Large G3BP-induced granules trigger eIF2α phosphorylation

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ABSTRACT Stress granules are large messenger ribonucleoprotein (mRNP) aggregates composed of translation initiation factors and mRNAs that appear when the cell encounters various stressors. Current dogma indicates that stress granules function as inert storage depots for translationally silenced mRNPs until the cell signals for renewed translation and stress granule disassembly. We used RasGAP SH3-binding protein (G3BP) overexpression to induce stress granules and study their assembly process and signaling to the translation apparatus. We found that assembly of large G3BP-induced stress granules, but not small granules, precedes phosphorylation of eIF2α. Using mouse embryonic fibroblasts depleted for individual eukaryotic initiation factor 2α (eIF2α) kinases, we identified protein kinase R as the principal kinase that mediates eIF2α phosphorylation by large G3BP-induced granules. These data indicate that increasing stress granule size is associated with a threshold or switch that must be triggered in order for eIF2α phosphorylation and subsequent translational repression to occur. Furthermore, these data suggest that stress granules are active in signaling to the translational machinery and may be important regulators of the innate immune response.

INTRODUCTION

In living cells messenger ribonucleoprotein (mRNP) complexes dynamically shuttle between actively translating polysomes and translationally silenced compartments, where they accumulate in stress granules and processing bodies, the latter being where RNA decay may occur (Rzeczkowski et al., 2011). Stress granules (SGs) are large mRNP aggregates that contain stalled translation initiation complexes and are formed when the cell encounters several types of stress. The stalled translation initiation complexes that concentrate in stress granules include many translation initiation factors (eukaryotic initiation factors [eIFs]), polyadenylated mRNAs, the 40S ribosomal subunit, and RNA-binding proteins, whereas the 60S ribosomal subunit is excluded (Anderson and Kedersha, 2002; Kedersha et al., 2002). Inhibition of translation at the initiation phase before ribosome subunit joining is well documented to drive the formation of stress granules. This observation is supported by translational inhibition with pateamine A, 4GE1, and edeine, which all induce SG formation, whereas knockdown of some eIFs, inhibition of ribosomal subunit joining, and even inhibition of the elongation phase of protein synthesis do not cause SG assembly (Thomas et al., 2005; Dang et al., 2006; Mokas et al., 2009). Of interest, overexpression of several RNA-binding proteins, including Tia1, CPEB1, cold-inducible RNA-binding protein, and RasGAP SH3-binding protein (G3BP), all induce SG formation (Tourriere et al., 2003; Gilks et al., 2004; Wilczynska et al., 2005; De Leeuw et al., 2007).

The heterotrimeric eIF2 (α, β, and γ subunits) functions in a ternary complex containing initiator methionyl-tRNA and GTP. Eukaryotic initiation factor 2 is responsible for delivery of initiator methionyl-tRNA to the ribosome in a GTP-dependent manner. Release of eIF2 from the translation initiation complex requires GTP hydrolysis, and the guanine nucleotide exchange factor eIF2B is responsible for recycling of GDP for GTP in eIF2 before subsequent rounds of translation.
initiation (Merrick, 2004). Many stresses that induce SG assembly cause translational repression by stimulating kinases that phosphorylate serine 51 of eIF2α (Kedersha et al., 1999; McEwen et al., 2005). This event blocks the nucleotide exchange cycle by causing stable association of eIF2 and eIF2B, thereby sequestering the limiting eIF2B (Dever et al., 1995). Disruption of the eIF2 nucleotide exchange cycle prevents delivery of initiator methionyl-tRNA to the ribosome, causing accumulation of initiation complexes lacking the ternary complex. There are four well-known eIF2α kinases that can respond to various stresses and repress translation: protein kinase R (PKR), which senses double-stranded RNA; protein kinase RNA-like endoplasmic reticulum kinase (PERK), which senses endoplasmic reticulum stress; heme-regulated inhibitor kinase (HRI), which senses oxidative stress and heme deficiency; and general control nonderepressible 2 (GCN2), which senses nutrient availability.

During the course of our studies, we found that G3BP overexpression induces stress granules in a dose-dependent manner. Using microscopic techniques, we analyzed individual cells and discovered that translational repression and eIF2α phosphorylation did not generally appear until large G3BP-induced stress granules were formed. Furthermore, we found that PKR was responsible for the induction of eIF2α phosphorylation by G3BP-induced stress granules. These data change the view of stress granules as inert depots for translationally silenced mRNPs to structures that may promote cellular signaling to the translational machinery for translational repression.

RESULTS
G3BP overexpression induces stress granules in a dose-dependent manner
Several proteins, including G3BP, have been shown to induce stress granule formation during conditions of overexpression (Tourriere et al., 2003; Gilks et al., 2004; Hua and Zhou, 2004; Kedersha et al., 2005). During our overexpression studies of G3BP we noted that many cells contained no stress granules despite significant G3BP expression, some cells contained small G3BP-induced stress granules, and others contained large G3BP granules. To understand the underlying causes for the differences in stress granule appearance and size, we titrated G3BP-green fluorescent protein (GFP-λN) expression plasmid into HeLa cells to investigate whether G3BP-induced granules are concentration dependent. We found that higher concentrations of G3BP could generally induce larger stress granules, as indicated by colocalization with another stress granule marker protein, Tia1 (Figure 1A, white arrows). This observation is due to the cellular function of G3BP and not a result of high concentrations of nucleic acids from the transfection procedure, because equivalent amounts of GFP-λN expression plasmid did not induce Tia1-positive stress granules (Figure 1B). Quantification of stress

FIGURE 1: G3BP granules are induced in a dose-dependent manner in HeLa cells. HeLa cells were transfected with the indicated amounts of either G3BP-GFP-λN (A) or GFP-λN (B) plasmid and stained for Tia1 as indicated in Materials and Methods. (C) Between 125 and 150 transfected cells were manually counted and scored for Tia1-positive stress granules and are presented as percentage transfected cells with stress granules. Error bars represent SD from three independent counts. (D) Immunoblots showing levels of GFP-G3BP-λN or GFP-λN transgene relative to endogenous G3BP and eIF2α levels.
G3BP overexpression induces eIF2α phosphorylation and translational inhibition

Because G3BP-induced SGs resemble canonical stress granules and phosphorylation of eIF2α is known to induce SG formation (Kedersha et al., 1999, 2000, 2002), we investigated whether cells containing G3BP-induced granules exhibit eIF2α phosphorylation. Deconvolution microscopy revealed that large G3BP-induced SGs did not undergo significant changes in size or shape upon arsenite treatment (unpublished data). To investigate whether other RNA-binding proteins and components of SGs could also induce eIF2α phosphorylation, we examined cells expressing PABP and eIF4G. In both cases, significant eIF2α phosphorylation was not present in overexpressing cells (Figure 5B). These data support results from GFP-λN expression indicating that transfection alone is not sufficient to induce SG assembly and support the specificity of G3BP in induction of SGs and eIF2α phosphorylation.

Because we observed different sizes of granules in cells overexpressing G3BP, we investigated whether granule size had any effect on the efficiency of G3BP-induced granule formation. A range of G3BP-GFP-λN concentrations were transfected into cells, and granule formation was evaluated by deconvolution microscopy. At the highest doses, many cells expressing G3BP do not contain granules, indicating that some cells are resistant to G3BP-induced granule formation. Analysis of levels of G3BP-GFP-λN by Western blotting followed by densitometric analysis indicates that approximately a threefold increase in G3BP over endogenous levels is sufficient to trigger stress granule formation.

By definition stress granules contain stalled translation initiation complexes comprising several translation initiation factors, mRNA, and the small, but not large, ribosome subunit. Because recent data suggest that the composition of stress granules may differ in a stress-dependent manner (Piotrowska et al., 2010; Buchan et al., 2011), we performed a comprehensive analysis of the components of G3BP-induced granules. Previous data indicated that eIF4G, poly(A)-binding protein (PABP), and eIF3b are all recruited to G3BP-induced stress granules (Kedersha et al., 2005). We confirmed localization of eIF4G and PABP, whereas eIF3b and Tia1 both colocalized with G3BP-induced SGs in HeLa cells as predicted (Figure 2). We were also able to detect the small ribosomal subunit marked by ribosome protein S6 (rps6) in G3BP-induced granules, as expected (Kedersha et al., 2002). The large ribosomal subunit was not efficiently recruited to G3BP-induced stress granules, as indicated by immunostaining for the 28S protein rpl36A (Figure 3). Fluorescence in situ hybridization analysis demonstrated that 5.8S rRNA, which strongly interacts with the 60S ribosomal subunit, and the 28S rRNA component of the large ribosomal subunit were also excluded from G3BP-induced granules (Figure 3, white arrows). Finally, the c-myc and β-actin mRNAs also localized to G3BP-induced granules (Figure 4). The specificity of these antibodies and RNA probes can be validated by consistent, diffuse staining in untransfected cells in the same fields as those with G3BP-induced SGs (Figures 2–4). These results extend previous data by other groups to show inclusion of additional markers of canonical stress granules in G3BP-induced granules (Kedersha et al., 2002; Tourriere et al., 2003).

From these data we conclude that G3BP-induced granules resemble canonical stress granules by all of the functional markers examined.

G3BP overexpression induces eIF2α phosphorylation and translational inhibition

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correlation with eIF2α phosphorylation by quantifying the intensity of eIF2α phosphorylation at the single-cell level. Strikingly, we found that levels of eIF2α phosphorylation were low or not apparent in many cells with smaller G3BP-induced SGs, similar to transfected cells lacking granules and untransfected cells (Figure 5, A, cell marked S, and C). Robust phosphorylation was not observed until stress granules reached a certain size, suggesting that a cellular switch or threshold regulates eIF2α phosphorylation. During arsenite stress, eIF2α phosphorylation precedes assembly of even small granules, suggesting that this mechanism is dependent on the stress signaling and SG assembly (unpublished data). Immuno-blot analysis of G3BP-transfected cells indicated that increased levels of eIF2α phosphorylation were evident at the population level compared with cells expressing GFP, though eIF2α phosphorylation was not as high as for cells treated with arsenite (Figure 5D).

This was consistent with our finding that only a maximum of 60% of cells expressing GFP-G3BP-λN form granules (Figure 1C).

Although eIF2α phosphorylation is generally associated with repressed translation, we sought to confirm that levels of eIF2α phosphorylation in cells with large G3BP-induced SGs correlate with translational repression in individual HeLa cells. Therefore we monitored translation using a short pulse of puromycin, which can be incorporated into nascent polypeptides and detected by immunofluorescence with an antibody directed against puromycin (ribopurinymicilation assay [RPA]; Schmidt et al., 2009; David et al., 2011). We found that cells containing large G3BP-induced SGs pronounced strident translational repression, whereas those with smaller granules had ongoing translation similar to cells lacking granules or those that were untransfected (Figure 6). Levels of translation in cells with large G3BP-induced SGs (Figure 6, cells marked with L)
G3BP stress granules can be induced in mouse embryonic fibroblasts containing mutant eIF2α.

Our data indicate that for at least G3BP-induced granules, eIF2α phosphorylation and translational repression could precede assembly of large stress granules. Therefore we reasoned that G3BP-induced SGs could be observed in cells without translational repression. To test this hypothesis, we expressed G3BP-GFP-αN in mouse embryonic fibroblasts (MEFs) expressing either serine 51 wild-type (S51) or a serine 51-to-alanine (S51A) nonphosphorylatable mutant of eIF2α. We confirmed that phosphorylation of eIF2α is indeed blocked in response to arsenite with the mutant MEFs by Western blotting (see Figure 9C later in the paper). With this system we were able to induce SGs containing Tiα1 and G3BP in both S51 and the nonphosphorylatable S51A mutant MEFs without arsenite stress (Figure 7, white arrows). This demonstrates that G3BP can induce SG formation independently of eIF2α phosphorylation. Arsenite induces translation shutoff and SG formation by activation of the eIF2α kinase HRI (McEwen et al., 2005). Thus, as expected, arsenite-induced granules were readily observed in the wild-type S51 MEFs but were absent in mutant S51A MEFs (Figure 7). In this case, only cells with G3BP-GFP-αN expression contained stress granules (Figure 7, yellow arrows). Immunofluorescence microscopy analysis indicated that SGs in S51A MEFs also contained elf3a and elf4G, similar to the G3BP-induced granules observed in HeLa cells (unpublished data).

To determine whether the G3BP-induced granules present in S51A MEFs were capable of translational repression through another pathway independent of eIF2α phosphorylation, we used RPA to measure translation in both the wild-type eIF2α S51 and mutant S51A MEFs. This analysis indicated that translation was blocked in S51A MEFs containing G3BP-induced granules as expected (Figure 8, white arrow) but, surprisingly, proceeded at normal levels in the S51A MEFs despite the presence of G3BP-induced SGs (Figure 8, yellow arrow). In fact, levels of translation were comparable to those for untransfected controls in the same fields as cells with G3BP-induced SGs. These data indicate that, indeed, in this case G3BP-induced SG assembly precedes translational repression since we can eliminate the later step of translational repression without significantly affecting assembly of these granules.

PKR mediates induction of eIF2α phosphorylation by G3BP-induced SGs.

In an effort to elucidate which eIF2α kinase mediates translational repression by G3BP-induced SGs, we used a panel of kinase-knockout MEFs. Using these MEFs, we could identify which kinase was capable of supporting G3BP-induced SG assembly without eIF2α phosphorylation at the single-cell level with fluorescence microscopy. As expected, we were able to induce eIF2α phosphorylation in wild-type MEFs in a dose-dependent manner as observed earlier in HeLa cells (unpublished data). Every MEF genotype was capable of forming G3BP-induced SGs in response to high concentrations of G3BP expression plasmid. Of interest, however, the only genotype lacking significant consequent eIF2α phosphorylation when SGs formed was the PKR-knockout cells (Figure 9A). Levels of eIF2α phosphorylation in PKR-knockout cells with granules resembled those observed in untransfected cells, indicating that PKR is the principal kinase that mediates eIF2α phosphorylation after G3BP-induced SGs assemble. Quantification of eIF2α phosphorylation in cells with G3BP-induced SGs indicated that the fold change in cells without G3BP-induced SGs versus cells with SGs was significant.
between all the other MEF genotypes. This suggests that minimal cross-talk occurs between eIF2α kinases in response to G3BP-induced SGs (Figure 9B). A possible minor role for PERK cannot be excluded since phosphorylation levels did not increase as robustly in response to G3BP-induced stress granule formation. The increased levels of eIF2α phosphorylation were statistically significant for all cell types examined, with the exception of PKR-knockout MEFs, in which G3BP SG-containing cells had lower levels of eIF2α phosphorylation than those cells without granules. These data suggest that PKR is the most prominent kinase in the signaling events responsible for translational repression after assembly of G3BP-induced granules. We were unable to distinguish between small and large granules in MEFs because MEF SGs are reproducibly smaller, and therefore only groups with and without G3BP-induced granules are represented (Figure 9B). We repeatedly observed cell line–specific variation in stress granule size, and so the inability to distinguish between small and large granules was not surprising (unpublished data). Because quantification of eIF2α phosphorylation in MEFs is dependent on the specificity of the antibody, we performed a Western blot with S51 and S51A MEFs during arsenite treatment and confirmed that the antibody is specific (Figure 9C). Under these conditions, eIF2α phosphorylation is strongly induced in only the S51 MEFs, whereas no signal was observed in S51A mutant MEFs. Here again, the specificity of the assay is indicated by the lack of puromycin/translation signal in arsenite-treated controls, where translation is abrogated. The results with PKR-knockout cells resemble those observed with eIF2α S51A mutant MEFs, in which G3BP-induced SGs were present without

**FIGURE 5:** Large G3BP-induced stress granules induce eIF2α phosphorylation. HeLa cells were transfected with G3BP-GFP-λN (A, C), GFP-λN (A), or PABP or eIF4G (B) and stained with antibodies that detect eIF2α phosphorylation before deconvolution imaging. Arsenite stress was applied as described in Materials and Methods. Cells with large or small granules are indicated with a yellow L or S, respectively. Color channels are switched for eIF4G staining because of antibody availability. (C) Intensities of eIF2α phosphorylation in G3BP-expressing HeLa cells were quantified with Pipeline Pilot analysis tools, and a Student’s t test was conducted. Untransfected and transfected cells (− or +) and stress granule size groups (−, no granules; +, small granules; ++, large granules) are indicated. The y-axis represents (phosphorylated eIF2α intensity [arbitrary units]/cell area × 1000). ***p ≤ 0.001. The minimum threshold size for large granules was ∼1.4 μM². (D) Immunoblots for eIF2α or phosphorylated eIF2α in cells expressing indicated transgenes. Cells were also treated with arsenite as indicated.
Our data indicate that G3BP-induced SGs can signal to the translation apparatus by stimulating eIF2α phosphorylation in a PKR-dependent manner (Figure 10B). This model explains how granule formation can be observed in both the PKR-knockout and S51A mutant MEFs that do not have induced eIF2α phosphorylation. Although we only have evidence that phosphorylated eIF2α is present at the same time as large SGs, we predict PKR activation and eIF2α phosphorylation as a consequence of large SG assembly because eIF2α phosphorylation is not observed with small SGs (Figure 10B). We hypothesize that the PKR activation and eIF2α phosphorylation may be involved in maintenance of SGs during other stresses after an initial phase of eIF2α phosphorylation by other kinases (e.g., HRI activation during arsenite stress).

Some points are not resolved within this model for induction of eIF2α phosphorylation (Figure 10B). For example, it is not known what is being sensed in the cell that triggers the coalescence of small granules into large granules. We conjecture that the depletion of initiation factors or RNA-binding proteins into small granules is sensed because it is unlikely that substantial protein synthesis can occur in the absence of accessible translation initiation factors. Sensing of small granules may also depend on localization of sufficient carrier proteins that allows high-affinity interaction between the small granule and molecular motors (Loschi et al., 2009). This would then permit coalescence of the small granule and initiation of subsequent signaling. Another point that is unresolved is how PKR is

![FIGURE 6: RPA analysis of cells containing G3BP-induced stress granules. G3BP-GFP-λN or GFP-λN plasmids were transfected into HeLa cells, and RPA analysis was conducted as indicated in Materials and Methods. Translation is represented by α-puro. For each transfected plasmid, cells were either treated without (no Ars) or with (+Ars) to control for antibody specificity. Cells with large or small granules are indicated with a yellow L or S, respectively. Cells were imaged with a deconvolution microscope.](image-url)
activated after large-granule assembly. We hypothesize that compaction of RNA in a granule that is sensed by PKR could result in eIF2α phosphorylation. Alternatively, derepression of PACT/Rax may occur, which is the only cellular activator of PKR, which in turn may phosphorylate PKR in the absence of double-stranded RNA (Patel and Sen, 1998; Ito et al., 1999; Garcia et al., 2006). Finally, stress granules could activate a signaling molecule upstream of PKR by such as MyD88 or IRAK1 (i.e., toll-like receptor 3, 4, or 9 signaling; Garcia et al., 2006).

Our finding that PKR is responsible for eIF2α phosphorylation is supported by earlier stress granule work in which Tia1 was overexpressed (Kedersha et al., 1999). Kedersha and colleagues showed that expression of either the nonphosphorylatable S51A mutant of eIF2α or the adenoviral VAI gene, which inhibits PKR activity, was sufficient to reduce induction of SGs by Tia1 overexpression. However, they concluded that overexpression of transgenes introduces so much exogenous RNA that PKR is activated and eIF2α is phosphorylated, resulting in SGs (Kedersha et al., 1999). Our data provide new insight that forces us to reexamine these conclusions. Specifically, expression of G3BP in MEFs expressing the nonphosphorylatable S51A mutant as the sole source of eIF2α still induced stress granules. Furthermore, expression of GFP-1N, PABP, and eIF4G do not induce stress granules despite similar expression from the cytomegalovirus (CMV) transcription promoter.

The observation that with high transgene expression eIF2α phosphorylation was only observed with large granules also argues against activation of PKR by exogenous RNA. Finally, we tested many deletion mutants of G3BP that do not induce eIF2α phosphorylation when expressed from a CMV promoter, indicating that a specific sequence in the exogenous RNA is not responsible for PKR activation and resulting SGs (unpublished data).

Based on the prominent role of SGs and PKR in antiviral innate immunity, the finding that PKR mediates eIF2α phosphorylation coinciding with large G3BP-induced SG assembly makes sense. Many viruses target SGs for disassembly and encode proteins that are known inhibitors of PKR activity (Garcia et al., 2006; White and Lloyd, 2012). Of interest, PKR participates in several toll-like receptor signaling pathways, including toll-like receptors 3, 4, and 9 (Horng et al., 2001; Ji et al., 2003). PKR activity also regulates NF-κB transcriptional activity (Kumar et al., 1997; Gil et al., 2001) and can induce c-Jun N-terminal kinase (JNK) activity (Goh et al., 2000; Taghavi and Samuel, 2012). Therefore it is reasonable that these proteins may be activated by assembly of large G3BP-induced granules.

JNK signaling has recently become a focal point in mRNP granule biology. Arimoto et al. (2008) documented that Rack1, an activator of JNK signaling and subsequently apoptosis, is sequestered in arsenite-induced SGs, thereby preventing JNK activity.

Rack1 is also sequestered in G3BP-induced stress granules, and G3BP inhibits activation of MTK1, an upstream kinase important for JNK activation. Active JNK has also been shown to be recruited to arsenite and heat-induced SGs along with a scaffolding molecule WDR62 (Wasserman et al., 2010). WDR62 and active JNK recruitment was not observed in G3BP-induced granules, whereas both Tia1 and TTP-induced granules colocalize with active JNK. It will be interesting for future work to examine Rack1, WDR62, and JNK activation in cells with small versus large G3BP-induced granules to determine whether differing localization and intensity exists. Phosphorylation of the decapping regulator Dcp1a by JNK regulates NF-κB and toll-like receptors 3, 4, and 9 (Horng et al., 2000; Taghavi and Samuel, 2012). Therefore it is reasonable that these proteins may be activated by assembly of large G3BP-induced granules.
**MATERIALS AND METHODS**

**Cell culture, transfections, and stress treatment**

HeLa Tet-On cells were maintained in 10% fetal bovine serum (FBS)/DMEM according to standard procedures. For all microscopy experiments, the cells were plated on glass coverslips at (1–1.3) × 10^5 cells per well in 12-well plates and grown overnight. Before transfections, medium was replaced with 2% FBS/DMEM and maintained throughout the experiment under those conditions. Transfections were conducted with FuGENE HD (Promega, Madison, WI) and 200 ng of plasmid per well for all experiments except DNA titration experiments. Eukaryotic initiation factor 2α, S/S and A/A homozygous mutants, and PKR−/− and HRI−/− MEFs were kind gifts from Randal Kaufman (Stanford Burnham Medical Research Institute, La Jolla, CA), Mauro-Costa Mattioli (Baylor College of Medicine, Houston, TX), and Scot Kimball (Pennsylvania State University, University Park, PA), respectively. GCN2−/−, PERK−/−, and corresponding wild-type control MEFs were originally developed in the laboratory of David Ron (University of Cambridge, Cambridge, United Kingdom) and were obtained from the American Type Culture Collection (Manassas, VA). Mouse embryonic fibroblasts were maintained under the same conditions as HeLa Tet-On cells. Before transfections with FuGENE HD, MEFs were plated at 5 × 10^4 cells per well of a 12-well plate and grown overnight for transfection the following day. A 2-μg amount of plasmid per well was transfected due to the low transfection efficiency in MEFs. One microgram each of pSR-myc-PABP (Zheng et al., 2008) and pSport6-eIF4G-HA plasmids was transfected into each well of a 12-well plate and immunostained as detailed later. Oxidative stress treatments on cells involved adding arsenite to a final concentration of 200 μM for 30 min before fixation.

**Plasmid construction**

pG3BP-GFP-λN was generated by subcloning the λN coding sequence from pCI-λN-V5 (Ivanov et al., 2008) into the NotI and XbaI sites of pG3BP-EGFP (White et al., 2007). pGFP-λN was constructed by digesting pG3BP-GFP-λN with EcoRI and BamHI and using Klenow polymerase to generate blunt-ended DNA fragments. These fragments were subsequently ligated, and DNA preparations were produced with standard procedures.

**In situ hybridization**

In situ hybridization was performed as described previously (White and Lloyd, 2011). Briefly, 5′ biotinylated DNA probes were used to detect 5.8S rRNA, 28S rRNA, β-globin, and c-myc RNA. Probe sequences are as follows: 5.8S rRNA, 5′-ggaaccgaggccgaaagt cgttc-3′; 28S rRNA, 5′-cggccgtgcccc-3′; β-globin, 5′-tatcctctcatt-3′; and c-myc, 5′-ttctcactct-3′.
Antibody was incubated with coverslips at a concentration of 1:400–1:500 at room temperature for 2 h. Subsequently, cells were stained in accordance with the procedures outlined in the next subsection.

Immunofluorescence staining
Preparation of coverslips, mounting on slides, and incubation with antibodies against PABP, eIF4G, eIF3A, and Tia1 were performed as previously described (White et al., 2007; White and Lloyd, 2011). Anti–phospho-eIF2α antibody (#3597; Cell Signaling Technology, Beverly, MA) was used at 1:200–1:400 dilution on coverslips. Detection of Myc-PABP was performed using anti-Myc antibody (Cell Signaling Technology) at 1:500. eIF4G-HA was detected with anti-hemagglutinin antibody (Roche, Indianapolis, IN) at 1:1000. All secondary antibodies (Molecular Probes) were used at 1:1000 dilution. Microscopy for figure preparation was performed on an Applied Precision DeltaVision deconvolution image restoration microscope (Applied Precision, Issaquah, WA), and images for quantification were captured on a Nikon TE-2000 (Nikon, Melville).

Ribopuromycilation assay
Cells were grown as described, and RPA was conducted essentially as described previously (David et al., 2011) using the 12D10 antibody directed against puromycin (Schmidt et al., 2009). Briefly, cells were pulsed with 50 μg/ml puromycin and 100 μg/ml cycloheximide for 5 min at 37°C before washing with permeabilization buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 25 mM KCl, 100 μg/ml cycloheximide, 10 U/ml RNase Inhibitor [NEB New England BioLabs, Ipswich, MA], and 1x protease inhibitor [ThermoFisher Scientific, Waltham, MA]) on ice and fixation with 4% formaldehyde at room temperature. The anti-puromycin antibody was incubated with coverslips at a concentration of 1:400–1:500 at room temperature for 2 h. Subsequently, cells were stained in accordance with the procedures outlined in the next subsection.

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granules, 4–8% for small granules, and >8% for large granules. The minimum threshold size for large granules corresponded to $\sim 1.4 \mu M^2$.

For MEF quantification experiments, an automated pipeline was generated with Pipeline Pilot, version 8.5 (Accelrys, San Diego, CA), to detect cell boundaries for each cell in each field (>20 fields per genotype per condition). The intensity of the red (phospho-eIF2$\alpha$ channel) was quantified and normalized to the cell area to produce $P-2\alpha/cell \ area$ for each genotype with and without arsenite. The $P-2\alpha/cell \ area$ value without arsenite was then normalized against the $P-2\alpha/cell \ area$ value with arsenite. Cells were then manually sorted based on the presence or absence of granules and examined for quality control of Pipeline Pilot output.

For statistical analysis, two-tailed, equivariant Student’s t tests were used to compare each group.

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