Cytoplasmic hGle1A regulates stress granules by modulation of translation

Aditi, Andrew W. Folkmann, and Susan R. Wente
Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232

ABSTRACT
When eukaryotic cells respond to stress, gene expression pathways change to selectively export and translate subsets of mRNAs. Translationaly repressed mRNAs accumulate in cytoplasmic foci known as stress granules (SGs). SGs are in dynamic equilibrium with the translational machinery, but mechanisms controlling this are unclear. Gle1 is required for DEAD-box protein function during mRNA export and translation. We document that human Gle1 (hGle1) is a critical regulator of translation during stress. hGle1 is recruited to SGs, and hGLE1 small interfering RNA–mediated knockdown perturbs SG assembly, resulting in increased numbers of smaller SGs. The rate of SG disassembly is also delayed. Furthermore, SG hGLE1-depletion defects correlate with translation perturbations, and the hGle1 role in SGs is independent of mRNA export. Interestingly, we observe isoform-specific roles for hGle1 in which SG function requires hGle1A, whereas mRNA export requires hGle1B. We find that the SG defects in hGle1-depleted cells are rescued by puromycin or DDX3 expression. Together with recent links of hGLE1 mutations in amyotrophic lateral sclerosis patients, these results uncover a paradigm for hGle1A modulating the balance between translation and SGs during stress and disease.

INTRODUCTION
Eukaryotic cells modulate gene expression to mount optimal stress responses and ensure cell survival (Lopez-Maury et al., 2008; de Nadal et al., 2011). Translation is one such mechanism that is rapidly and reversibly regulated during stress to inhibit global protein synthesis and selectively translate mRNAs necessary for adaptation and damage repair (Kedersha and Anderson, 2009; Spriggs et al., 2010). The global decrease in protein synthesis is mediated by phosphorylation of the eukaryotic initiation factor 2 alpha subunit (eIF2α), which results in stalled translation initiation (Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2009; Spriggs et al., 2010). Moreover, translationally stalled, mRNA–protein complexes (mRNP)s are redirected to non membrane-bound reversible aggregates in cytoplasmic foci known as stress granules (SGs; Buchan and Parker, 2009; Kedersha and Anderson, 2009; Thomas et al., 2011). Current working models view SGs as sites for sequestering stalled mRNP{s} from active translation machinery, with specific mechanisms controlling storage, decay, or reinitiation of translation (Kedersha and Anderson, 2002; Kedersha et al., 2005; Buchan and Parker, 2009; Thomas et al., 2011). Whether common factors modulate SG dynamics and active translation is not fully resolved.

Several pieces of evidence support the hypothesis that mRNP{s} in SGs are in dynamic equilibrium with active translation complexes. Treatment of cells with drugs that immobilize polysomes (such as emetine and cycloheximide) prevents SG assembly. In contrast, puromycin-induced disassembly of polysomes promotes SG assembly (Kedersha et al., 2000). A recent study also showed that hepatitis C virus–infected cells oscillate between active and repressed phases of translation, defined by the absence and presence of SGs, respectively (Ruggieri et al., 2012). Aberrant SG formation is linked with various neurological diseases, including amyotrophic lateral sclerosis (ALS) (Bosco et al., 2010; Dewey et al., 2011; Wolozin, 2012; Vanderbiltweyde et al., 2013). In addition, diverse viruses hijack SG machinery to effectively bias active translation for their own protein...
synthesis (Bosco et al., 2010; Ariumi et al., 2011; Khaperskyy et al., 2012; Lloyd, 2012, 2013). Further work is required to better understand how interplay between translation and SG dynamics is regulated at the molecular level.

Importantly, the molecular composition of SGs is variable and dependent upon the type of stress (Kedersha et al., 1999; Cuesta et al., 2000; Stoecklin et al., 2004; Balagopal and Parker, 2009). Prototypical SG constituents include poly(A)+ mRNA, 40S ribosomal subunits, translation initiation complexes, RNA-binding proteins, RNA-dependent ATPases known as DEAD-box proteins (Dbps), motor proteins, and cell-signaling molecules (Anderson and Kedersha, 2008; Buchan and Parker, 2009; Kedersha and Anderson, 2009). mRNA-protein rearrangements are likely required for changes in mRNPs necessary for SG association versus active translation, and such mRNP remodeling is potentially facilitated by Dbp5. However, the molecular mechanisms of how Dbps are regulated to modulate SG exchange of mRNPs requires further investigation.

Gle1 is a conserved essential protein required for regulation of Dbp function during both mRNA export and translation. Originally identified in the budding yeast Saccharomyces cerevisiae (y; Murphy and Wente, 1996), yGle1 functions in mRNA export in association with inositol hexakisphosphate to stimulate the ATPase activity of Dbp5 for mRNP remodeling that confers export directionality at the nuclear pore complex (NPC; Alcazar-Roman et al., 2006; Weirich et al., 2006; Tran et al., 2007; Montpetit et al., 2011). In the cytoplasm, yGle1 interacts with translation initiation factor elf3 and modulates the function of a different Dbp, Ded1 (for which the human orthologue is DDX3; Yedavalli et al., 2004; Bolger et al., 2008; Bolger and Wente, 2011). In contrast, during translation termination, yGle1-inositol hexakisphosphate interacts with Sup45 (eRF1) to again regulate Dbp5 (Bolger et al., 2008; Bolger and Wente, 2011). Given Gle1’s regulation of multiple Dbps at distinct steps in the gene expression pathway, we speculated that its function might be linked to SG dynamics.

In human (h) cells, the GLE1 gene is alternatively spliced to generate at least two protein isoforms—hGle1A and hGle1B (Kendirgi et al., 2003). The hGle1A and hGle1B isoforms shuttle between the nucleus and cytoplasm and differ by only a 39-amino-acid carboxy(C)-terminal extension in hGle1B. These additional residues contain the binding domain for the NPC protein (nucleoporin, Nup) hCG1 (Kendirgi et al., 2003, 2005), and at steady state, hGle1B localizes predominantly at the NPC (Kendirgi et al., 2003, 2005; Rayala et al., 2004; Folkmann et al., 2013). We showed that hGle1B is necessary and sufficient for mRNA export in human tissue culture cells (Folkmann et al., 2013). In contrast, hGle1A is mainly localized in the cytoplasm (Kendirgi et al., 2003), and discrete cellular functions for hGle1A have not been directly tested.

In this paper, we define a specific role for hGle1A during SG formation and translation regulation during environmental stress responses. hGle1A localizes to SGs, and hGle1 depletion causes SG-assembly and SG-disassembly defects. We also find that SG defects in hGle1-depleted cells strongly correlate with deregulation of translation and are only rescued by expression of hGle1A. Additionally, hGle1 interacts with DDX3, and DDX3 is linked to the hGle1 role in SG dynamics. We propose that hGle1A acts to modulate the distribution of mRNPs between active translation and repressed translation in SGs through regulation of DDX3. We also recently reported unexpected links between hGLE1 mutations and ALS (Kaneb et al., 2014), wherein ALS-linked hGLE1 mutations alter the cellular pools of hGle1A and hGle1B. Thus defining critical hGle1A functions in modulating the balance between active translation and SGs reveals connections between pathophysiology and cellular stress responses.

RESULTS

hGle1A and hGle1B isoforms are differentially required during mRNA export

To follow up on our previous study showing hGle1B rescues mRNA export defects in hGle1-depleted cells (Folkmann et al., 2013), we investigated whether hGle1A is sufficient using our established small interfering RNA (siRNA)-mediated knockdown add-back system in HeLa cells. Immunoblotting confirmed the hGle1 depletion and respective expression of enhanced green fluorescent protein (EGFP)-hGle1A or EGFP-hGle1B (Figure 1A). For an assay for mRNA export, the nuclear versus cytoplasmic (N/C) distribution of bulk poly(A)+ mRNA was detected and quantified by oligo-dT in situ hybridization. Consistent with our published report (Folkmann et al., 2013), poly(A)+ RNA accumulated in the nucleus of hGle1A siRNA-treated cells (Figure 1, B and C). Expression of EGFP alone, EGFP-tagged siRNA-resistant (R) hGle1A (EGFP-hGle1A(R)), or EGFP-tagged siRNA-resistant hGle1B (EGFP-hGle1B(R)) plasmids in scrambled control (CTRL) siRNA cells showed a widespread distribution of poly(A)+ RNA in the nucleus and cytoplasm with similar mean N/C ratios of ~0.8 (Figure 1, B and C). As reported (Folkmann et al., 2013), expression of EGFP-hGle1B(R) in hGle1 siRNA-treated cells rescued the nuclear poly(A)+ RNA accumulation (mean N/C ratios ~1.0). In contrast, neither EGFP-hGle1A(R) (mean N/C ratios ~1.25) nor EGFP alone (mean N/C ratios ~1.25) were sufficient to rescue the nuclear poly(A)+ RNA accumulation in hGle1A siRNA cells (Figure 1, B and C).

The steady-state enrichment of hGle1 at the NPC requires hGle1 self-association and interaction with both hNup155 and hCG1 (Kendirgi et al., 2003, 2005; Rayala et al., 2004; Folkmann et al., 2013). We speculated that hGle1A might not rescue mRNA export defects because it lacks the hCG1-binding region and is not recruited to the NPCs (Kendirgi et al., 2003, 2005). For testing this hypothesis, the localization of EGFP-hGle1A(R) and EGFP-hGle1B(R) in CTRL and hGle1A siRNA-treated cells was monitored using three-dimensional (3D) structural illumination microscopy in living cells coexpressing the NPC protein Pom121-mCherry. Interestingly, in CTRL siRNA cells, EGFP-hGle1B localized at the cytoplasmic face of the nuclear envelope, whereas EGFP-hGle1A was not enriched at the nuclear envelope (Figure 1D). However, in hGle1A siRNA cells, both the EGFP-hGle1A and EGFP-hGle1B isoforms were localized at the cytoplasmic face of the nuclear envelope (Figure 1D). This indicated that endogenous hGle1B competes with EGFP-hGle1A for recruitment to the NPC in CTRL siRNA cells. Furthermore, hGle1A localization at the NPC was not sufficient for efficient mRNA export, suggesting a specific role for the hGle1-hCG1 interaction during the export mechanism. Overall we concluded that hGle1B and hGle1A play distinct cellular roles.

hGle1 is recruited to stress granules

Given yGle1’s roles in translation initiation and termination, we speculated that cytoplasmically localized hGle1A was involved in regulating translation. Because SGs and translation are in dynamic equilibrium, and Dbps such as DDX3, DDX1, and elf4A that play a role in translation are recruited to SGs (Low et al., 2005; Mazroui et al., 2006; Lai et al., 2008; Onishi et al., 2008; Shih et al., 2012), we asked whether hGle1A and hGle1B are recruited to SGs upon heat shock stress. To test this, we subjected HeLa cells coexpressing mCherry-G3BP and either exogenous EGFP, EGFP-hGle1A(R), or EGFP-hGle1B(R) to heat shock at 45°C for 60 min and imaged them using live-cell microscopy. EGFP-hGle1A and EGFP-hGle1B, but not EGFP, were colocalized to
cytoplasmic foci with the SG marker mCherry-G3BP (Tourriere et al., 2003; Supplemental Figure S1A). Indirect immunofluorescence microscopy with anti-hGle1 and anti-G3BP antibodies was also used to evaluate endogenous hGle1 localization. At 37°C in HeLa cells, endogenous hGle1 localization was pancellular with distinct nuclear rim staining, whereas G3BP was uniformly distributed throughout the cytoplasm (Figure 2A). On heat shock, hGle1 was detected at cytoplasmic foci that colabeled with G3BP (Figure 2A). Line-scan analysis of individual SGs indicated that anti-hGle1 staining overlapped with anti-G3BP (Figure 2A). Moreover, the hGle1 cytoplasmic foci also colocalized with other SG components, including DDX3, HuR, and FMRP (Gallouzi et al., 2000; Mazroui et al., 2002; Lai et al., 2008; Shih et al., 2012; Supplemental Figure S2A). HeLa cells were treated for 60 min at 37°C with thapsigargin, which induces SGs by causing endoplasmic reticulum (ER) stress, and hGle1 localization to SGs was observed.
hGle1 specifically functions in SG assembly in response to cellular stress

For determining whether hGle1 plays an active role modulating SGs and/or is sequestered in SGs to regulate its own activity during stress, the effect of hGle1 depletion on SGs was assayed. If hGle1 is involved in SG formation, the absence of hGle1 should perturb SG dynamics; however, if hGle1 is simply sequestered, differences in SG dynamics would not be expected (as is found for sequestered signaling molecules; Kim et al., 2005; Arimoto et al., 2008). The presence of SGs was monitored after 60 min of heat shock in CTRL and hGLE1 siRNA-treated HeLa cells by indirect immunofluorescence with anti-G3BP antibodies. The majority of the hGle1-depleted cells exhibited an increased number of small G3BP-positive SGs compared with CTRL cells (Figure 3A). Other hGle1-depleted cells showed either diffuse cytoplasmic distribution of G3BP or localization to a few disorganized foci (Figure 3A). Similar phenotypes were observed when other SG markers were analyzed (DDX3, HuR, and FMRP; Supplemental Figure S2A). As controls for off-target effects, two independent siRNA sets were used that target different regions.
FIGURE 3: hGle1 is required for SG assembly and SG disassembly. (A) hGle1-depleted cells show SG-assembly defects. HeLa cells transfected with CTRL or hGLE1 siRNAs were subjected to heat shock at 45°C for 60 min and processed for immunofluorescence using anti-hGle1 and G3BP antibodies. hGle1-depleted cells show either increased numbers of SGs or fail to assemble SGs. Scale bar: 10 μm. (B) Expression of EGFP-hGle1A but not EGFP-hGle1B rescues SG-assembly defects in hGle1-depleted cells. CTRL or hGLE1 siRNA-treated HeLa cells were transfected with EGFP, EGFP-hGLE1A<sup>R</sup>, or EGFP-hGLE1B<sup>R</sup> plasmids, heat shocked, and processed for immunofluorescence detection of G3BP and hGle1. Scale bar: 10 μm. (C) Quantification of SG numbers in CTRL and hGLE1 siRNA cells expressing indicated plasmids. (D, E) Analysis of SG formation in CTRL and hGLE1 siRNA-treated samples. hGLE1 siRNA or CTRL siRNA-treated HeLa cells were heat shocked at 45°C. Samples were fixed across a time course of 0–60 min and processed for immunofluorescence detection of G3BP and hGle1. Error bar represents mean ± SE from three independent experiments. **, p < 0.001.
of the hGLE1 gene. A similar increase perturbation of SGs was observed with both, indicating that the phenotype is specific to hGle1 depletion (Supplemental Figure S2D). SG changes were also observed in hGle1-depleted HeLa cells treated with thapsigargin and in hGle1-depleted U2OS and RPE-1 cells upon heat shock (Supplemental Figure S2, B and C). Thus hGle1 depletion altered SG assembly, and the effects were not limited to stress or cell types.

To analyze whether the SG defects observed with hGle1 depletion were due to altered mRNA export, we conducted knockdown experiments for two other NPC-associated essential mRNA export factors: DDX19B (yDbp5 homologue) and NXF1 (Grüter et al., 1998; Kang and Cullen, 1999; Herold et al., 2000; Hodge et al., 2011). siRNA-mediated knockdown of NXF1 or DDX19B resulted in nuclear accumulation of bulk poly(A)+ RNA indicative of an mRNA export defect (Supplemental Figure S3A). However, neither NXF1 siRNA nor DDX19B siRNA-treated cells showed perturbations of SGs like that seen in hGle1-depleted cells (Supplemental Figure S3, A and B). Thus inhibition of mRNA export was not sufficient for perturbing SG formation.

Next the hGle1 siRNA knockdown and add-back approach was used to test for hGle1A versus hGle1B roles at SGs. CTRL siRNA or hGle1 siRNA cells were transfected with either EGFP, EGFP-hGLE1A8, or EGFP-hGLE1B8 plasmids. After 24 h, the cells were subjected to heat shock, and G3BP localization was assessed as an indicator of SG formation. Expression of EGFP, EGFP-hGLE1A8, or EGFP-hGLE1B8 plasmids had no impact on the number of SGs formed in CTRL siRNA-treated cells (Figure 3, B and C), and the number of SGs formed was significantly higher in hGle1 siRNA-treated cells expressing EGFP (Figure 3, B and C). Strikingly, the expression of EGFP-hGLE1A8 but not EGFP-hGLE1B8 led to a significant rescue of the SG defect induced upon hGle1 depletion (Figure 3, B and C). These results further supported the fact that the SG defects in hGle1 siRNA-treated cells were not due to off-target effects (based on the hGle1A-alone rescue) or mRNA export defects (based on hGle1B being sufficient for rescuing mRNA export). Overall we concluded that the hGle1A isoform specifically functions in SG assembly.

hGle1 is required for early assembly and disassembly of SGs

SGs first assemble as numerous small cytoplasmic foci, and in response to prolonged stress, the foci coalesce to form a smaller number of large SGs (Kedersha et al., 2000). For pinpointing the role for hGle1 in early or later stages of SG assembly, time-course analysis was conducted by heat shocking in 10-min increments, followed by fixation and indirect immunofluorescence with anti-G3BP antibodies to assess SG formation. CTRL siRNA cells assembled many small SGs after 20 min of heat shock. With increasing time of heat shock treatment, the SGs were larger in size and fewer in number (Figure 3, D and E). Quantification of SG size and number at each time point revealed several important distinctions between CTRL and hGle1 siRNA-treated cells. First, the number of SGs detected in hGle1 siRNA-treated cells was significantly higher compared with CTRL siRNA-treated cells at all time points during heat shock (Figure 3D). Second, the average SG size was significantly smaller in hGle1-depleted cells compared with CTRL cells at all time points (Figure 3E). Finally, SG number or size did not change much over the entire course of heat shock for the hGle1-depleted cells compared with CTRL cells (in which SGs became bigger and fewer over time; Figure 3, D and E). These results indicated that hGle1 is involved in early SG-assembly steps that regulate SG number and granule size.

We further investigated whether hGle1 is also required during SG disassembly. Following heat shock for 60 min, CTRL siRNA or hGle1 siRNA cells were allowed to recover for 10, 20, 30, 40, and 60 min at 37°C, and SG disassembly was monitored by indirect immunofluorescence with anti-G3BP antibodies. Cells were scored as having SGs if G3BP foci were detected. Interestingly, hGle1-depleted cells disassembled SGs. However, SG disassembly occurred more slowly than in CTRL cells, with significantly higher percentages of cells still containing SGs at later time points of recovery (Figure 3F). Collectively hGle1 activity was required for proper SG assembly and disassembly.

hGle1 depletion-induced SG defects persist upon microtubule perturbation

Intact and dynamic microtubule (MT) networks are required for proper SG assembly; thus MT disruption by drugs that depolymerize (nocodazole) or stabilize (Taxol) MTs results in smaller numbers of SGs (Ivanov et al., 2003; Chernov et al., 2009; Kolobova et al., 2009; Nadezhdina et al., 2010). Given the similar phenotype, we tested whether the SG phenotypes in hGle1-depleted cells were linked to changes in the MT network. If SG defects in hGle1-depleted cells were dependent upon MTs, we predicted that disrupting MT dynamics in hGle1-depleted cells would not have an additive effect. Conversely, if hGle1-dependent SG defects were independent of MTs, a further increase in the number of SGs should result from both hGle1 depletion and disrupted MT networks. CTRL and hGle1 siRNA cells were treated with vehicle alone, Taxol, or nocodazole, and the number of SGs was determined based on G3BP localization. Indirect immunofluorescence staining with anti–α-tubulin antibodies confirmed that MT networks were disrupted after treatments with Taxol or nocodazole compared with vehicle alone (Figure 4, A–C). Similar to previous studies (Chernov et al., 2009), treatment of CTRL cells with either Taxol or nocodazole led to smaller and numerous SGs. Addition of Taxol and nocodazole with hGle1 siRNA treatment led to a further increase in the number of SGs (Figure 4, A–C). Interestingly, quantification of SG numbers revealed a significantly higher level in hGle1 siRNA cells compared with CTRL cells in the presence of either Taxol or nocodazole (Figure 4D). These results supported the conclusion that hGle1-dependent SG assembly defects are independent of an effect on MTs.

The function of hGle1 in SG assembly is linked to translation

SG assembly is regulated by the available pool of free nontranslated mRNPs (Kedersha et al., 2000). Thus, altering translation could lead to perturbations in SG assembly. Given this and that yGle1 plays a role in translation, we next asked whether hGle1 modulates translation and whether this function is linked with SG formation. For measuring translation in CTRL and hGle1 siRNA-treated HeLa cells under nonstress and stress conditions, metabolic labeling with L-azidohomoalanine (AHA), a methionine analogue, was conducted for 30 min, and newly synthesized AHA-containing proteins were detected with Alexa Fluor-488 alkyne using click chemistry (Dieterich et al., 2006, 2007). Following the labeling reaction, indirect immunofluorescence microscopy was used to assess hGle1 and G3BP. In CTRL siRNA cells, there was strong signal for AHA-labeled proteins under nonstress conditions (Figure 5A). Strikingly, hGle1 siRNA-treated cells exhibited reduced AHA-488 signal as compared with CTRL siRNA-treated cells. A 35S metabolic labeling assay was used as an alternative measure of protein synthesis, and again a reduction in nascent protein synthesis was observed in hGle1 siRNA-treated cells compared with CTRL siRNA-treated cells (Figure 5D). Reduced protein synthesis under nonstress conditions was expected, due to the role of hGle1 in mRNA export. Therefore we tested whether depletion of NXF1 also led to reduced protein synthesis under
Thus the AHA signal detected was due to nascent protein synthesis. As a control, NXF1 siRNA-treated cells were tested, and there was no increased AHA labeling compared with CTRL siRNA-treated cells upon heat shock (Supplemental Figure S3C). Therefore the deregulation of translation during heat shock in hGle1-depleted cells was not due to global perturbations in mRNA export. To confirm AHA-labeling results by an alternative method, we measured nascent protein synthesis upon heat shock in CTRL and hGLE1 siRNA cells by a 35S metabolic assay. Consistent with AHA-labeling results, we observed increased incorporation of [35S]methionine/cysteine in hGle1-depleted cells (Figure 5D).

Finally, upon evaluation of the SG defects in the hGle1-depleted cells, the deregulation in translation was found to be strongly correlated with the SG defects. Approximately 70% of hGle1-depleted cells with increased AHA-488 labeling either did not assemble SGs or

FIGURE 4: hGle1-dependent SG defects are not linked with microtubules. (A–C) HeLa cells transfected with CTRL and hGLE1 siRNAs were treated with (A) vehicle alone, (B) 5 μM nocodazole, or (C) 100 nM Taxol for 120 min at 37°C followed by heat shock at 45°C for 60 min. Cells were processed for immunofluorescence with anti-G3BP, α-tubulin, and hGle1 antibodies. Scale bar: 10 μm. (D) Quantification of SG numbers in hGLE1 and CTRL siRNA cells treated with indicated drugs after heat shock.
FIGURE 5: hGle1 modulates SG assembly by regulating translation. (A, B) Nascent protein synthesis is deregulated in hGle1-depleted cells. HeLa cells treated with CTRL and hGLE1 siRNAs were subjected to heat shock at 45°C or left untreated. After 15 min, AHA was added to the incubations, and heat shock treatment was continued for an additional 30 min. Samples were processed by Alexa Fluor-488 alkyne staining followed by immunofluorescence with anti-G3BP and hGle1 antibodies. Scale bar: 10 μm. (C) Quantification of AHA-488 staining. Mean fluorescence intensity of AHA-488 staining in individual cells was calculated in CTRL and hGLE1 siRNA cells using ImageJ. (D) CTRL or hGLE1 siRNA-treated HeLa cells were either heat shocked at 45°C or left untreated followed by metabolic labeling with 100 μCi/ml [35S]methionine/cysteine for 30 min at either 37°C or 45°C. Cells were lysed, and 35S incorporation was measured by liquid scintillation counter. Counts per minutes (cpm) are shown for hGLE1 and CTRL siRNA-treated cells. (E) hGle1-depleted cells have polysome profile defects under normal conditions. CTRL or hGLE1 siRNA cells were lysed, and polysome profiles were generated by subjecting cells to a 7-47% sucrose gradient centrifugation. (F) Expression of hGle1A but not hGle1B rescues translation defect in hGle1-depleted cells. CTRL or hGLE1 siRNA-treated HeLa cells were transfected with mCherry, mCherry-hGLE1A*, or mCherry-hGLE1B* plasmids, heat shocked, and processed for metabolic labeling using AHA. AHA incorporation was detected with Alexa Fluor-488 alkyne using click chemistry. Scale bar: 10 μm. (G) Quantification of AHA-488 staining in CTRL and hGLE1 siRNA cells expressing the indicated plasmids.
had an increased number of SGs (Figure 5B). Because hGle1 deple-
tion resulted in defects in translation, we next investigated whether
the hGle1A and/or hGle1B isoform rescues translation defects in
hGle1-depleted cells. CTRL or hGle1 siRNA-treated cells were trans-

tected with mCherry alone, mCherry-hGle1A, or mCherry-hGle1B
plasmids. Twenty-four hours after plasmid transfection, cells were
heated shock and processed for AHA labeling. Interestingly, expres-
sion of mCherry-hGle1A but not mCherry-hGle1B or mCherry res-
cued translation defects in hGle1-depleted cells upon heat shock (Figure 5, F and G). Together these results suggested that hGle1A modulates SG formation by regulating translation upon heat shock.

During cell-stress conditions, various signaling pathways con-
verge to result in the phosphorylation of elf2α at Ser-51, which
inhibits elf2 recycling and results in a global reduction in transla-
tion (Wek et al., 2006; Spriggs et al., 2010). To gain insight into the mechanisms for how hGle1 depletion alters translation, we ana-
lyzed the phosphorylation levels of elf2α. Immunoblotting with
anti-elf2α P [Ser-51] antibody revealed an increase in elf2α phos-
phorylation in CTRL siRNA-treated cells after 30 min of heat shock,
which persisted to the 60-min time point (Figure 6, A and B). Strik-

ngly, the relative elf2α phosphorylation level was reduced modestly (~36%) but significantly in hGle1-depleted cells compared with
CTRL siRNA cells (Figure 6, A and B). These results indicated that reduced elf2α phosphorylation might be linked to translation deregulation in hGle1-depleted cells upon heat shock.

DDX3 suppresses loss of hGle1 function
Based on the published connections between yGle1 and Ded1 dur-
ing translation initiation (Alcazar-Roman et al., 2006; Bolger et al.,
2008; Bolger and Wente, 2011; Folkmann et al., 2014), the roles for Ded1 in releasing mrna from SGs and promoting translation (Hilliker et al., 2011), and the recruitment of the Ded1 human homologue DDX3 to SGs (Lai et al., 2008; Hilliker et al., 2011; Shih et al., 2012), we evaluated whether hGle1 function in SGs is mediated through interaction with DDX3. First, communoprecipitation experiments were conducted with HeLa cell lysates from untreated or heat-
shocked cultures. hGle1 and DDX3 were cosolated in the presence
or absence of stress (Figure 8A). We also examined whether expres-
sion of DDX3 rescues hGle1-dependent SG defects. CTRL or hGle1 siRNA-treated cells were transfected with EGFP alone, EGFP-
DDX19B, or DDX3-EGFP. DDX3-EGFP was expressed at low levels, so the cells did not form constitutive SGs (Supplemental Figure S6). Cells were subjected to heat shock for 60 min at 45°C. Notably, only
DDX3-EGFP expression partially rescued SG defects in hGle1-de-
plicated cells (Figure 8, B and C). Next we tested whether DDX3 also
rescues translation defects in hGle1-depleted cells upon heat shock.

hGle1 modulates the dynamic balance between translation
and SGs upon stress
Several pharmacological agents are known to change the dy-
amic equilibrium between SGs and translation. For example,
cycloheximide treatment prevents SG formation, whereas puromy-
cin results in larger SGs upon stress (Kedersha et al., 2000). If hGle1 regulates the equilibrium between SGs and active translation, hGle1 depletion might shift the balance of mRNPs toward active transla-
tion, limiting the free mRNP pool and inhibiting SG assembly. In this model, SG-assembly defects induced by hGle1 depletion should be rescued by shifting the mRNP balance toward SG formation by add-
tion of puromycin. The number of SGs in CTRL or hGle1 siRNA-
treated cells was determined in the presence of either vehicle alone
or puromycin. Treatment of CTRL siRNA cells with either puromycin
or vehicle alone did not change the number of SGs significantly
(Figure 7, A and B). Notably, treatment of hGle1-depleted cells with
either 0.1 mg/ml or 0.5 mg/ml puromycin significantly reduced the
number of SGs (Figure 7, A and B). Thus puromycin partially rescued
the SG defects in hGle1-depleted cells upon heat shock. As a con-
trol, we tested whether puromycin rescues the increase in SGs ob-
served in nocodazole-treated cells and found it did not (Supplemental
Figure S5, A and B). Because puromycin failed to rescue MT-dependent SG defects, puromycin and nocodazole were affect-
ing SG formation at distinct steps. We concluded that puromycin
rescues the SG phenotype in hGle1-depleted cells by increasing the
free mRNP pool.

If hGle1 levels impact the free mRNP pool by changing the dis-
tribution of mRNPs between active translation and SGs, then over-
expression of hGle1 should increase the free mRNP pool and result
in larger SGs (similar to puromycin treatment alone). HeLa cells
transfected with either EGFP, EGFP-hGle1A, or EGFP-hGle1B
plasmids were heat shocked and analyzed for SG formation. G3BP-
positive SGs assembled in cells expressing either EGFP, EGFP-
hGle1A, or EGFP-hGle1B plasmids (Figure 7D). Overexpression
of hGle1A caused a modest but significant increase in SG size (mean
= 1.34 μm²) compared with EGFP (mean = 1.16 μm²) or hGle1B
(mean = 1.15 μm²) (Figure 7D). Thus hGle1A but not hGle1B overexp-
ression resulted in larger SG formation. TTP, TIA-1, and G3BP pro-
teins play a role in early assembly of SGs, and their overexpression
drives constitutive SG formation under nonstress conditions (Touri-
ere et al., 2003; Gilks et al., 2004). However, in nonstress conditions,
expression of either EGFP, EGFP-hGle1A, or EGFP-hGle1B plas-
mids in HeLa cells showed pancellular cytoplasmic staining for
G3BP, suggesting that hGle1 cannot nucleate SG formation in the
absence of stress (Figure 7C).
CTRL or hGLE1 siRNA cells were transfected with mCherry-alone or mCherry-DDX3 plasmids and heat shocked; this was followed by metabolic labeling with AHA. Importantly, addition of mCherry-DDX3 but not mCherry-alone partially rescued the translation defect in hGle1-depleted cells (Figure 8, D and E). Overall hGle1 function in SG assembly and translation might be linked to DDX3 regulation.

DISCUSSION

Localization of mRNA in SGs plays a role in temporal and spatial regulation of gene expression. In this report, an essential role for hGle1 in SG biology is revealed. This function is distinct from hGle1's action during mRNA export at the NPC. hGle1 is recruited to SGs upon stress and also interacts with the translation initiation factor DDX3. Moreover, hGle1 depletion results in both defective SG assembly and altered translation during stress conditions, suggesting that hGle1 depletion shifts the distribution of mRNPs toward translation. This is further supported by our observation that hGle1-dependent SG defects are rescued by addition of the translational inhibitor puromycin. Finally, hGle1A overexpression does not induce SGs in the absence of stress but does increase the size of SGs in response to stress. Similarly, puromycin is not sufficient alone to nucleate SGs but induces larger granule formation in the presence of...
Previously we defined functions for Gle1 as a regulator of Dbps during mRNA export and translation (Murphy and Wente, 1996; Tran et al., 2007; Bolger et al., 2008; Bolger and Wente, 2011; Noble et al., 2011). Given the effects here on translation in the hGle1-depleted stress. Thus hGle1A induces the formation of larger SGs by modulating translation. In sum, hGle1 plays a key role in the exchange of mRNPs between SGs and the active translation machinery, thereby serving as a critical factor during the cellular stress response.
cells and the interactions between hGle1 and DDX3, a role for Gle1 in translation is likely conserved between S. cerevisiae and human cells. Multiple lines of evidence suggest that the impact of hGle1 on SGs is not due to its role in mRNA export. Depletion of the mRNA export factor DDX19B or NFX1 does not result in SG defects. Moreover, hGle1A rescues SG defects yet fails to rescue the mRNA export defects. It is clear that the hGle1A and hGle1B isoforms play distinct separable functions in the cell: hGle1A in cytoplasmic SG function and hGle1B in mRNA export at the NPC.

This work supports a model wherein hGle1 exists in at least two distinct pools in the cell, allowing for a repertoire of independent functions and an ability to simultaneously regulate multiple steps in the gene expression pathway. It is possible that isoform-specific interactions with their unique protein partners might determine localization and/or functions. For instance, hGle1B but not hGle1A interacts with hCG1. Interestingly, only EGFP-hGle1B localizes to the NPC when transiently expressed in HeLa cells. EGFP-Gle1A does not localize to the NPC in the presence of endogenous hGle1. However, we find here that EGFP-hGle1A can in fact localize to NPCs when endogenous hGle1 is depleted; yet mRNA export is not efficient with hGle1A alone. We conclude that hGle1B binding to hCG1 is required for a step in mRNA export that is distinct from strict localization at NPCs. Indeed, our very recent studies support this conclusion, as interactions between the C-terminal domain of Nup42 (human homologue hCG1) and yGle1 are required for mRNP remodeling in yeast (Adams et al., 2014).

How mRNPs move in and out of SGs is a long-standing question in the field (Kedersha et al., 2005; Anderson and Kedersha, 2008; Zhang et al., 2011). To transition between these different states, mRNP rearrangements potentially occur, and thus RNA-binding proteins and mRNP remodelers are expected to play a role in directing mRNPs for storage in SG or translation. In yeast, the Dbp Ded1 regulates release of mRNA from SGs and promotes translation (Hilliker et al., 2011). We propose that hGle1 might function in distribution of mRNPs between SGs and translation through its regulation of DDX3. We find hGle1 interacts with DDX3, and the hGle1-dependent SG and translation defects are rescued by expression of DDX3. Interestingly, Ded1 and DDX3 assemble SGs in an ATP-independent manner (Hilliker et al., 2011; Shih et al., 2012). However, Ded1’s ATPase activity is required for disassembly of SGs and re-entry into translation (Hilliker et al., 2011). Here we show that hGle1 depletion results in SG-assembly defects and slows disassembly of SGs. Moreover, hGle1 is involved in regulating the exchange between SGs and translation in a manner similar to that reported for Ded1. Thus lack of proper hGle1-mediated control of DDX3 could lead to improper SG dynamics.

Surprisingly, elf2α phosphorylation at Ser-51 is modestly reduced in hGle1-depleted cells upon stress. This is consistent with the continued translation observed upon heat shock in hGle1-depleted cells. As expression of nonphosphorylatable serine to alanine mutant at residue 51 of elf2α allows continued protein synthesis upon heat shock in CHO cells (Murtha-Riel et al., 1993), elf2α phosphorylation is likely one of the primary mechanisms to inhibit global protein synthesis upon stress (Gebauer and Hentze, 2004; Wek et al., 2006; Sonenberg and Hinnebusch, 2009). It is intriguing that hGle1 depletion affects elf2α phosphorylation and that cells maintain translation under stress. The elf2 kinase, PKR is often activated by its association with double-stranded RNAs, and upon heat shock, Alu RNA can form stable complexes with PKR that result in its activation (Chu et al., 1998; Williams, 1999). Thus, as hGle1 regulates Dbps for mRNP remodeling, this could be a link to a potential activation of PKR kinase. Alternatively, there might be other mechanisms that play a role in regulating elf2α phosphorylation. Additionally, translation perturbations observed in hGle1-depleted cells could be due to other regulatory pathways that may or may not be linked with elf2α. Further studies are required to determine precisely how hGle1 levels influence elf2α and modulate translation.

Deregulation of SG assembly, disassembly, and their clearance is associated with various neurological diseases. An emerging theme is that hyperassembly of SGs acts as an intermediate for disease progression. For instance, ALS-linked TDP-43 and FUS mutants form larger and more stable SGs under normal stress conditions (Dewey et al., 2011; Parker et al., 2012; Wolozin, 2012; Buchan et al., 2013; Vance et al., 2013). The SG phenotype during hGle1A overexpression is remarkably similar to that for the ALS-linked TDP-43 and FUS mutants. Our very recent work has linked mutations in hGle1 with ALS (Kaneb et al., 2014). These ALS-linked hGle1 mutants lack the hCG1 binding site and fail to localize at the NPC in a manner similar to the hGle1A isoform. Thus the ALS-linked hGle1 mutations alter the cellular pools of hGle1A and hGle1B isoforms, and this may contribute to disease phenotypes. Our work here presents further evidence that deleting specific hGle1 isoforms differentially affects mRNA export and translation. Additionally, mutations in hGle1 linked with inherited human diseases LCCS1 and LAADD (Noussain et al., 2008) are due to deregulated mRNA export (Folkmann et al., 2013, 2014).

Taking these observations together, we speculate that the ALS hGle1 mutations could impact both mRNA export (by decreasing the pool of hGle1B available) and translation (by altering the pool of hGle1A and SG dynamics). Moreover, other disease states could arise from alterations in hGle1A function specifically that impact translational regulation. Investigating the hGle1 mechanisms that drive the assembly and disassembly of SGs under normal conditions will guide the understanding of how pathological aggregates form.

MATERIALS AND METHODS

Cell culture, treatments, and plasmids

HeLa, U2OS, and RPE-1 cells were grown in complete DMEM or DMEM-F12 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) at 37°C in 5% CO2. Cells were incubated at 45°C in a non-CO2 air incubator for 60 min to induce heat stress unless indicated otherwise. For ER-induced stress, cells were exposed to 10 μM thapsigargin (Sigma-Aldrich, St. Louis, MO) for 60 min at 37°C. Puromycin (Sigma-Aldrich) was used at 0.1 mg/ml or 0.5 mg/ml for 60 min. Cycloheximide (Sigma-Aldrich) was used at 10 μg/ml. For MT perturbations, cells were incubated with 5 μM nocodazole (Sigma-Aldrich) or 500 nM Taxol (Sigma-Aldrich) for 120 min at 37°C; this was followed by heat shock at 45°C for 60 min. Plasmids expressing EGFP-C1, EGFP-hGle1B8 (pSW3908; Folkmann et al., 2013), EGFP-hGLE1A (pSW1409; Kendirgi et al., 2003), and Pom121-mCherry (Mor et al., 2010) were described previously. siRNA-resistant EGFP-hGLE1A8, mCherry-hGLE1A8, and mCherry-hGLE1B8 were generated by introduction of three silent mutations at the hGle1 siRNA-targeting regions of EGFP-hGLE1A (pSW3909), mCherry-hGLE1A, and mCherry-hGLE1B plasmids by site-directed mutagenesis, respectively. DDX3-EGFP plasmid was PCR amplified from a cDNA (MGC:2129) purchased from GeneCopoeia (Rockville, MD) and cloned into pEGFP-N3 vector using Xhol and BamHI sites (pSW4105). mCherry-DDX3 was generated by PCR amplification of DDX3 cDNA and cloned into pmCherry-C1 vector.

siRNAs and plasmids transfections

Negative control siRNA and hGle1 siRNAs were purchased from Qiagen (Valencia, CA). NXX1 and DDX19B SMARTpool siRNAs

Volume 26 April 15, 2015 hGle1A regulates SG dynamics 1487
were purchased from Dharmacon (Lafayette, CO). Cells were reverse-transfected with indicated 20 nM siRNAs using HiPerFect (Qiagen) following the manufacturer’s instructions. Plasmid transfections were performed using Fugene 6 (Promega, Madison, WI) following the manufacturer’s instructions. For rescue of SG and translation phenotype, siRNA-resistant (‘R’) EGFP, EGFP-hGLE1B, and EGFP-hGLE1A expression constructs were transfected after 48 h of siRNA transfections.

Coimmunoprecipitation
HeLa cells were plated in 100-mm dishes (Fisher Scientific, Pittsburgh, PA) and either left untreated or heat shocked for the indicated times at 45°C. After treatment, cells were washed with 1× phosphate-buffered saline (PBS) and lysed in buffer containing 0.5% NP-40, 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche, Nutley, NJ). Cell lysates were spun at 13,000 rpm for 10 min at 4°C, and the supernatants were incubated in inhibitory cocktail (Roche, Nutley, NJ). Cell lysates were spun at 14,000 rpm for 10 min at 4°C, and the supernatants were incubated with anti-hGle1, 1:100 (ASW48), and anti-DDX3, 1:1000 (Bethyl Laboratories, Montgomery, TX), antibodies.

Immunoblotting and immunofluorescence
For immunoblotting, cells were plated in 60-mm dishes (Fisher Scientific) and reverse-transfected with 20 nM indicated siRNAs. After 72 h post-siRNA transfections, cells were lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), and protease and phosphatase inhibitor cocktail. For add-back experiments, indicated plasmids were transfected after 24 h of siRNA treatments. Proteins were resolved on 7.5% SDS-PAGE and immunoblotted with anti-hGle1, 1:1000 (Folkmann et al., 2013), anti-GFP, 1:1000 (Life Technologies), anti-actin, 1:5000 (Sigma-Aldrich), anti-EB1, 1:1000 (Cell Signaling Technology, Danvers, MA), and anti-phospho-EB1 (Ser51), 1:1000 (Cell Signaling Technology). Far-red dye-conjugated secondary antibodies were used for detection, and blots were scanned using a Li-Cor Odyssey scanner. For immunofluorescence, cells were plated on 1.5-mm round coverslips in a 24-well plate (Fisher Scientific). Following treatment, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100. Cells were washed with 2% BSA/PBS three times, and Alexa-labeled proteins were detected with 1 μM alkyne-tagged Alexa Fluor-488 (Life Technologies) using Click-iT cell detection reagent (Life Technologies) for 45 min at room temperature. Cells were further processed for immunofluorescence as described above. For 35S metabolic labeling, CTRL and hGle1 siRNA-treated cells were washed with DMEM without cysteine and methionine and incubated in the same medium for 45 min at 37°C. Radioactive [35S]methionine/cysteine (100 μCi/ml) (Perkin Elmer, Waltham, MA) was added to the medium, and cells were incubated for an additional 30 min. Following treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were washed with 2% BSA/PBS three times, and AHA-labeled proteins were detected with 1 μM alkyne-tagged Alexa Fluor-488 (Life Technologies) using Click-iT cell detection reagent (Life Technologies) for 45 min at room temperature. Cells were further processed for immunofluorescence as described above. For 35S metabolic labeling, CTRL and hGle1 siRNA-treated cells were washed with DMEM without cysteine and methionine and incubated in the same medium for 45 min at 37°C. Radioactive [35S]methionine/cysteine (100 μCi/ml) (Perkin Elmer, Waltham, MA) was added to the medium, and cells were incubated for an additional 30 min at 37°C. Cells were lysed in RIPA buffer and protease and phosphatase inhibitor cocktail. Protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, CA). For liquid scintillation counts, equal amounts of labeled cell lysates were precipitated with 1 ml of 10% trichloroacetic acid (TCA) and BSA as a carrier protein for 30 min on ice. TCA precipitates were filtered onto glass microfiber filter disks grade GF/C in a filtration apparatus under vacuum. Disks were washed with 10% TCA followed by 100% ice-cold ethanol. Filters were air dried, and then counts were determined using a liquid scintillation counter (Beckman Coulter, Brea, CA).

Measurement of SG size and number
Post–image processing was done using ImageJ software. ImageJ plug-in 3D objects counter was used to calculate size and numbers of SGs of individual cells. The 3D objects counter measurement
parameters were set to “surface,” and the minimum size filter was set to 3. Surface measurements were used to calculate sizes of SGs. SG sizes and numbers were represented using box plot generated in Prism6 (GraphPad, La Jolla, CA). The percentages of cells with SGs were calculated as 100 × [number of cells with G3BP-positive foci]/(total number of cells)]. All data are the result of at least three independent experiments. Statistical significance was determined using Student’s t test (Microsoft Excel, Redmond, WA) and Fisher’s exact test (GraphPad) where appropriate.

Polysome profiles
Cells were grown in 10-cm dishes and lysed in 10 mM Tris-Cl (pH 7.5), 100 mM NaCl, 30 mM MgCl₂, 200 μg heparin/ml, 100 μg/ml cycloheximide, and 2 mM MgCl₂, 10 mM NH₄Cl, and 12 mM MgCl₂ 100 μg/ml cycloheximide in ultracentrifugation at 5000 rpm for 5 min. Cleared lysates were layered on 7–47% sucrose gradients cast in 50 mM Tris (pH 7.5), 50 mM NH₄Cl, and 12 mM MgCl₂ 100 μg/ml cycloheximide in ultracentrifuge tubes (Beckman). Gradients were centrifuged in a Beckman SW-41 rotor for 3 h at 28,800 rpm. An absorbance profile was collected from the gradients at 254 nm.

ACKNOWLEDGMENTS
We thank Renee Dawson for technical assistance and critical reading of the manuscript; the Wente laboratory for discussions; Yaron Shav-Tal for the Pom121-mCherry plasmid; Andrew Link for assistance with polysome profiling; and James R. Goldenring for the mCherry-Tal for the Pom121-mCherry plasmid; Andrew Link for assistance with response to stress. Nat Rev Genet 12, 833–845.


Lloyd RE (2013). Regulation of stress granules and P-bodies during RNA export II: nucleotide cycling and mRNP remodeling by Dbp5 are conserved mechanisms in eukaryotes: mechanisms and biological targets. Cell 136, 737–745.