involved in mRNA transport in both oocytes
and somatic cells in vertebrates, as well as in invertebrates (Ferrandon et al., 1994; Broadus et al., 1998; Kiebler et al., 1999;
Micklem et al., 2000; Tang et al., 2001; Belanger et al., 2003; Yoon and Mowry, 2004; Gautrey et al., 2005; Lebeau et al., 2008;
Vessey et al., 2008). When bound to the 3′UTR, mammalian Stau1 triggers mRNA decay by recruitment of UPF1, a key molecule
for mRNA degradation (Kim, Y. K. et al., 2005). By contrast, it enhances CAP-dependent translation when tethered to the 5′UTR
(Ferrandon et al., 1994; Kiebler et al., 2005). In addition to these cytoplasmic functions, nuclear roles for mammalian Stau1 molecules have
begun to emerge (Kiebler et al., 2005). Whether Stau1 functions contribute to stress granule physiology is unknown.

In this work we show that, although mammalian Stau1 is always present in stress granules, either induced by cellular stress or by
pharmacological inhibition of translation initiation, is not an essential component of these foci. Moreover, we found that Stau1
depletion facilitated stress granule assembly, whereas transfection of Stau1 constructs inhibited their formation. Processing body
integrity was moderately affected by Stau1, according to our finding that Stau1 is scarcely recruited to these structures. We show
that Stau1 associates with polysomes, protecting them from stress-induced breakdown. We propose that downstream of the stress
signaling, the breakdown of polysomes and concomitant stress granule formation are regulated by Stau1.

Results Presence of Stau1 in stress granules and processing bodies

We have previously shown that Stau1 is recruited to stress granules in brain primary cell cultures upon induction of oxidative stress
(Thomas et al., 2005). Here, we analyzed the presence of Stau1 in stress granules induced in transformed and non-transformed cell
lines of distinct origin. Using a specific antibody against Stau1 (Craig et al., 2005), we found that this molecule was present in stress
granules induced in NIH 3T3, HeLa, H9, COS-7, WI-38, H1299 and U2OS cells, upon exposure to the calcium-pump inhibitor thapsigargin – a known inductor of endoplasmic reticulum stress – as well as in stress granules induced by arsenite, which induces oxidative stress (Fig. 1 and M.G.T., L.J.M.T. and G.L.B., unpublished data). Stau1 was observed to localize in every stress
granule identified by staining of the specific marker TIAR or of the eukaryotic initiation factor 4E (eIF4E), also known to be
recruited to these foci (Fig. 1A,B). Confocal slicing along the z-axis confirmed the presence of Stau1 inside stress granules and Stau1
was also detected in stress granules induced by heat shock or DTT (G.L.B. and Mariela Loschi, unpublished data). Stress granules are
known to be transient, and thus we investigated whether the recruitment of Stau1 to these structures varies during their assembly and
dissolution. We found that although thapsigargin-induced stress granules persisted longer than those induced by oxidative stress (see
below), Stau1 was always detected in stress granules during their assembly and dissolution (not shown). By contrast, distinct

molecules were incorporated to thapsigargin-induced stress granules in a time-dependent manner (see below).

Finally, we tested whether the stress-induced Stau1 aggregates were sensitive to polysome-stabilizing drugs, a distinctive feature of
stress granules. As expected, we found that stabilization of polysomes by cycloheximide prevented the formation of foci containing TIAR and Stau1, whereas puromycin elicited no effect or slightly enhanced stress granule formation (Fig. 1C). Thus, stress granule assembly and incorporation of Stau1 to these foci are concomitant with polysome disruption.

Similarly to stress granules, processing bodies represent dynamic storage compartments for untranslated mRNAs, and are likewise
in equilibrium with translating polysomes. In addition, stress granules and processing bodies share certain protein components
(reviewed by Eulalio et al., 2007; Anderson and Kedersha, 2008). Here, we investigated the recruitment of Stau1 to normal or stress-
induced processing bodies identified by the presence of Dep1a, which is a marker for processing bodies. First, we investigated the
presence of processing bodies upon induction of oxidative or ER stress. In both stress models, stress granules were frequently
detected in close contact with processing bodies (Fig. 2), as similarly reported for stress granules induced by the overexpression
of G3BP or TIA-1 (Kedersha et al., 2005) (reviewed by Anderson and Kedersha, 2008). As reported before, we found that treatment
with arsenite increased the size and number of processing bodies, as well as the number of cells containing processing bodies with a
time-course that paralleled that of stress granules (not shown). At all time points analyzed upon arsenite exposure, we found that
Dep1a was excluded from stress granules, whereas the processing
body components GW182 and Xm1 were present in both types of foci (M.G.T. and G.L.B., unpublished results). By contrast, upon continuous treatment with 250 nM thapsigargin, Dcp1a incorporated to stress granules and free processing bodies were no longer detected (4 and 8 hours in Fig. 2A,B). Strikingly, during the stress granule dissolution phase of thapsigargin treatment, processing bodies also vanished; the Dcp1a signal remaining dispersed in the cytoplasm.

All these observations suggest that a regulated transfer of molecules between stress granules and processing bodies occurs, and that these foci are differentially modulated by different stressors.

Next, we followed the distribution of endogenous or transfected Stau1 and Dcp1a molecules in NIH 3T3 and U2OS cells under resting or oxidative stress conditions. Expression of transfected molecules was allowed for short times, because overexpression of Stau1 or Dcp1a affects stress granules and processing bodies (Fenger-Gron et al., 2005) (see below). Endogenous Stau1 was barely detected in processing bodies identified as Dcp1a-RFP foci under resting conditions (Fig. 2C,D), and was scarcely recruited to processing bodies upon stress induction. Similarly, tagged Stau1 was sporadically detected in processing bodies identified by staining of endogenous Dcp1a, and oxidative stress induction moderately increased the proportion of processing bodies containing Stau1-V5 (Fig. 2C,D). As expected, the exoribonuclease Xm1 was always detected in Dcp1a-RFP foci (Fig. 2D), whereas PABP was completely excluded from processing bodies in any condition tested (Fig. 2C).

In summary, in resting conditions, the ubiquitously expressed Stau1 is mostly associated with polysomes (Marion et al., 1999; Luo et al., 2002; Dugré-Brisson et al., 2005; Thomas et al., 2005) and is mobilized to stress granules concomitantly with polysome disruption upon stress. A proportion of Stau1 remains non-aggregated and is probably retained in the fraction of polysomes that were not disassembled by the stressor (see below).

**Stau1 silencing enhances stress granule formation**

We evaluated the effect of Stau1 depletion on stress granule formation. The selected siRNAs against Stau1 or Stau2 (siStau1 and siStau2) specifically diminished the expression of their target molecules without affecting the expression of the other Staufen homologue or of ECFP constructs (Fig. 3A,B and M.A.D., L.J.M.T. and G.L.B., unpublished observations). Reduction of endogenous Stau1 in NIH 3T3 cells by siStau1 was approximately 75%, as judged by western blot analysis (Fig. 3B). We then analyzed whether silencing of Staufen molecules affects stress granule formation. We found that upon oxidative stress induction, stress granules were assembled in cells with reduced levels of Stau1, and remarkably, Stau1 was barely detectable or completely absent from these stress granules, as visualized by line-scan analysis (Fig. 3C). A quantitative analysis performed at different times upon arsenite exposure revealed that the formation of stress granules was significantly facilitated by Stau1 depletion (Fig. 3D,E). Silencing of Staufen 2 provoked a minor stimulation of stress granule formation in NIH 3T3 cells (M.A.D., M.G.T. and G.L.B., unpublished observations), probably reflecting the fact that this Staufen homolog is expressed at low levels in these cells. We found that siRNAs against human Stau1 also enhanced stress granule formation in U2OS cells (M.G.T. and G.L.B., unpublished observations). For comparison, the effect of siRNA-mediated depletion of selected processing body components on stress granule formation was evaluated. In contrast to the effect of Stau1 knockdown, depletion of Hedls, or of RCK/p54 – a processing body component also recruited to stress granules – moderately impaired the formation of stress granules upon oxidative stress induction (supplementary material Fig. S1A). Staining for Dcp1a showed processing body disruption in resting conditions and partial recovery of processing bodies upon arsenite exposure (supplementary material Fig. S1B). Thus, the enhancing effect on stress granule formation was restricted to the siRNA-mediated knockdown of Staufen molecules.
We then analyzed whether stress granules induced by ER stress were similarly affected. We found that silencing of Stau1 significantly enhanced the formation of thapsigargin-induced stress granules. As for the oxidative stress model, we found that cells with stronger Stau1 silencing showed a higher incidence of stress granules (supplementary material Fig. S1C). The size of the stress granules was also affected by Stau1 depletion. An increase in the average size from 5.8 μm² in control cells to 7.2 μm² in siStau1-treated cells was observed 60 minutes after stress induction (Fig. 3F). Finally, as for the arsenite-stress model, thapsigargin-induced stress granules lasted longer in Stau1-depleted cells (Fig. 3G).

Altogether, these observations indicate that Stau1 is not an essential component of stress granules; this molecule rather impairs stress granule formation and facilitates their dissolution. We also analyzed the effect of Stau1 depletion on processing bodies. Both basal and arsenite-induced processing bodies were weakly enhanced by Stau1 depletion (M.G.T. and G.L.B., unpublished results). It has been proposed that stress granules help cell survival in several ways (Kim, W. J. et al., 2005; Kim, W. J. et al., 2007; Mazroui et al., 2007; Yu et al., 2007; Arimoto et al., 2008; Eisinger-Mathason et al., 2008) (reviewed by Anderson and Kedersha, 2008). We assessed cell survival upon induction of ER stress in Stau1-depleted cells. We found that the exposure of siNR-treated cells to 100 nM thapsigargin resulted in a non-lethal stimulus, whereas it provoked the death of Stau1-depleted cells (Fig. 3H). These results highlight the relevance of Stau1 as a protective molecule against the stress stimulus.
Stau1 modulates stress granule dynamics

Stau1 overexpression impairs stress granule formation

Next, we evaluated the effect of increasing Stau1 levels on stress granule formation. First, we confirmed that transfected tagged Stau1 was incorporated into stress granules. Murine or rat Stau1 fused to V5 or ECFP colocalized with the marker proteins TIA-1 and TIAR, the small ribosomal subunit marker S6, the translation initiation factors elF4E and elF4G1, and the RBPs HuR and G3BP in low-expressing cells exposed to arsenite (supplementary material Fig. S2A). Strikingly, we found that a moderate overexpression of Stau1 inhibited stress granule formation upon oxidative stress induction. The stress granule marker molecules TIA-1 and TIAR were detected in the cytoplasm of Stau1-overexpressing cells and showed a non-granular distribution (Fig. 4A). The absence of stress granules was confirmed by the uniform distribution of PABP and elF4G1 (Fig. 4B), indicating that aggregates containing polyadenylated mRNA or initiation factors were not formed. As expected, overexpression of ECFP had no effect on stress granule formation and, as previously shown (Gilks et al., 2004), transfection of TIA-1-GFP was not inhibitory but rather stimulatory (supplementary material Fig. S2B). The inhibitory capacity of Stau1 correlated with the expression levels. Levels of transfected Stau1 relative to the endogenous protein were estimated as indicated in the Materials and Methods. After 24 hours of expression, cells with three times the normal amount of Stau1 showed a reduction from...
86% to 22% in arsenite-induced stress granule formation (supplementary material Fig. S2C). By contrast, and as reported before (Gilks et al., 2004), we found that inhibition of stress granule formation by a fragment containing the prion-like domain of TIA-1 required a massive overexpression obtained after 3 days of transfection (C.C.L. and G.L.B., unpublished results). Staun-mediated inhibition was partially overridden by increasing concentrations of arsenite (L.J.M.T. and G.L.B., unpublished data), suggesting that the formation of stress granules depends on the balance between Staun levels and the strength of the stress stimulus.

We also analyzed the effect of Staun transfection on the formation of stress granules induced by ER stress. A time-course analysis indicated that cells overexpressing Staun had a reduced response to thapsigargin relative to non-transfected cells or ECFP-transfected cells (Fig. 4C). Thus, the regulation of stress granule formation by Staun was independent of which elf2α kinase was involved in triggering their assembly.

The inhibitory capacity of several Staufen constructs was compared. Murine Staun fused to V5 or rat Staun fused to ECFP had comparable effects (Fig. 4A-D). There were no differences between the inhibitory capacity of a mutant Staun that was unable to bind protein phosphatase 1 (Monshausen et al., 2002) and that of the wild-type construct (L.J.M.T. and G.L.B., unpublished results). Staun2 had a moderate effect on the assembly of stress granules induced by arsenite or thapsigargin (L.J.M.T., C.C.L. and G.L.B., unpublished results). Staun1 is a modular protein with four dsRNA-binding domains (RBD2 to RBD5) and a tubulin-binding domain (TBD) (Fig. 4D). The N-terminal half of the molecule mediates the association with polysomes (Luo et al., 2002), and we found that this region, including up to RBD4 (RBD234) (Fig. 4D) retained the inhibitory capacity of the full-length molecule. The formation of stress granules upon treatment with arsenite or thapsigargin was significantly impaired in NIH 3T3 or U2OS cells expressing this construct (Fig. 4D). By contrast, a construct lacking this region, named TBD-RBD5, had no significant effect on the assembly of stress granules induced either by oxidative or ER stress (Fig. 4D). Staun-ECFP, RBD234-EGFP and TBD-RBD5-EGFP were expressed at similar levels as judged by western blot analysis (results not shown). We found that, like the full-length Staun, the RBD234 fragment was recruited to the stress granules in the reduced number of cells that succeed in forming these foci (Fig. 4E). By contrast, the TBD-RBD5 was not recruited to stress granules (Fig. 4E), in agreement with the observation that the RBD5 has a very low RNA-binding activity (Wickham et al., 1999). Altogether, these results suggest that the Staun-mediated inhibition of stress granule formation is linked to its association with polysomes.

Staun regulates stress granule formation downstream of the phosphorylation of elf2α

We have previously shown that Staun-containing stress granules are induced by edeine, a drug that blocks the recruitment of the large ribosome subunit (Thomas et al., 2005). More recently, it was demonstrated that unlike stress-induced stress granules, the phosphorylation of elf2α is not required when stress granules are assembled by initiation blockers such as hippuristanol (Mazour et al., 2006; Anderson and Kedersha, 2008). Here, we evaluate the effect of Staun on the formation of stress granules triggered by this inhibitor. We found that endogenous or transfected Staun was recruited to hippuristanol-induced stress granules and that the assembly of stress granules in Staun-transfected cells was dramatically reduced from 95% to 46% (Fig. 5A,B). Altogether, these results suggest that both types of stress granules – phospho-elf2α dependent as well as phospho-elf2α independent – are similarly downregulated by Staun.

Accordingly, we found that the stress-induced phosphorylation of elf2α was not affected by Staun. First, we analyzed the temporal correlation between elf2α phosphorylation and stress granule formation. We found that both processes were rapidly triggered by arsenite and reached their maximal induction at about the same time (60-90 minutes) (Fig. 5C). Thereafter, phosphorylation rapidly decayed below basal levels, whereas more than 50% of the cells still contained stress granules (120 minutes in Fig. 5C), suggesting that sustained phosphorylation of elf2α is not required once stress granules are formed. Next, we evaluated the phosphorylation of elf2α in single cells by immunofluorescence at 60 minute after stimulus. We found that elf2α was phosphorylated in cells transfected with Staufen1-ECFP as well as in non-transfected cells, whether or not they contained stress granules (Fig. 5D; supplementary material Fig. S3). Finally, siRNA-mediated depletion

![Fig. 5. Staun modulates stress granule assembly downstream of elf2α phosphorylation.](image)

**A** Hippuristanol

**B** Hippuristanol

**C** Time (minutes)

**D** Levels of phosphorylated or total elf2α were determined by immunofluorescence in single cells expressing Staun-ECFP under control conditions (C; n=26) or upon arsenite exposure (Ars; n=51) and in neighboring non-expressing cells (NT) in control (n=32), or stress conditions (n=64).
of Stau1 had no effect on eIF2α phosphorylation triggered by arsenite (M.G.T., M.A.D. and G.L.B., unpublished results).

Stress granule dissolution is mediated by the stress-induced expression of Hsp70, which helps to revert the aggregation of stress granule-core proteins (Mazroui et al., 2007; Anderson and Kedersha, 2008). We found that Stau1 transfection did not alter Hsp70 levels under resting conditions and allowed arsenite-induced upregulation of this chaperone as in non-transfected cells (C.C.L. and G.L.B., unpublished results). We conclude that the Stau1-dependent regulation of stress granule formation does not involve stress sensing, impairment of the inactivation of the key factor eIF2α or upregulation of the stress granule-dissolving factor Hsp70.

Stau1 stabilizes polysomes against stress-induced breakdown
Stau1 is known to associate to polysomes and ribosomal subunits under resting conditions and is recruited to stress granules, which exclude polysomes, upon stress induction. Thus, we investigated the association of Stau1 to polysomes upon induction of stress granule formation. Cell extracts were separated by sedimentation in sucrose gradients and the distribution of Stau1 and of selected marker proteins was analyzed by western blot. As expected, 60 minutes after arsenite exposure, the ribosomal proteins P0 and S6 shifted towards fractions of lower sedimentation (fractions 1-15 in Fig. 6A,B). PABP accompanied the shift (Fig. 6A), reflecting the fact that translation of most messengers is impaired upon stress induction.

Fig. 6. Stau1 associates with stress-resistant polysomes. (A) NIH 3T3 cells were exposed to 0.5 mM arsenite for 1 hour, polysomes were separated in sedimentation gradients and fractions were analyzed by western blot to detect P0, a marker for large ribosomal subunits, S6, a marker for small subunits, PABP, TIAR and Stau1. (B) Distribution of P0 and Stau1 in pooled fractions from the gradient shown in A were evaluated by western blot and relative abundances are represented in the graphs. (C) Three independent experiments were performed as in A, and the distribution of polysomes was evaluated by following the P0 and S6 distributions in the gradient. Left column pair, total number of fractions = 13; polysome-containing fractions = 10-13. Middle and right column pairs, total number of fractions = 24; polysome-containing fractions = 16-24. The content of Stau1 in the polysomal fraction was measured by western blot analysis and is expressed normalized to S6 (left column pair) or P0 (middle and right column pairs). Duplicate western blot analysis of each gradient showed variations less than 10%. On average, the polysomes that remain upon stress induction contain twice the amount of Stau1 than found in polysomes under resting conditions. (D,E) Cells were transfected with ECFP or Stau1-V5 and exposed to 0.5 mM arsenite for 1 hour. The polysome profile was evaluated by monitoring the distribution of P0 in the gradient and the relative amount of polysomes is expressed as the percentage of P0 in the polysomal fraction relative to total. The amount of polysomes recovered after stress induction was increased from 10 to 20% in the presence of Stau1-V5. (F) A model for the modulation of stress granules by Stau1. Under resting conditions, Stau1 is associated with polysomes by binding to mRNAs and ribosomal subunits. Cellular stress or pharmacological inhibition of 60S ribosomal recruitment provokes the breakdown of polysomes and concomitant accumulation of abortive translation initiation complexes that are aggregated by specific RBPs including TIA-1, TIAR and G3BP and by the O-glycosylation of ribosomal proteins (reviewed by Anderson and Kedersha, 2008; Ohn et al., 2008). Stau1 is recruited to growing stress granules by a piggyback mechanism. Formation of stress granules is counterbalanced by Stau1, which stabilizes polysomes against stress-induced breakdown, thus helping stress granule dissolution. In addition, Hsp70 contributes to stress granule disassembly (Mazroui et al., 2007).
induction. A similar shift of the global polysome profile was described in related sublethal stress models (Koritzinsky et al., 2006). A quantitative analysis indicated that, upon exposure to arsenite, the proportion of P0 and S6 in the fraction containing large polysomes (fractions 16 to 24) was reduced to 35% of control levels (Fig. 6A,B). In striking contrast, the distribution of Stau1 was much less affected by the exposure to arsenite, the abundance of this molecule in the fast-sedimenting polysomes being moderately reduced to 80% relative to that observed in non-stressed cells.

The stress-granule marker protein TIAR was analyzed simultaneously and, as previously described (Kedersha et al., 2002), it was detected mostly in the fractions containing small RNRs, also co-migrating with free ribosomal subunits but never beyond 80S monosomes (Fig. 6A). This observation rules out the possibility that the fast-sedimenting fractions contained intact or fragmented stress granules to which Stau1 would associate. In addition, as Stau1 includes a tubulin-binding domain that associates with microtubules in vitro (Wickham et al., 1999), we investigated the presence of supramolecular complexes containing microtubules. Tubulin (Fig. 6A) and actin (results not shown) were not detected beyond the fractions corresponding to small polysomes, thus excluding the possibility that the fast-sedimenting fractions represent Stau1 complexes associated to cytoskeletal structures.

The mobilization of Stau1 relative to that of the ribosomal markers S6 and P0 upon arsenite was measured in three independent experiments performed similarly. In all cases we found that most polysomes are disrupted upon oxidative stress induction and that the fraction of polysomes recovered after the stress insult contained twice the amount of Stau1 than polysomes extracted from non-stressed cells (Fig. 6C). Next, we evaluated the effect of overexpressed Stau1 on polysome disruption upon stress. U2OS cells were transfected with tagged Stau1 or with ECFP, exposed to arsenite and the polysome profile was analyzed as before, by monitoring the distribution of P0 in sucrose gradients. As expected, arsenite provoked a partial breakdown of polysomes in all cases. However, we found that the amount of polysomes recovered upon stress was significantly larger in Stau1-transfected cells than in ECFP-transfected cells (Fig. 6D,E).

Altogether, our observations suggest that Stau1 associates with polysomes, preventing their disruption and downregulating stress granule formation. To investigate whether this has an effect on the translational blockade triggered by stress, we evaluate the protein synthesis rate in Stau1-depleted cells exposed to oxidative or ER stress. Incorporation of radio-labelled amino acids was measured as indicated in the Materials and Methods at several time points during stress granule formation and dissolution. We found no significant differences in the inhibition of protein synthesis that occurs simultaneously with stress granule formation (supplementary material Fig. S4) (1-2 hours after oxidative stress; 2 hours after ER stress). In addition, the partial recovery of protein synthesis that correlates with stress granule dissolution (supplementary material Fig. 4) (3-5 hours after oxidative stress; 4-8 hours after ER stress) and with Hsp70 translation (M.A.D., C.C.L. and G.L.B., unpublished results) was not significantly affected by Stau1 knockdown. These data indicating that polysome stabilization by Stau1 does not correlate with higher translation rates are suggestive of polysome stalling.

**Discussion**

Here, we report the downregulation of stress granule formation by the double-stranded RBP Stau1. We found that this ubiquitously expressed protein is recruited to stress granules induced in cell lines, oligodendrocytes and neurons by several stress stimuli, including oxidative stress, ER stress or heat shock (Thomas et al., 2005). Given that Stau1 binds to mRNAs and ribosomal subunits (Kiebler et al., 1999; Marion et al., 1999; Duchaine et al., 2002; Luo et al., 2002; Baez and Boccaccio, 2005; Thomas et al., 2005), we propose that Stau1 is mobilized to stress granules in association with mRNAs and/or small ribosomal subunits, which are always present in stress granules.

Although stress granules always contained Stau1, we found that stress granules lacking this protein were formed in Stau1-depleted cells, indicating that Stau1 is not an essential component of these foci. Moreover, Stau1 knockdown facilitates the formation of stress granules induced by oxidative or ER stress. Conversely, we found that a moderate overexpression of Stau1 impaired stress granule formation. Stau1 is the first stress granule component reported to negatively regulate the assembly of these foci. The modulating effect was observed on stress granules induced either by a stress signal or by huppirlanol, an initiation blocker that does not induce the phosphorylation of eIF2αe. Thus, the regulation of stress granule formation by Stau1 occurs downstream of this event.

We propose that upstream of the assembly step mediated by the aggregation domains of TIA-1, TIAR and G3BP, and by post-translational modifications of ribosomal proteins, stress granule formation is regulated by Stau1, probably by affecting the equilibrium between silencing foci and polysomes (Fig. 6F). Supporting this model, a significant proportion of endogenous or overexpressed Stau1 is associated with polysomes (Kiebler et al., 1999; Marion et al., 1999; Duchaine et al., 2002; Luo et al., 2002; Thomas et al., 2005; Baez and Boccaccio, 2005; Dugré-Brisson et al., 2005). In addition, the present studies revealed that the polysomes that remain after the stress-induced breakdown are enriched in Stau1. Our results suggest a direct effect of Stau1 in mediating polysome stability against stress. Supporting this notion, the Stau1 domains directly interacting with ribosomes (RBD2 to RBD4) (Luo et al., 2002) elicited an effect comparable with that of the full-length molecule. How Stau1 modulates polysome stability remains to be investigated. Polysome breakdown upon stress is the consequence of impaired initiation and continuing elongation and termination. Our data suggest that Stau1 leads to polysome stalling by unknown mechanisms. Stau1 may slow elongation, so that loss of ribosome subunits is reduced. Alternatively, Stau1 may impair ribosome release. Interestingly, stalled polysomes have been recently described to accumulate during mitosis and this impairment in elongation is determinant of the lack of stress granules during cell division (Sivan et al., 2007). Whether Stau1 is involved in polysome slowdown and stress granule inhibition during mitosis is unknown.

In addition to its interaction with ribosomes, Stau1 is a general mRNA binding factor and this might contribute to the global effect on polysome stability described here. Stau1 ribonucleoparticles comprise at least 7% cellular messengers, a fraction of them involved in cellular metabolism and ubiquitylation (Furic et al., 2007). The binding motifs and the regulatory relevance of this direct or indirect association have not been addressed and thus, their putative contribution to stress granule regulation remains unsolved. It has been shown that Stau1 triggers mRNA degradation when it is bound downstream of the STOP codon (Kim, Y.K., et al., 2005). The importance of the decay of putatively relevant targets including TIA-1 (Kim, Y.K., et al., 2007) and ARF1 (Kim, Y.K., et al., 2005) requires further investigation. In addition, Stau1 stimulates...
translational efficiency are required for stress granule disruption, and thus, this may contribute to stress granule regulation.

Finally, it has been reported that Stau1 molecules interact with molecular motors and the cytoskeleton, mediating the transport and anchorage of ribonucleoparticles (Ferrand et al., 1994; Broadus et al., 1998; Wickham et al., 1999; Kiebler et al., 1999; Micklem et al., 2000; Tang et al., 2001; Belanger et al., 2003; Yoon and Mowry, 2004; Gautrey et al., 2005; Vessey et al., 2008). We believe that the anchorage of ribosomes or mRNAs to the cytoskeleton is not a major factor in impeding the mobilization of the translational apparatus to stress granules, because Staun construct excludes the protein domains thought to be involved in the interaction with the cytoskeleton are still able to inhibit stress granule formation.

It has been proposed that stress granule aggregation requires the activity of motor molecules (Kwon et al., 2007; Anderson and Kedersha, 2008), so it might be that stress granule dissolution also requires active transport. Staufen molecules were implicated in RNA transport in several cell types and thus, mammalian Stau1 might help to the motor-mediated transport of mRNPs from discrete silencing foci to dispersed polysomes.

Given that Stau1 is a modulator of stress granule formation, whether Stau1 is regulated by cellular stress is a relevant issue. We found that Stau1 levels remained constant during acute oxidative stress in NIH 3T3 cells (M.A.D. and G.L.B., unpublished results). Whether Stau1 expression responds to other stress models remains to be investigated. Mammalian Staufen molecules contain several putative phosphorylation sites, and it has been shown that phosphorylation of Xenopus Staufen modulates its binding to intracellular structures (Allison et al., 2004). Whether differences in the expression levels, splicing variants or phosphorylation state of Stau1 and Stau2 modulate stress granule formation remains an open question.

The formation of stress granules is usually transient and stress granules and related silencing foci containing polyadenylated mRNA form persistently when cell viability is compromised (Kayali et al., 2005; Jamison et al., 2008; Anderson and Kedersha, 2008). We found that cell survival under stress is affected in Staun-depleted cells and this might be connected to the differential persistence of stress granules. In addition to their relevance in regulating translation and mRNA stability upon stress (Stohr et al., 2006; Mazroui et al., 2007; Anderson and Kedersha, 2008), stress granules are believed to indirectly affect transcription, inflammation and cell survival by sequestration of specific molecules (Yu et al., 2007; Kim, W. J. et al., 2005; Jamison et al., 2008; Anderson and Kedersha, 2008). We found that Stau1 levels remained constant during acute oxidative stress in NIH 3T3 cells (M.A.D. and G.L.B., unpublished results). Whether Staun found that Stau1 levels remained constant during acute oxidative stress in NIH 3T3 cells (M.A.D. and G.L.B., unpublished results).

Materials and Methods

Plasmids

The following plasmids were used: Stau1-ECFP, pECPF-N1 (Clontech, Palo Alto, CA) carrying a cDNA encoding rat Stau1 (AF290989), gift from Stefan Kindler (University of Hamburg, Germany); Staun-V5, murine Stau1 (AF061942) cloned in pCDNA6.0 (Invitrogen, Carlsbad, CA); RBD2343-EGFP and TBD-RBDS-EGFP generated by PCR-cloning of the 1-816 nt. and the 814-1464 nt. fragments respectively from the murine AFO61942 in the Xhol and BamHI sites of pECPF-N1.

Cell transfection, drug treatment and immunochemistry

NIH 3T3, COS-7 and HeLa cells from ATCC were grown in DMEM or MEM (Sigma) supplemented with 10% fetal bovine serum (Natocor, Córdoba, Argentina), penicillin and streptomycin (Sigma). Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen). Thapsigargin (used at 250 nM unless otherwise indicated) from DMDO stock, sodium arsenite (used at 0.25 mM unless otherwise indicated), cycloheximide and puromycin (used at 1.0 mg/ml) and rifamycin (used at 10 μM) from stock aqueous solutions were added to conditioned medium. Stress stimuli were pulsed (1 hour) or continuous as indicated.

For immunofluorescence, cells were fixed in 4% formaldehyde, 4% sucrose in PBS at 37°C; permeabilized in 0.1% Triton X-100 in PBS and blocked in 1% Blocking Reagent (Boehringer Mannheim). Primary antibodies were diluted as follows: rabbit polyclonal antibodies against Staun, RLS1 (Thomas et al., 2005), anti-PAB (providing by Evita Mohr, University of Hamburg, Germany), anti-Dep1 and anti-Xrn1 (gift from Jens Lykke Andersen, University of Colorado, CO), 1:500; anti-S6 (Cell Signaling, Beverly, MA), anti-eIF2G1 (Abcam, Cambridge, UK); anti-eIF2γ and anti-phospho-eIF2α (Stressgen, Victoria, British Columbia, Canada) 1:100; goat polyclonal anti-TIA-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:100. Monoclonal antibodies anti-TIA; anti-eIF4E and G3BP (BD Biosciences, San José, CA); anti-Hur and anti-hPABP (Immunostain, Cleveland, UK), 1:100, and anti-V5 (Invitrogen), 1:500. Secondary antibodies were from Molecular Probes or Jackson ImmunoResearch Laboratories (West Grove, PA). Cells were mounted in Mowiol 4-88 (Calbiochem, EMD Biosciences, San Diego, CA).

siRNA treatment

The following siRNA sequences were used: anti-Stau1 (siStau1), 5'-aactgacattgagccgca-3'; anti-Stau2 (siStau2), 5'-aacaagatggagctga-3'; anti-Hef1ds (siHefds, Cat. No. 004397, Dharmacon, Chicago IL); anti-Rckp5 (siRckp54, Cat. No. 0143295, Dharmacon), and non-relevant siRNA (siNR), 5'-tagaccaauaucaauu-3'; siStau1 and siNR carrying the siSTABLE chemical modification (Dharmacon). Cells grown at 50% confluence were treated with 100 nM siRNA and TransIT-TKO (Dharmacon) following manufacturer’s instructions, and analyzed 48 hours later. When simultaneous plasmid transfection was required, the siRNA was incorporated into the lipofectamine-transfection procedure and cells were analyzed 16 hours later.

Confocal microscopy, cell counting, stress granule size and intensity measurements

Images were acquired with a LSM 5 PASCAL confocal microscope (Carl Zeiss, Oberkochen, Germany). Equipment adjustment was assessed by using 1 μM FITC-labeled retinol binding protein microspheres (Molecular Probes). Linear intensity profiles were plotted with the ‘Intensity Profile’ tool. Pictures were exported to Adobe Photoshop software for cropping. Neither filters nor gamma-adjustments were applied. To estimate the expression levels of ECFP-tagged Staun, NIH 3T3 cells were transfected with ECFP or Staun-ECFP, and stained with the RLS1 antibody and a Cy3-labeled secondary antibody. Relative expression levels were determined in single cells where the Staun immunofluorescence intensity was linear with the fluorescence intensity of the ECFP moiety. Stress granules were identified by TIA staining and cells were scored as positive when they had at least three foci of a minimal size of 0.5 μm, because cells with less than five stress granules were infrequent in all tested conditions and granules are usually 0.5-5 μm in size. Cell counting was performed manually with the help of the ‘Cell Counter’ tool of the ImageJ software (NIH) on ×40 or ×100 micrographs. P-values according to Fisher’s exact test were determined using Instat software (GraphPad Software, San Diego, CA). Error bars represent s.e. from at least two replicates. Stress granule size was measured with the ‘Analyze Particles’ tool of the ImageJ software on ×100 confocal micrographs.

Sedimentation velocity centrifugation

Cells were harvested in CSK buffer (Thomas et al., 2005) supplemented with 0.25 mM sucrose, 1% Triton X-100, and protease inhibitor cocktail (Sigma), homogenized and centrifuged for 10 minutes at 5000 r.p.m. to obtain post-nuclear extracts. Phosphatase inhibitors (50 mM orthovanadate, 2.5 mM dicypermethrine, 0.2 mM odaic acid, 1 mM β-glycerophosphate and 1 mM NaF) were included when required. Samples of 0.5 to 1.5 mg protein (determined by the Bicinchoninic Acid Protein Kit assay, Sigma) were loaded onto continuous 13 ml sucrose gradients (linear 20-60% w/v in CSKB) and centrifuged at 220000 g for 2 hours. The polynuclear profile was monitored by absorption at 254 nm. Protein from 0.5 ml fractions was precipitated in 10% trichloroacetic acid, washed twice with cold acetone and analyzed by western blot.

Western blot

Protein from total, post-nuclear cell extracts or gradient fractions were analyzed. Briefly, protein was resuspended in Laemmli sample buffer, separated by SDS-PAGE, and electrotransferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA). Primary antibodies were used as follows: anti-Stau1 RLS1, 1:500; rabbit anti-PABP, anti-S6, anti-eIF2α and anti-phospho-eIF2α, 1:1000, mouse monoclonal anti-TIAR, anti-β actin (Sigma) and anti-β tubulin (Sigma), 1:1000; human anti-P0 (ImmunoVision, Springdale, AR) 11:000; Chemiluminescence was monitored by X-ray film (Immunovision, Springdale, AR) 11:000.
for quantitative measurements, using the ‘volume report’ and the ‘local average’ tools. Alternatively, autodischargings were scanned and signal intensities were compared with ImageJ (NIH) software.

**Protein synthesis assay**

L-aminobenzyl [34C][U] (Perkin Elmer, Boston MA) were added to cells plated in 24-well plates. At the indicated times, supernatant was carefully removed and cells washed once with warm PBS. Cells were lysed in 0.2 ml RIPA buffer containing protease inhibitor cocktail and the trichloroacetic acid (TCA)-insoluble radioactivity was determined in duplicates. Trichloroacetic acid was precipitated at 2 hours between 37°C until the formation of dark formazan crystals. After lysis with acid isopropyl alcohol (0.04 N HCl in isopropanol) absorbance at 570 nm was measured. Linearity of the response was evaluated in each experiment by measuring serial dilutions of all cells. All measurements were done in triplicate wells.

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**MTT assay for cell viability**

Cell viability was evaluated using MTT [3(4,5-cimethylthiazol-2-yl)-2,5-diphenil tetrazolium bromide]. At the end of the indicated treatment, cells were incubated with 0.5 g/ml MTT during 2 hours at 37°C until the formation of dark formazan crystals. After lysis with acid isopropyl alcohol (0.04 N HCl in isopropanol) absorbance at 570 nm was measured. Linearity of the response was evaluated in each experiment by measuring serial dilutions of all cells. All measurements were done in triplicate wells.

**References**


Osteogenesis goes with the flow
There is mounting evidence that the mechanical microenvironment within a tissue can influence stem-cell fate; much remains unknown, however, about how particular mechanical signals are transduced. On page 546, Emily Arnsdorf and colleagues investigate how one such signal – oscillatory fluid flow (which promotes osteogenesis in mature bone) – determines the fate of mesenchymal stem cells. Using C3H10T1/2 progenitor cells as a model, the authors analyse the effect of oscillatory fluid flow on the expression of the transcription factors Runx2, PPARγ and SOX9 (markers of osteogenic, adipogenic and chondrogenic differentiation, respectively). They show that all three proteins are upregulated, as are the GTPase RhoA and its effector ROCKII (which probably enhance tension in the actin cytoskeleton). In addition, chemical activation of RhoA acts additively with flow to upregulate expression of Runx2, whereas disruption of the actin cytoskeleton downregulates flow-induced Runx2 expression. Lastly, they show that – unlike Runx2 – SOX9 and PPARγ are upregulated by actin-cytoskeleton disruption in the absence of flow, and RhoA activation also downregulates PPARγ expression. They conclude, therefore, that oscillatory fluid flow regulates the osteogenic differentiation of progenitor cells via the RhoA-ROCKII pathway.

Drebrin A – actin’ up at synapses
Most of the excitatory synaptic transmissions in the CNS are received by dendritic spines. These specialised neuronal protrusions change shape through reorganisation of the F-actin cytoskeleton – this appears to modulate synaptic transmission, but the mechanism is not well understood. Now, Lotfi Ferhat and colleagues (p. 524) investigate the role of drebrin A (DA), a neuron-specific F-actin-binding protein, in regulating spine morphology and synaptic transmission. Using high-density cultures of mature hippocampal neurons, the authors first show that overexpression of GFP-tagged DA increases the length and density of dendritic spines, and that this is mediated by the actin-binding domain of DA. Notably, overexpression of DA augments transmission at glutamatergic (excitatory) synapses, probably by increasing their density, but does not affect the efficacy of inhibitory (GABAergic) synaptic transmission. By contrast, downregulation of DA causes a decrease in the activity of both GABAergic and glutamatergic synapses. Thus, modification of the actin cytoskeleton by DA might, the authors suggest, alter synaptic transmission. Their data highlight the intimate relationship between form and function in CNS neurons.

SIRT7 gets rDNA transcription going
Over half of the RNA synthesis in the cell takes place during transcription of ribosomal DNA (rDNA) – a nucleolar process that is tightly regulated in response to cell-cycle stage, ageing, starvation and other factors. For instance, rDNA transcription is repressed during mitosis, but we lack a complete picture of how this is controlled. Now, Valentina Sirri and colleagues (p. 489) investigate the mitotic activity of SIRT7, a nucleolar histone deacetylase that has been reported to help regulate rDNA transcription. The authors show that, in contrast to previous reports, SIRT7 associates with nucleolar organizer regions (NORs; the chromosomal sites at which rDNA genes cluster) even when rDNA transcription is repressed, and that this localisation is mediated by a direct interaction between SIRT7 and the rDNA transcription factor UBF. They next show that SIRT7 is phosphorylated by the CDK1-cyclin-B pathway during mitosis, then dephosphorylated before rDNA transcription resumes after mitosis. Moreover, the resumption of transcription requires SIRT7 activity, and the SIRT7 C-terminus becomes more reactive to cognate antibodies before transcription begins. The authors propose, therefore, that dephosphorylation of SIRT7 promotes a change in its conformation, and that this derepresses transcription. Their data underscore the complexity of rDNA transcriptional regulation.

Connexins – taking the scenic route?
Connexins, the transmembrane proteins that make up gap junctions (GJs), form hexameric hemichannels (or connexons) in the Golgi before they are trafficked to the cell membrane. Several mechanistic aspects of connexin trafficking have been well characterised, but their targeting remains controversial – for instance, are connexins targeted exclusively to GJ-rich cell-surface membrane domains, or are they also trafficked to other domains? Using rapid time-lapse imaging of GFP-tagged connexin 43 (Cx43), Dale Laird and colleagues (p. 554) now show that Cx43 is present both in GJ-like clusters and non-GJ membranes (including membrane protrusions and cell surfaces that lack an adjacent cell) on the surface of rat mammary-tumour cells. The authors next use FRAP to analyse lateral mobility of Cx43 within the cell membrane, and show that it is most mobile in non-GJ regions. Within GJ-like clusters, they observe a spectrum of Cx43 mobility, and show that a Cx43 variant that lacks a C-terminus tends to remain more mobile within GJ-like clusters. The authors conclude that Cx43 resides at all cell-surface membrane domains in the cells studied, and propose that Cx43 mobility reflects the assembly state of GJs. Their work contributes to our understanding of the trafficking and assembly dynamics of GJs.

Stressing out with Staufen
In response to stress, mammalian cells halt the translation of mRNA, and instead form stress granules – mRNA-containing cytoplasmic foci that harbour abortive initiation complexes. Stress granules are in dynamic equilibrium with translating polysomes – but how is stress-granule assembly regulated? On page 563, Graciela Boccaccio and colleagues establish a role for Staufen 1 (a polysome-associated RNA-binding protein) in the dissolution of stress granules. Using several cell types, the authors show that Sta1 is recruited to stress granules when cells are subjected to ER stress or oxidative stress. Sta1 is not, however, an essential component of stress granules, which still form in Sta1-depleted cells. The authors note that knocking down Sta1 promotes stress-dependent granule formation, but that this granule formation is impaired when a construct encoding Sta1 is transfected. They show that the N-terminal half of Sta1 is responsible for this inhibitory capacity. Finally, the authors observe that polysomes that remain following stress induction are enriched in Sta1. They propose, therefore, that Sta1 helps to stabilise polysomes, tipping the polysome-stress-granule balance in favour of granule dissolution. These results help to clarify how the cellular stress response can be modulated.

Disease Models & Mechanisms in press
Shedding light on chronic Listeria infection
The pathogenic bacterium Listeria monocytogenes is the third most common cause of bacterial meningitis in neonates, and causes abortion and stillbirth. Additionally, sporadic outbreaks of listeriosis continue to claim lives, particularly in the very young and the elderly. In a paper published in Disease Models & Mechanisms, Christopher Contag and colleagues use in vivo bioluminescent imaging to visualize L. monocytogenes in mice. The authors show that the live form of the bacterium, as well as attenuated forms that have defective intracellular replication, reside in the bone marrow and persist for weeks following acute infection. The results draw attention to the bone marrow as a site of residual Listeria infection, both during and after treatment. Additionally, this study demonstrates that growth mechanisms that enable Listeria to colonise the bone marrow still function in attenuated Listeria strains; this is important because these attenuated strains are currently being tested for their ability to induce anti-tumour immune responses in cancer patients.