Uncovering the roles of an essential mRNA regulatory factor Gle1 in stress response and disease

By

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To my loving husband and my parents

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LIST OF ABBREVIATIONS

4E-BP	eIF4E-binding protein
AHA	L-azidohomoalanine
ALS	Amyotrophic lateral sclerosis
Dbp	DEAD-box protein
eIF2α	Eukaryotic initiation factor alpha 2
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
H	Human
HSF1	Heat shock factor 1
IP_6	Inositol-hexakisphosphate
IRES	Internal ribosome entry sites
JNK	c-Jun NH_2-terminal kinase
LCCS1	Lethal congenital contracture syndrome 1
MAPK	Mitogen-activated protein kinases
mRNP	mRNA-protein complexes
MT	Microtubule
NPC	Nuclear pore complexes
Nup	Nucleoporin
P-TEFb	Positive transcription elongation factor b
S. cerevisiae	*Saccharomyces cerevisiae*
SG	Stress granule
uORF	Upstream open reading frame
UPR	Unfolded-protein response

CHAPTER 1

Introduction

Overview of cellular stress response

All living organisms experience a variety of stresses throughout their life. These insults must be appropriately dealt with to maintain proper cellular homeostasis. Therefore, cells have evolved diverse adaptive mechanisms to mount the optimal stress response and ensure cell survival. Gene expression changes are the central components of stress response along with alterations in metabolism, cell cycle progression and protein homeostasis (López-Maury et al., 2008; de Nadal et al., 2011). These stress responses allow for considerable fine-tuning, and the type of stress response depends upon the nature and intensity of stimulus. There are two types of pathways; core stress response pathways elicited in response to various stresses and specific stress pathways that are activated in response to a specific stress. Core response pathways are highly conserved among various organisms and provide cross-protection, such that cells exposed to one type of mild stress will show resistant to a different type of stress. In general, stress response pathways utilize post-translational control to provide an immediate response, followed by rewiring of the gene expression programs that deliver slower but long-term adaptation (Fulda et al., 2010; de Nadal et al., 2011). However, under conditions where these survival strategies fail, signaling pathways are activated to initiate cell death by various mechanisms. Thus, there exists a fine balance between these cell decisions- to live or to die- and this balance is influenced by the type and severity of stress (Fulda et al., 2010; Kültz, 2005; López-Maury et al., 2008; de Nadal et al., 2011; Richter et al., 2010).

Mechanisms of stress response

Sensing: To execute an appropriate response under stress, a cell needs to detect stress. Diverse and highly specialized stress sensors are founds in cells to sense and communicate signals in response to various types of stresses (Lamech and Haynes, 2015; Török et al., 2014) (Figure1.1). For instance, a very well characterized molecule IRE1α acts as a sensor of endoplasmic reticulum (ER) stress. IRE1α is a trans-membrane ER-resident protein consisting of a bifunctional kinase and RNase domain. It senses the misfolded proteins generated due to ER stress and activates the unfolded-protein response (UPR) pathway. Under basal conditions, IRE1α is ubiquitylated and degraded by the ERAD complex. ER stress triggers release of the IRE1α-ERAD complex thus reducing ubiquitylation of IRE1α (Sun et al., 2015). In addition, accumulation of misfolded proteins also promotes IRE1α disassociation from the Bip chaperone leading to IRE1α oligomerization and activation. Activated IRE1α initiates the UPR pathway resulting in activation of many ER chaperones, lipogenic and ERAD genes (Chen and Brandizzi, 2013).

Signal transduction: Signals received by a cell need to be effectively transmitted to ensure a rapid and appropriate stress response. Signal transduction pathways integrate, amplify and relay incoming signals through a signaling cascade using a network of enzymes that act upon one another (Armbruster et al., 2014) (Figure1.1). Mitogen-activated protein kinases (MAPKs) are one of the best-characterized evolutionarily conserved signal transduction pathways. Various types of stresses including heat shock, oxidative stress and ultraviolet light activate these pathways (Morrison, 2012; Zhang and Liu, 2002). In mammals, the MAPK family consists of p38, extracellular-regulated kinase (ERK) and c-Jun NH_2- terminal kinase (JNK). Each MAPK

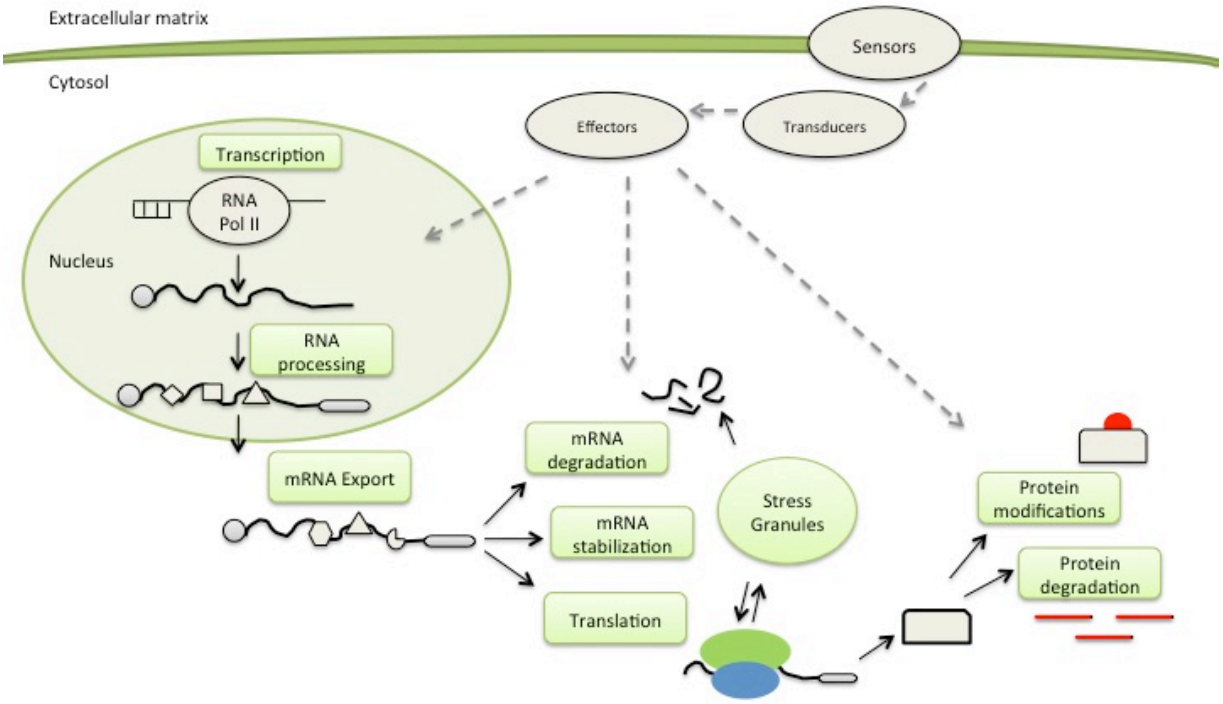


Figure 1.1: Mechanisms of stress response in eukaryotes.

Under stress conditions, cells sense signals through sensor molecules and communicate signals to transducers. Transducers amplify and relay signals to effector molecules. Gene expression changes are central to the stress response and are regulated at multiple levels under stress conditions as detailed in the text.

kinase cascade consists of three components: MAPK kinase kinase (MAPK3), MAPK kinase (MAPK2) and MAPK. MAPK3 phosphorylates MAPK2, which in turn phosphorylates MAPK. Phosphorylated MAPKs have diverse substrates that regulate a variety of cellular processes such as translation, cell growth, metabolism, cell division and others (Kim and Choi, 2010; Kyriakis and Avruch, 2001; Morrison, 2012; Zhang and Liu, 2002). Taken together, signal transduction pathways integrate and relay information from sensors to target molecules for a proper stress response.

Cellular responses: Once a signal is relayed, effector molecules execute programs that allow cells to initiate efficient adaptive responses and ensure cell survival (Figure1.1). Initial effector processes are rapid and provide very short-term protection. These initial responses do not depend upon new RNA or protein synthesis, but are mainly executed at the level of post-translational control. This includes utilizing pre-existing molecules for cellular defense, modulation of enzyme activity, and permeability of ion channels or transporters (Bensaude et al., 1996; Hohmann et al., 2007; Luyten et al., 1995; Proft and Struhl, 2004). Regulation at transcriptional and post-transcriptional levels such as inhibition of protein synthesis, formation of stress granules and inhibition of mRNA export occur after few minutes of the given stress (Figure1.1). This regulation provides long-term adaptation and is crucial for cell survival. In addition, gene expression changes are reversible. This reversibility allows cells to return to their basal level after removal of stress (Buchan and Parker, 2009; Gasch et al., 2000; López-Maury et al., 2008; de Nadal et al., 2011; Sørensen et al., 2005). For example, heat shock proteins are immediately synthesized after heat shock. These proteins restore cellular homeostasis by facilitating folding and synthesis of proteins. In addition, they participate in trafficking, protein degradation and regulation of transcription factors. Interfering with heat shock protein functions result in

impaired stress response and apoptosis (Feder and Hofmann, 1999; Nollen and Morimoto, 2002; Richter et al., 2010).

Regulation of gene expression in response to stress

Gene expression reprogramming is essential for adaptive stress response and thus regulated tightly through modulation of each step in mRNA metabolism- from transcription to export to translation (Figure1.1). Discussed below are various mechanisms that allow cells to modulate their gene expression programs in response to stress.

Regulation of gene expression at the level of mRNA synthesis and processing

Synthesis of an mRNA represents the first step in the flow of information –DNA to RNA to protein- and is one of the principal control points for gene expression. In eukaryotes, the majority of protein-coding genes are transcribed by RNA polymerase II (Hahn, 2004). At the beginning of transcription, DNA binding proteins and chromatin modifying enzymes remodel chromatin to allow access to the transcription machinery. RNA polymerase II, along with general transcription factors, is recruited to the promoter region of a gene and forms a closed complex known as the pre-initiation complex. In the subsequent steps, melting of two DNA strands occurs allowing positioning of the template strand to the active site of RNA polymerase. Using the template strand as a guide, RNA polymerase II begins synthesizing RNA. After successful synthesis of an pre-mRNA, RNA polymerase II falls off, marking the end of a transcription cycle (Shandilya and Roberts, 2012; Weake and Workman, 2010). Pre-mRNA processing occurs simultaneously with transcription in a 5′ to 3′ direction. These processing reactions, such as addition of 5′ cap, splicing, editing and 3′ end processing, are performed by factors associated

with the carboxy-terminal domain of RNA polymerase II. Thus, transcription and RNA processing events are interdependent upon each other (Bentley, 2014).

Modulation of mRNA synthesis and processing during stress

Inducible gene expression is required for mounting an appropriate stress response. Thus, transcription and mRNA processing machineries are redirected to the synthesis of genes involved in the stress response, while transcription of housekeeping genes is inhibited. In this way a cell ensures that limited resources are not improperly utilized during stress (Lelli et al., 2012; Weake and Workman, 2010). This regulation occurs at multiple levels. First, access to DNA by transcription machinery is inhibited. In eukaryotes, DNA is packaged into nucleosomes and is further condensed into chromatin. Therefore, nucleosome positioning and accessibility of chromatin have profound effects on transcription in response to stress. This regulation is exemplified by the work done in various laboratories demonstrating that stress alters chromatin dynamics, modulates the accessibility of RNA polymerase II to genes, and ultimately influences transcription (Huebert et al., 2012; Shivaswamy and Iyer, 2008; Smith and Workman, 2012; Zanton and Pugh, 2006). Notably, some of the chromatin modifying enzymes do not necessarily assume the same function under normal conditions as they do in stress. For example, Set1 methyltransferase adds a methyl group to histone H3 at lysine 4 (H3K4) and this modification is usually associated with transcriptional activation. In contrast, Set1-dependent H3K4 methylation represses transcription during stress, especially of genes involved in ribosome biogenesis (Weiner et al., 2012). Additional controls are exerted at the level of recruitment of various transcriptional factors. Synthesis of an mRNA by RNA polymerase II depends largely upon the precise orchestrated recruitments of transcription factors and is also modulated by co-activators,

co-repressors and mediator complex. Thus, combinatorial action of various transcription factors, cofactors and co-repressors determines transcriptional response under stress conditions (Estruch, 2000; Weake and Workman, 2010).

Since mRNA synthesis and mRNA processing events are coupled, it is not surprising that various mRNA processing events are also regulated during stress (Yost et al., 1990). For instance, a transcriptome wide study demonstrated that splicing is inhibited globally during heat stress with unspliced transcripts are retained in the nucleus (Shalgi et al., 2014). Furthermore, various splicing factors including SR proteins and hnRNPs are re-localized to nuclear stress bodies and thus modulate splicing (Biamonti and Vourc'h, 2010).

Selective synthesis and processing of mRNAs during stress

As mentioned, rapid induction of gene expression is needed to promote synthesis of mRNAs involved in stress survival. One of the widely employed mechanisms is regulation of chromatin dynamics by kinase signaling pathways (Miotto, 2013; Whitmarsh, 2007). For instance, in *Saccharomyces cerevisiae* (*S. cerevisiae*), Hog1 is phosphorylated upon osmotic stress and translocates from the cytoplasm to the nucleus (Brewster and Gustin, 2014; Ferrigno et al., 1998). The Hot1 transcription factor recruits Hog1 to the promoters of osmoresponsive stress genes, which stimulates rapid transcription of osmoresponsive genes by recruiting mediator complex, general transcriptional machinery and members of RISC complex (Brewster and Gustin, 2014; Whitmarsh, 2007). Similarly, in human cells, p38 MAPK binds to and stimulates transcription of stress-responsive genes upon various stresses (Ferreiro et al., 2010).

RNA polymerase II pausing at the promoters of stress-responsive genes is an additional mechanism to allow for rapid induction of gene expression. Extensive work in flies and human

cells shows that heat shock-responsive genes are bound to RNA polymerase II under non-stress conditions (Adelman and Lis, 2012; Brannan et al., 2012; Jonkers and Lis, 2015; Maxwell et al., 2014; Sawarkar et al., 2012; Zhou et al., 2012). However, RNA polymerase II pauses after transcribing 20-40 nucleotides. This RNA polymerase II pausing is mediated by two factors: negative elongation factor and DRB sensitivity-inducing factor. Under stress conditions, paused RNA polymerase II is released, resulting in synthesis of heat shock-responsive transcripts. Notably, this RNA polymerase II release is dependent upon activation and recruitment of the transcription factor heat shock factor 1(HSF1). Under basal conditions, HSF1 is found as a monomer associated with Hsp70 and Hsp90 proteins that negatively regulates HSF1 activity. Upon stress, HSF1 dissociates from the chaperones, homo-trimerizes and binds to DNA. After its promoter accumulation, it recruits mediator complex and positive transcription elongation factor b (P-TEFb). PTEF-b phosphorylates the carboxy-terminal domain of RNA polymerase II and DRB sensitivity-inducing factor, enabling the release of negative elongation factor and thus allowing elongation of RNA polymerase II (Anckar and Sistonen, 2011; Vihervaara and Sistonen, 2014).

Taken together, these studies highlight the regulation of transcription and pre-mRNA processing events to mediate the expression of stress-responsive genes during changing conditions.

Regulation of gene expression at the level of mRNA export

In eukaryotes, genetic information is compartmentalized in the nucleus, which enables mRNA and protein synthesis in two distinct compartments in the cell. Although this provides another complex means of regulation and ensures that gene expression is spatially and

temporally regulated, it poses a physical barrier for the movement of mRNA(Hatch and Hetzer, 2014). In the nuclear envelope, the presence of specialized channels known as nuclear pore complexes (NPCs) allows the regulated movement of most RNA and proteins between the nucleus and cytoplasm. NPCs are highly conserved large proteinaceous assemblies consisting of multiple copies of ~30 nucleoporins (Nups). The NPC structure consists of a central channel and peripheral structures, namely nuclear basket and cytoplasmic filaments. Selective, bidirectional transport of molecules through the central channel is established through specific required interactions between cargo receptors and unstructured phenylalanine-glycine (FG) domain-containing nucleoporins known as FG Nups. Unidirectional transport through the NPC is achieved by regulated association and dissociation of transport factors on both sides of NPC (Grünwald et al., 2011; Köhler and Hurt, 2007; Strambio-De-Castillia et al., 2010).

In case of mRNAs, export competency is achieved only after completion of pre-mRNA processing events and association with export factors. Bulk mRNAs are exported by the mRNA export factors consisting of a heterodimer NXF1/NXT1 (also known as TAP/p15), and this process is not dependent upon Ran gradient needed for protein transport (Herold et al., 2000). The NXF1/NXT1 complex is recruited to the mRNA through an adaptor protein ALY (Hautbergue et al., 2008)(Figure 1.2). Notably, a subset of transcripts do not use the NXF1 pathway, but instead rely on the karyopherin Crm1 for their export (Kimura et al., 2004). Following the formation of an export-competent mRNA, export reeptors interact with FG Nups, leading to docking and translocation through NPC (Bonnet and Palancade, 2014; Carmody and Wente, 2009; Terry and Wente, 2007). The unidirectionality of mRNA export is primarily maintained by the actions of two essential conserved factors known as Gle1 and DEAD-box protein Dbp5. Work in *S. cerevisiae* shows that Gle1 in association with inositol-

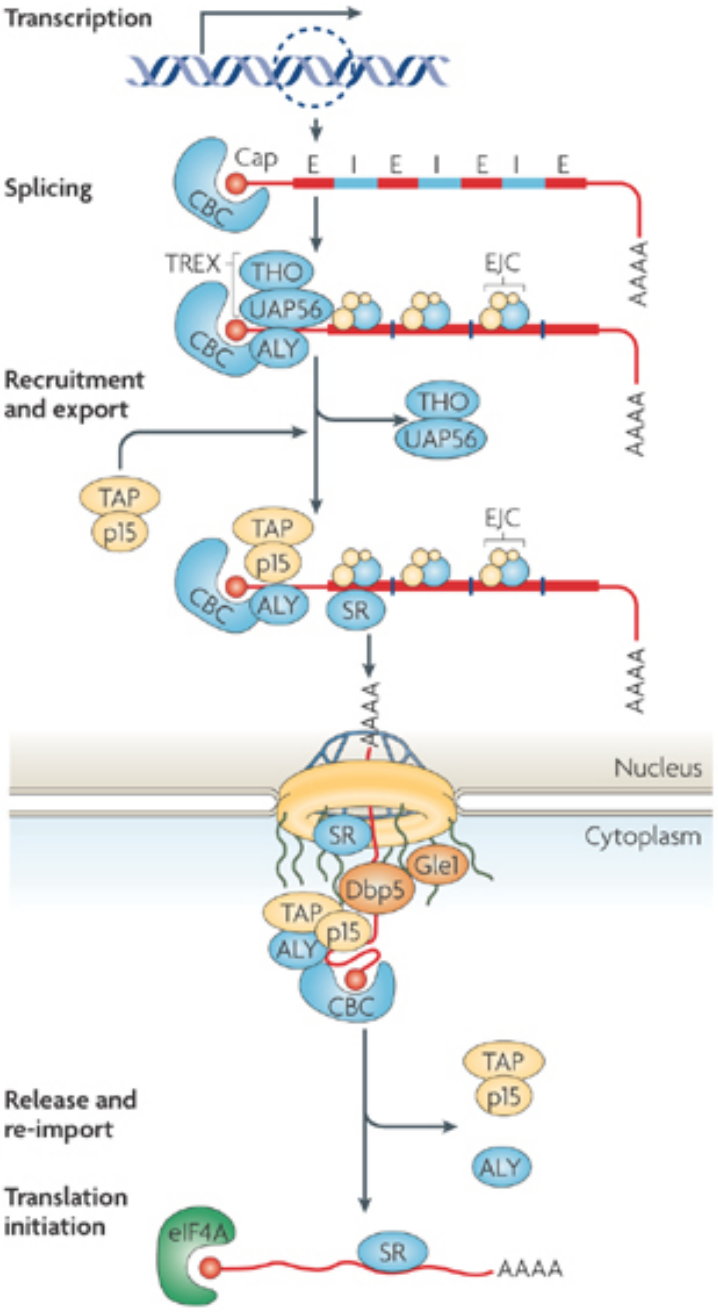


Figure 1.2: A schematic of mRNA export in mammalian cells.

RNA pol II transcripts are co-transcriptionally assembled into pre-mRNP complexes followed by their association with the NXF1/NXT1 (labeled as TAP/p15 in the figure) mRNA export factors as described in detail in text. These mRNPs are ready to be exported through the NPCs. This figure is adapted from (Köhler and Hurt, 2007).

hexakisphosphate (IP_6) stimulates the ATPase activity of Dbp5, which triggers the removal of a subset of proteins from mRNAs, including the export receptor Mex67 (*S. cerevisiae* homologue of NXF1) (Figure 1.2). Thus selective removal of export factors changes the composition of mRNA-protein complexes (mRNPs), preventing mRNA translocation back into the nucleus (Alcázar-Román et al., 2006; Lund and Guthrie, 2005; Tran et al., 2007; Weirich et al., 2006a). Once in the cytoplasm, mRNPs can be translated. Interestingly, Gle1, Dbp5 and IP_6 also function in translation (Alcázar-Román et al., 2010; Bolger et al., 2008). This suggests a close coupling between mRNA export and protein synthesis processes that ensures proper gene expression.

Modulation of mRNA export during stress

To prevent protein synthesis of housekeeping genes during stress, mRNA export of bulk poly $(A)^+$ mRNA is inhibited. Work in *S. cerevisiae* provides important insight into this mechanism. Bulk poly $(A)^+$ mRNA export is inhibited in yeast following ethanol and heat stress (Saavedra et al., 1996). It is proposed that the heterogeneous nuclear protein, Npl3p, dissociates from mRNAs, making them export incompetent under stress conditions (Krebber et al., 1999). Interestingly, the block of mRNA export is also facilitated by Slt2 kinase in yeast. Slt2 phosphorylates mRNA export adaptor Nab2 upon stress. Following stress, Nab2 association with Mex67 is reduced. In contrast, Nab2's interaction is enhanced with Yra1 and Mlp1, causing relocalization of these proteins to nuclear foci and promoting retention of bulk mRNAs in the nucleus (Carmody et al., 2010). In addition to these mechanisms, post-translation modifications of NPC components and their compositions affect mRNA export during stress (Izawa et al., 2004; Regot et al., 2013; Takemura et al., 2004).

Selective export of stress-responsive mRNAs during stress

While bulk export is inhibited, export of stress specific transcripts is supported under stress. In yeast, the export of heat shock mRNAs requires Nup42 and Mex67 and is independent of Npl3 and Crm1(Hurt et al., 2000; Saavedra et al., 1996, 1997). This mechanism allows selective export of heat shock mRNAs. Likewise, in mammalian cells, translation factor eEF1A1 facilitates export of *HSP70* mRNAs from the nucleus to translationally active polysomes. Depletion of eEF1A1 results in poor heat shock response and reduced thermo-tolerance (Vera et al., 2014).

Together, these studies indicate that selective export of mRNAs from the nucleus to cytoplasm is an important mechanism for the regulation of gene expression during stress. This importance is further highlighted by a series of recent studies that implicate altered nucleocytoplsmic transport as one of the potential mechanisms for neurological diseases, especially amyotrophic lateral sclerosis (ALS) (Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015a). However, we lack a clear picture of how this process is regulated during stress in higher eukaryotes. Defining the molecular aspects of import/export mechanisms in mammalian cells will be instrumental in understanding how gene expression is regulated during stress and diseases.

Regulation of gene expression at the level of translation

Translation of an mRNA into a protein represents the final step in gene expression. Protein synthesis is a dynamic process and is the primary level of control to alter the proteome of a cell. As such, translational control allows for rapid and reversible regulation of gene expression by altering the composition and abundance of proteins (Sonenberg and Hinnebusch, 2009).

The translation process consists of four steps –initiation, elongation, termination and recycling. Initiation begins with the formation of a ternary complex containing eIF2-GTP and the initiator Met-tRNA$_i^{Met}$. This ternary complex binds to the 40S small ribosomal subunit with the help of initiation factors eIF1, eIF1A, eIF3 and eIF5 to form the 43S pre-initiation complex (Kong and Lasko, 2012; Sonenberg and Hinnebusch, 2009) (Figure 1.3). Meanwhile, mRNA is bound to the cap-recognition complex known as eIF4F and PABP. The eIF4F complex consists of cap binding protein eIF4E, eIF4G and RNA helicase eIF4A. Next, mRNA bound to eIF4F complex and PABP is recruited to the 43S pre-initiation complex through an association between eIF4G and eIF3 (Figure 1.3) (Aitken and Lorsch, 2012; Gebauer and Hentze, 2004; Kong and Lasko, 2012; Sonenberg and Hinnebusch, 2009). After binding to mRNA at the 5′ end, 43S pre-initiation complex scans for an initiator AUG codon. Recognition of the start codon triggers release of eIF1. eIF5 mediates binding of the large ribosomal subunit 60S, resulting in formation of 80S initiation complex. Start codon recognition and large subunit joining trigger GTP hydrolysis of eIF2 and eIF5B, respectively. eIF5 is released and the 80S initiation complex is ready to enter into the elongation phase (Figure 1.3) (Aitken and Lorsch, 2012; Gebauer and Hentze, 2004; Kong and Lasko, 2012; Sonenberg and Hinnebusch, 2009).

Elongation begins with the delivering of amino-acyl tRNA by the elongation factor eEF1A-GTP to the A-site of the ribosome. When the correct tRNA is deposited, GTP is hydrolyzed and eEF1A-GDP dissociates. Following this, peptide bond formation occurs and the nascent peptide is transferred onto the A-site tRNA. The reaction is catalyzed by a peptidyltransferase center consisting mainly of conserved ribosomal RNA of the 60S subunit. The eEF2 shifts the peptidyl-tRNA into the P-site and deacylated tRNA into A-site. With this, the ribosome is ready for another round of elongation cycle (Dever and Green, 2012).

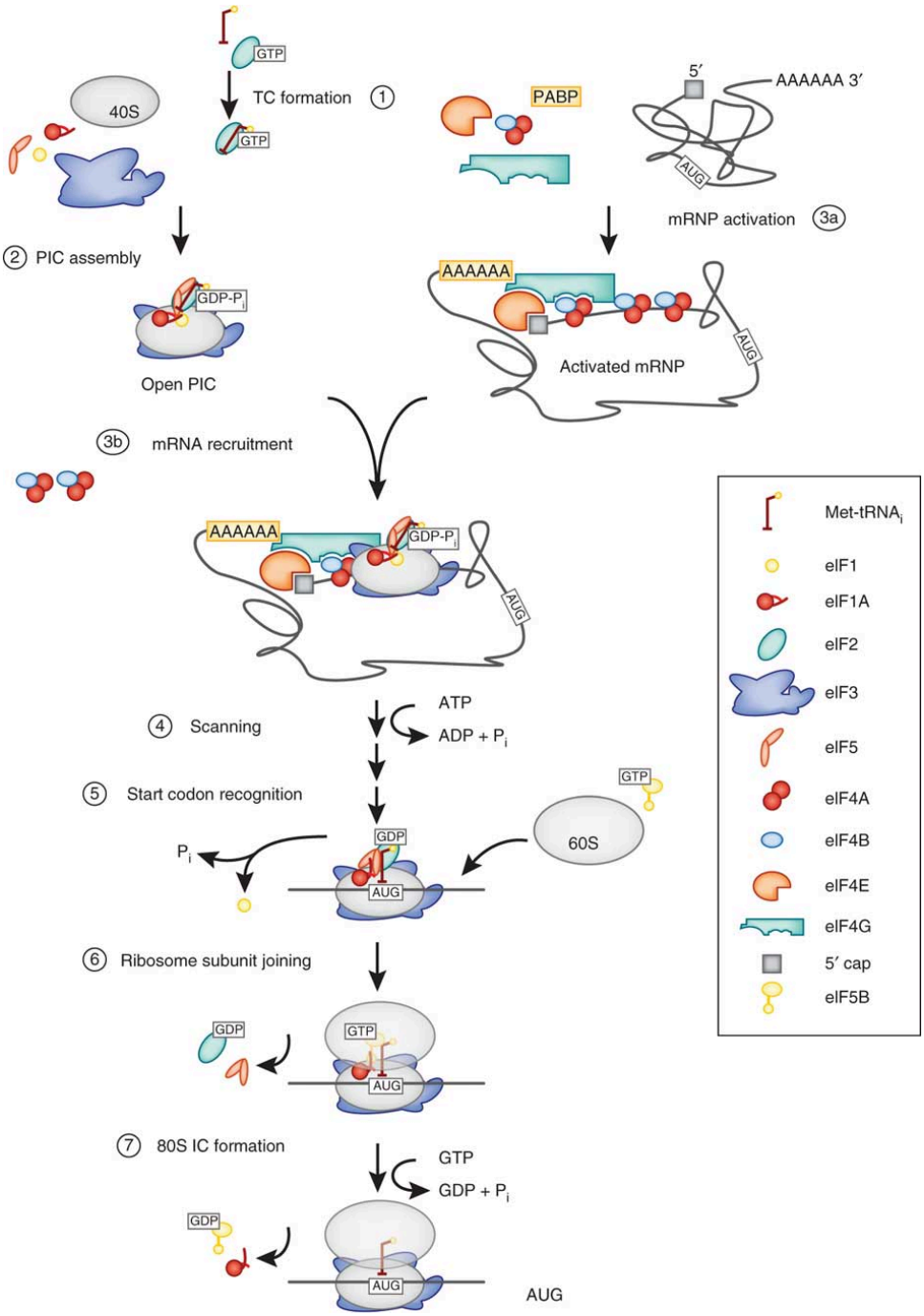


Figure 1.3: A schematic of cap-mediated translation initiation in eukaryotes.

Translation initiation is a complex process that involves assembly of a pre-initiation complex (PIC) containing the 40S subunit, initiator tRNA and initiation factors. The mRNA associated with initiation factors is recruited to the pre-initiation complex, followed by start site recognition. Finally, the 60S subunit joins and ribosomes are primed for translation initiation. Detailed steps are discussed in the text. TC= ternary complex IC= initiation complex. Figure is reprinted from (Aitken and Lorsch, 2012).

Translation termination occurs when the ribosome encounters a stop codon (UAA, UAG or UGA). Two termination factors, eRF1 and eRF3, are involved in this process. Encounter of a stop codon results in binding of a eRF1-eRF3-GTP ternary complex. This stimulates the transfer of peptide from the tRNA in the P-site to H_2O, coupled with hydrolysis of GTP. Following GTP hydrolysis, polypeptide is released from the ribosome (Dever and Green, 2012; Jackson et al., 2012; Mitkevich et al., 2006).

After the release of polypeptide, 80S ribosomes are still bound to mRNA. The recycling process involves release of mRNA and disassociation of 80S subunit so that ribosomes can start another round of translation. This process is very well studied in bacteria and ribosome-recycling factors are found in bacteria (Hirokawa et al., 2005; Janosi et al., 1994; Kiel et al., 2007). However, how ribosomes are recycled in eukaryotes is not well understood. Studies by various laboratories suggest that ABCE1 family members are likely players involved in the recycling process (Barthelme et al., 2011; Pisarev et al., 2010; Shoemaker and Green, 2011). In summary, ribosome recycling connects translation termination and initiation together.

Modulation of translation during stress

Translation is a complex and highly energy consuming process. To meet the demands of changing conditions, translation is reprogrammed to selectively synthesize proteins needed for defense mechanisms, while global protein synthesis is downregulated. This regulation occurs at various steps of translation (discussed below).

Initiation step: One of the mechanisms widely employed by cells to globally inhibit protein synthesis is by phosphorylation of initiation factor eIF2α at serine 51 (Wek et al., 2006). As mentioned above, eIF2-GTP is a part of the ternary complex. After GTP hydrolysis, eIF2-GDP is

released from the complex (Kong and Lasko, 2012; Sonenberg and Hinnebusch, 2009). eIF2B, a guanine nucleotide exchange factor, recycles eIF2 by recharging it with GTP. The eIF2 protein consists of three subunits- α, β, γ. Phosphorylation of eIF2α at serine 51 serves as a competitive inhibitor of eIF2B and prevents exchange of GDP to GTP. This inhibition results in an overall reduction of ternary complex formation and therefore global protein synthesis is reduced (Figure 1.4) (Rowlands et al., 1988). Phosphorylation of eIF2α at serine 51 is reliant upon four kinases – GCN2, PERK, HRI, and PKR. These kinases are usually activated in a stress specific manner. Thus, various pathways converge on eIF2α phosphorylation to regulate protein synthesis (Haro et al., 1996; Wek et al., 2006).

A second mechanism to reduce general translation is by interfering with the cap recognition complex. eIF4E, as a part of eIF4F complex, recognizes the m^7G cap of mRNA. Several eIF4E-binding proteins (4E-BPs) hinder eIF4F binding to the cap by sequestering eIF4E (Richter and Sonenberg, 2005). The strength of the binding depends upon the phosphorylation state of 4E-BPs. In a hypophosphorylated state, 4E-BPs bind to eIF4E strongly. However, in a hyperphosphorylated state, a conformational switch releases eIF4E, allowing the formation of eIF4F (Brunn et al., 1997; Gingras et al., 1998; Lin et al., 1994; Pause et al., 1994; Richter and Sonenberg, 2005) (Figure 1.4). Notably, the phosphorylation status of 4E-BPs is regulated by various stress and growth signals (Richter and Sonenberg, 2005; Teleman et al., 2005). Furthermore, eIF4E activity is controlled by phosphorylation. Phosphorylation by MAPK kinase Mnk1 at serine 209 promotes cap binding and translation efficiency (Pyronnet et al., 1999). Proteolytic cleavage of translation factors is another way to regulate translation. eIF4G and PABP are cleaved by caspase 3 during stress and this cleavage event interferes with the translation process.

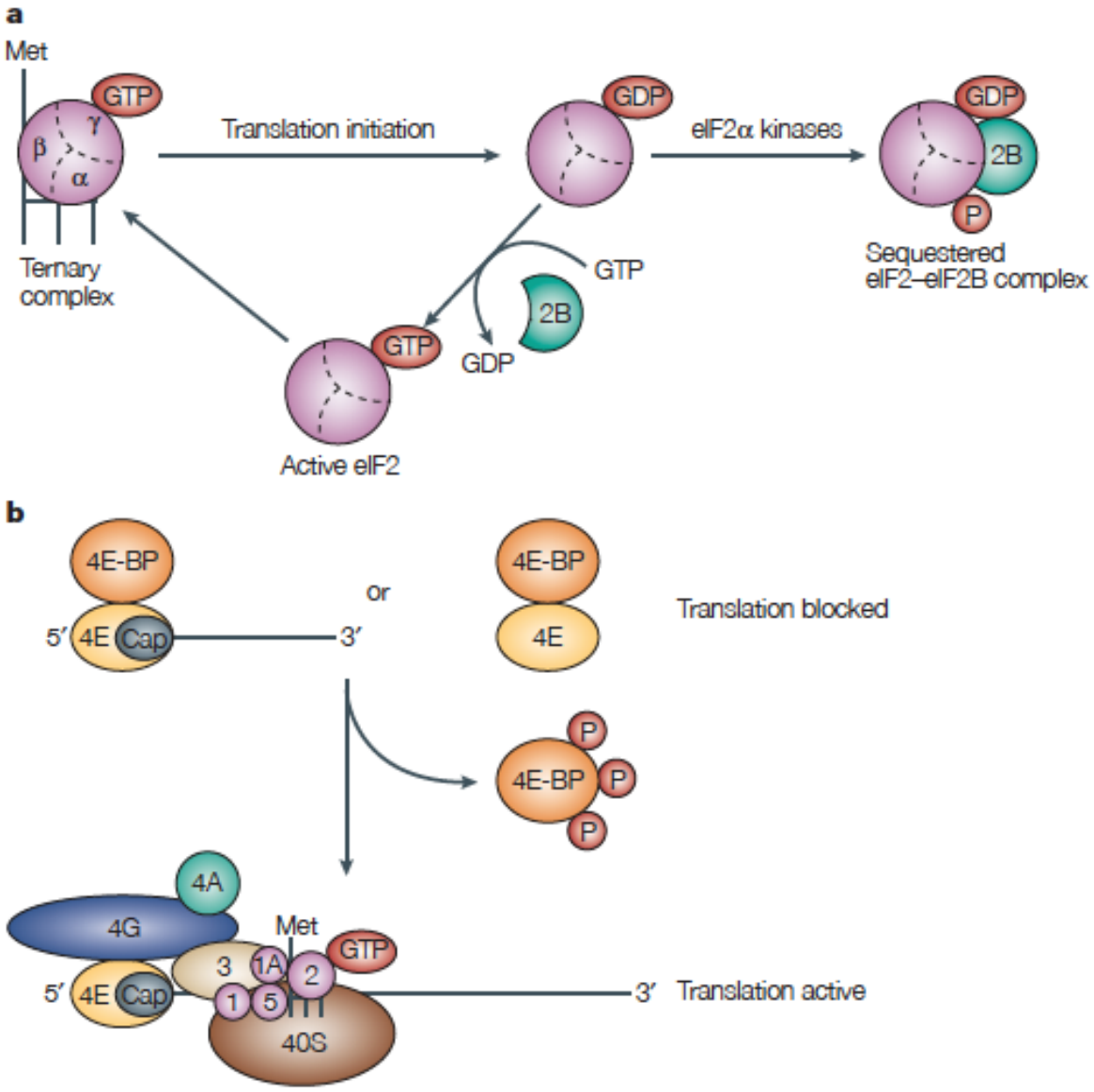


Figure 1.4: Regulation of global translation under stress conditions.

(A) Global control of protein synthesis by phosphorylation of eIF2α at serine 51 position. Phosphorylated eIF2α sequesters eIF2B resulting in limited ternary complex formation. (B) 4E-BPs inhibits translation by sequestering eIF4E and interfering with eIF4F complex formation. Figure is reprinted from (Gebauer and Hentze, 2004).

However, cleavage is only observed during apoptosis (Marissen and Lloyd, 1998; Marissen et al., 2004).

Elongation step: In addition to extensive regulation at the initiation step, translation is also modulated at the elongation stage. For example, elongation factor eEF2 is phosphorylated at threonine 56 in response to oxidative stress (Ryazanov, 1987; Ryazanov and Davydova, 1989). This residue lies in the GTP binding domain and phosphorylation prevents eEF2 binding to the ribosomes, thus inhibiting ribosome translocation. Atypical calmodulin-dependent kinase eEF2 phosphorylates eEF2 at threonine 56 (Ryazanov, 1987; Ryazanov and Davydova, 1989). eEF2 kinase activity itself is regulated extensively by its phosphorylation at various residues. For instance, mTORC kinase inhibits its activity in response to growth signals (Browne and Proud, 2004). In contrast, phosphorylation by AMPK kinase promotes its activation under hypoxic, ER and oxidative stress conditions (Browne et al., 2004; Horman et al., 2002). During recovery phase from stress, eEF2K is degraded by the ubiquitin-proteasome system, thus allowing resumption of translation. Therefore, eEF2 activity is tightly regulated during growth and stress conditions (Kruiswijk et al., 2012; Wiseman et al., 2013).

In addition to regulation of elongation factors, growing evidence also suggests that ribosomes pause during heat oxidative and proteotoxic stress. Using ribosome profiling, it was shown that pausing generally happens in the first 100 bases of the codon. Moreover, association of Hsp70 with the ribosomes and translation machinery is reduced in the presence of stress (Shalgi et al., 2013). Based on these observations, it is proposed that ribosome-associated chaperones assist in nascent peptide folding near the exit tunnel, but this association is reduced during stress. Thus, exposed nascent peptides emerging from the ribosomes signal translation pausing (Shalgi et al., 2013). Although the molecular basis for translation elongation pausing is

not fully understood, widespread elongation pausing helps to maintain protein homeostasis under stress.

RNA modifications: Most tRNAs and rRNAs are extensively post-transcriptionally modified on the four canonical bases. Although most of these modifications are not essential, increasing evidence suggests that they play regulatory roles, especially during stress (Karijolich and Yu, 2015; Kirchner and Ignatova, 2015; Yacoubi et al., 2012). In tRNA, the greatest diversity is found at position 34 (the wobble position) and position 37 (3′ side of the anticodon). These positions are critical for codon-anticodon pairing and thus modifications at these sites alter translation accuracy (Gu et al., 2014; Hou et al., 2015). For instance, upon oxidative stress, the distribution of 5-methylcytosine modification increases at the wobble position of tRNA Leu (CAA). This modification allows for selective translation of mRNAs from genes enriched in the TTG codon (Chan et al., 2012). In addition to tRNA modifications, stress-induced fragmentation of tRNA is also observed. Stress induced nucleases such as angiotensin specifically cleave tRNAs within the conserved 3′ CCA termini, thus inhibiting translation. Cleaved -CCA ends are repaired by a CCA-adding enzyme during the stress recovery phase (Czech et al., 2013).

Similar to tRNA, rRNAs are dynamically modified in response to stress. Methylation of 28S by methytransferase NSUN5 results in more efficient translation of stress responsive mRNAs under oxidative conditions (Schosserer et al., 2015). Together, these studies highlight the stress induced reprogramming of RNA modifications and their role in translation control.

Selective translation during stress

Eukaryotic cells preferentially translate proteins needed for survival and cellular homeostasis during stress. Discussed below are various mechanisms that allow cells to

synthesize proteins during stress conditions.

Non-canonical cap independent translation: All cellular mRNAs do not contain a 5' cap. These mRNAs are preferentially translated in a cap-independent mode of translation initiation known as IRES mediated translation, where 40S ribosome is directly recruited to the vicinity of the start codon without the requirement of some or all initiation factors (Hellen and Sarnow, 2001; Komar and Hatzoglou, 2011). The mRNA regions required for this 40S ribosome recruitment are known as internal ribosome entry sites (IRES). IRES sequences form complex secondary and tertiary structures that allow them to interact with a subset of canonical translation initiation factors and mediate cap-independent translation. In addition, a number of RNA-binding proteins known as IRES trans-acting factors also modulate IRES-mediated translation (Hellen and Sarnow, 2001; Komar and Hatzoglou, 2011; Martínez-Salas et al., 2012). A growing body of evidence indicates that IRES mediated translation is required for the synthesis of key regulatory proteins in situations when cap-dependent translation is impaired, such as mitosis, apoptosis or cellular stress. In support of this, stress-responsive transcripts coding for Hsp70, p53, c-Myc, Bcl2, XIAP, VEGF, HIFα etc. contain IRES elements in their 5′ UTR and their translation is believed to be IRES-dependent (Bornes et al., 2007; Holčík et al., 2003; Lang et al., 2002; Sherrill et al., 2004; Spriggs et al., 2008; Yang et al., 2006a). Collectively, IRES-mediated translation provides a mean to escape global decline in protein synthesis by allowing selective synthesis of key regulatory genes involved in cell cycle, stress response and apoptosis.

Regulatory upstream ORFs: Many cellular mRNAs contains one or many upstream open reading frames (uORFs) that precede the initiation codon of the main coding regions. These uORFs have been shown to impact gene expression by either regulating the translation efficiency of the main ORF or by modulating mRNA decay (Barbosa et al., 2013; Morris and Geballe, 2000). One of

the best-studied examples is the yeast transcription factor Gcn4. The 5'UTR of *GCN4* contains four uORFs that directly regulate translation of *GCN4* mRNA. In the absence of stress, uORFs are efficiently translated due to rapid loading of translation initiation factors and ribosome recruitment to the mRNA. However, ribosomes fail to reinitiate at the main ORF due to termination at these uORFs and thus Gcn4 protein is not made. Under stress condition, due to limitation of ternary complex, ribosomes scan and bypass through uORFs and reach the *GCN4* initiator codon (Abastado et al., 1991; Dever et al., 1992; Holcik and Sonenberg, 2005; Mueller and Hinnebusch, 1986). This mechanism allows rapid translation of *GCN4* mRNA in response to stress. In addition to *GCN4*, transcripts coding for ATF4, GADD34 and CHOP (All proteins involved in stress response) have uORFs and their protein synthesis is regulated by eIF2α phosphorylation (Barbosa et al., 2013).

Ribosome heterogeneity: Ribosomes are traditionally viewed as protein synthesis machines with little or any regulatory roles. However, emerging evidence challenges this view and suggests that ribosomes can regulate gene expression by modulating the initiation and elongation rate of specific mRNA in response to various stimuli. Ribosome heterogeneity is thought to be generated by altering the composition as well as post-transcriptional modification of ribosomal protein and/or rRNA (Filipovska and Rackham, 2013; Slavov et al., 2015; Xue and Barna, 2012). For instance, a recent study found that mitochondrial ribosomal protein MRPL-18 contains a hidden CUG start codon downstream of the main initiation codon. Under heat stress conditions, a cytosolic isoform of MRPL-18 is generated by alternative translation initiation at CUG codon. This cytosolic isoform is incorporated into the 80S ribosome complex and facilitates synthesis of Hsp70 protein (Zhang et al., 2015b). Thus 'specialized ribosomes' ensure efficient translation of stress-responsive mRNAs under unfavorable conditions.

In summary, these studies reveal that translation is reprogrammed at various levels to ensure that proteins are synthesized at the right time and at the right place and that cellular homeostasis is maintained during changing conditions.

Regulation of gene expression by stress granules

Stress reprograms translation to inhibit global protein synthesis and to selectively translate mRNAs necessary for adaptation and damage repair. This global inhibition of protein synthesis results in translationally stalled mRNA and protein complexes that are redirected to cytoplasmic foci known as stress granules (SGs) (Buchan and Parker, 2009; Kedersha and Anderson, 2009). SGs are cytoplasmic, non-membrane-bound reversible aggregates of mRNA and protein complexes that are observed in a wide variety of cells and organisms (Figure 1.5A and 1.5B). SGs assemble when translation is impaired either due to stress, drugs that inhibit translation initiation or due to overexpression of certain RNA-binding proteins that act as translation repressors (Anderson, 2006; Buchan and Parker, 2009; Kedersha and Anderson, 2002, 2009) (Figure 1.5A and 1.5B). SG formation is initiated by and dependent upon phosphorylation of eIF2α at serine 51 in most cases. Thus, expression of a phosphomimetic mutant of eIF2α (S51D) induces SG assembly, while expression of a phosphodead eIF2α (S51A) prevents translation suppression and SG assembly (Kedersha et al., 1999; McEwen et al., 2005). However, drugs such as pateamine and hippuristanol, that suppress translation by inhibiting eIF4A, independent of eIF2α, also initiate SG assembly (Mazroui et al., 2006). Thus, SG formation requires a non-translating stalled pool of mRNPs.

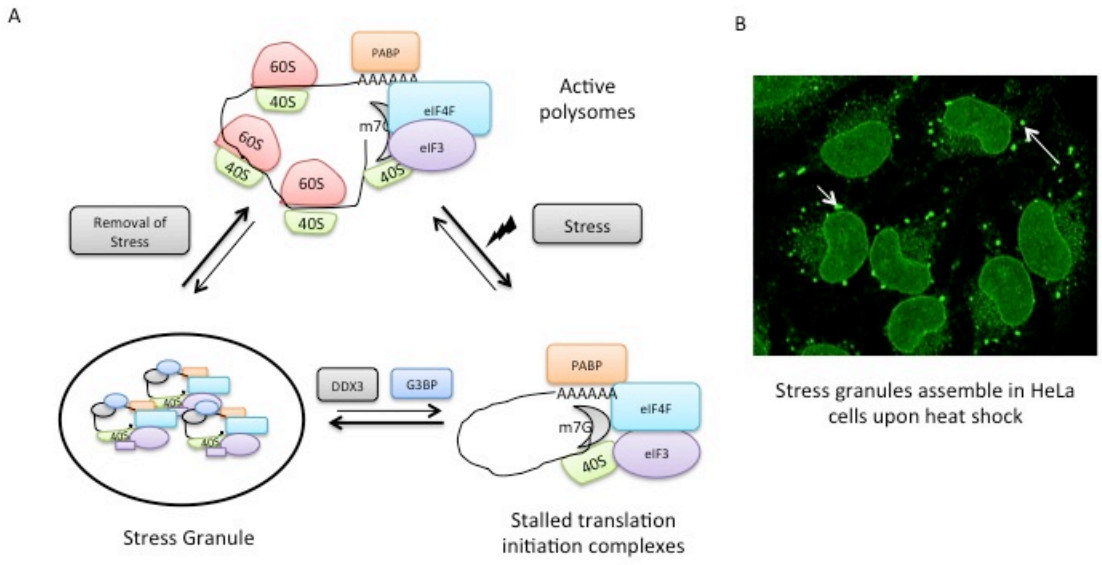


Figure 1.5: SGs are assembled upon translation arrest under stress conditions.

(A) Global inhibition of translation initiation results in accumulation of stalled translation initiation complexes. These complexes with the help of RNA-binding proteins are redirected to cytoplasmic foci known as SGs. SG are dynamic in nature and they disassemble after the removal of stress. (B) Assembly of SGs in Hela cells upon heat stress. HeLa cells were heat shocked for 60 min at 45^0C and processed for immunofluorescence using anti-hGle1 antibodies. Arrows point to SGs.

Compositions of SGs

The prototypical SG constituents are mainly poly $(A)^+$ mRNA, 40S ribosomal subunit, translation initiation complexes (eIF2, eIF3, eIF4A, eIF4E and eIF4G) and RNA-binding proteins involved in mRNA structure and function (Buchan and Parker, 2009; Kedersha and Anderson, 2002, 2009; Kedersha et al., 1999). In addition, several cell signaling molecules such as TRAF2, mTORC, stress-activated JNK kinase, HDAC6 etc. are found in SGs (Kedersha et al., 2013) . Transcripts coding for housekeeping genes are retained in SGs, while *HSP70* mRNA encoding for stress-specific chaperones are excluded from SGs (Kedersha and Anderson, 2002; Zurla et al., 2011). However, the molecular composition of SGs is variable and is dependent upon the type of stress. For example, TTP and Hsp27 are found in SGs upon heat shock but are excluded during oxidative stress (Cuesta et al., 2000; Kedersha et al., 1999; Stoecklin et al., 2004). Interestingly, recent studies also suggest that SG morphology and composition change during the course of the stress response (Yang et al., 2006b).

Regulation of SGs assembly and disassembly

SG assembly process begins as numerous small microscopically visible foci, which progressively coalesce together to form several large complexes. The complexes that comprise these SGs disperse once the stress is over. SGs are highly dynamic structures and their assembly and disassembly is regulated by several mechanisms. One of the mechanisms is by regulation through posttranslational modifications of proteins, as it provides a rapid response and new protein synthesis is not required. Recent studies from various laboratories show that SG components undergo various types of protein modifications such as acetylation, phosphorylation, ubiquitylation and methylation, and these modifications influence the recruitment and function of

SG components (De Leeuw et al., 2007; Kwon et al., 2007; Stoecklin et al., 2004). For example, phosphorylation of RNA-binding proteins TTP and G3BP reduce their localization in SGs (Stoecklin et al., 2004; Tourrière et al., 2003). Importantly, protein modifications were also shown to determine a protein's propensity to remain soluble or assemble into the SGs and hence regulate granule assembly in a spatial and temporal manner (Kato et al., 2012).

A second aspect that influences assembly and disassembly of SGs is the dynamic protein-protein and RNA-protein interactions. Recent studies suggest that SGs are liquid droplets and assemble by liquid-liquid phase transition. Many of the RNA-binding proteins have conserved prion or QN rich domains that allow proteins to self-aggregate and promote phase separation, thus facilitating SGs assembly (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). Loss of these domains result in inhibition of SG assembly (Gilks et al., 2004; Kedersha et al., 1999). Similarly, a recent study identified low complexity amino-acid sequences that are necessary and sufficient for reversible assembly and disassembly of granules (Kato et al., 2012). Notably, this observation suggests that differential homotypic and heterotypic interactions could promote partitioning of components into granules with distinct compositions that coexist in the cytoplasm.

Another regulatory mechanism to control SG formation is via cytoskeletal elements. Microtubules facilitate fusion of smaller granules into larger complexes by actively transporting SG components. Therefore, disruption of either microtubules or knockdown of microtubule motor proteins like kinesin and dynein inhibit SG assembly and disassembly (Ivanov et al., 2003; Loschi et al., 2009; Nadezhdina et al., 2010). Taken together, the nature of the stress response and the interactions of various mRNPs likely define a granule and determine the assembly order for SGs.

SGs are reversible complexes and disassemble after removal of stress (Figure 1.5). SG disassembly is thought to be promoted by re-initiation of translation (Kedersha et al., 2000). Certain mRNAs in the SGs enter into translation during stress recovery and thus promotes SG disassembly. Moreover, mRNAs can also be exchanged with P bodies for decay. This mechanism is supported by observations that inhibiting mRNA decay machinery led to an increased number of SGs in yeast and various mRNA decay components such as TTP and XRN1 are found in SGs (Buchan et al., 2008; Kedersha et al., 2005; Stoecklin et al., 2004). Therefore, there exists a dynamic exchange of mRNPs between SGs and P bodies. Furthermore, various chaperones influence SG disassembly (Cherkasov et al., 2013; Gilks et al., 2004; Kedersha et al., 1999; Mazroui et al., 2007). Finally, recent studies suggest that SG clearance could be promoted by autophagy. Several components that play a role in autophagy are found in SGs. Moreover, SG disassembly is inhibited by the silencing of these components or the expression of altered proteins arising from disease-linked mutations, suggesting that autophagy plays a critical role in disassembly of SGs (Buchan et al., 2013; Seguin et al., 2014).

Functions of SGs

The SGs are proposed to function in global translation repression by storing translationally repressed mRNAs since their formation correlates with global reduction of protein synthesis. However, recent studies suggest that SG formation is not required for global inhibition of protein synthesis and could be uncoupled from translation by disruption of SG formation (Hofmann et al., 2012; Loschi et al., 2009; Ohn et al., 2008). Thus, translation regulates SG formation but SGs are not necessary for translation arrest. This raises the question as to why mRNPs aggregate into SGs? SG formation results in higher local concentration of various SG

proteins. SGs also contain stalled mRNAs- translation initiation complexes. This is thought to facilitate mRNP assembly or rearrangement driven by these factors. Therefore, SGs could promote assembly of translation initiation complexes, thus enabling efficient translation re-initiation after the stress is over (Moeller et al., 2004). Another consequence of mRNP aggregation is to stabilize mRNAs during stress. Several RNA binding proteins that stabilize mRNAs such FMRP, Staufen1, HuR and TIA1 are found in SGs and it is proposed that sequestration in SGs may prevent decay of mRNAs (Gallouzi et al., 2000; Gilks et al., 2004; Mazroui et al., 2002; Thomas et al., 2009). For instance, recruitment of *p21* mRNA and its co-activator CUGBP1 to SGs stabilizes *p21* during oxidative stress. (Lian and Gallouzi, 2009). Despite this, SGs are dynamic entities whose components move in and out of SGs. Various studies have demonstrated that SG proteins like TTP, TIA-1, G3BP and poly $(A)^+$ mRNAs have a short half-life inside SGs (Kedersha et al., 2005). Moreover, SGs are in dynamic equilibrium with the translation complexes (Kedersha et al., 2000). Thus, SGs do not just store mRNA, but are proposed to act as mRNA triage centers where mRNPs are remodeled, packaged and sorted for storage, decay and re-initiation for translation.

SGs are also recently proposed as RNA-centric signaling hubs analogous to receptor mediated multi-protein signaling networks. Various cell-signaling molecules are found in SGs including adaptor/scaffold proteins, kinases, phosphatses, helicases, ribonucleases, GTPases, nucelocytoplasmic shuttling proteins, and ubiquitin modifying enzymes (Kedersha et al., 2013). Localization of these signaling molecules in SGs reduces their cytosolic/nuclear concentration and likely alters the cellular signaling. For example, mTORC localization in SGs inhibits cell growth (Sahoo et al., 2012; Wippich et al., 2013) while Wnt recruitment influences cell polarity.

Therefore, by sequestering various signaling molecules, SGs also serve to modulate signaling pathways and impact growth, metabolism and cell polarity.

Finally, SG formation is linked to cell survival during a stress response. For example, sequestration of TRAF2 and RACK1 molecules in the SGs inhibits apoptosis. TRAF2 is an adaptor protein that links the TNFα signaling cascade to NF-kB activation in the nucleus, resulting in an inflammatory response and apoptosis. In response to heat stress, eIF4G recruits TRAF2 to the SGs and thus prevents NF-kB activation (Kim et al., 2005). Similarly, RACK1 acts as a scaffold and facilitates activation of MTK1. MTK1 is a mitogen-activated kinase that acts upstream of JNK and promotes apoptosis. Sequestration of RACK1 in SGs prevents activation of MTK1 and cell survival is promoted (Arimoto et al., 2008). Thus, SGs link mRNP regulation to cell survival by sequestering and nullifying key apoptosis-promoting factors.

Aberrant SG formation links with diseases

SGs are highly dynamic structures that play an important role in RNA metabolism, cell growth and survival. Therefore, it is not surprising that SGs are also observed in various disease states. For example, certain viruses like HTLV exploit SGs to support chronic infection by switching SG assembly on and off with the help of the viral protein Tax (Legros et al., 2011). Similarly, cancer cells form SGs in response to hypoxia to sequester HIF1α regulated transcripts. Upon reoxygenation, these transcripts are rapidly translated and promote radio-resistance in cancer cells upon irradiation (Moeller et al., 2004). Moreover, SG formation in cancer cells promotes cell survival against chemotherapeutic agents (Gareau et al., 2011; Kaehler et al., 2014; Somasekharan et al., 2015). In addition to above-mentioned diseases, SGs have also gained considerable attention due to their link with various neurological diseases (Li et al., 2013;

Ramaswami et al., 2013; Vanderweyde et al., 2013). Mutations in genes encoding for several SG components like FMRP, FUS, ataxin, VCP and TDP43 are associated with various neurological diseases (Bosco et al., 2010; Elden et al., 2010; Johnson et al., 2010; Millecamps et al., 2010; Sreedharan et al., 2008; Verkerk et al., 1991). It is proposed that aberrant or persistent SG formation is one of the underlying causes of these diseases. This hypothesis is supported by observations that 1) Cytoplasmic foci or aggregates are often seen in affected cells that resemble compositionally to SGs (Johnson et al., 2009; Wolozin, 2012). 2) RNA binding proteins linked with neurodegenerative diseases such as hnRNPA1 or TDP-43 promote a hyper-aggregated state and aberrant SG assembly (Johnson et al., 2009; Kim et al., 2013). 3) Expression of altered proteins arising from disease-linked mutations, that are required for clearance of SGs, lead to failure of SG clearance in cells and promotes neurodegeneration (Buchan et al., 2013). 4) Knockdown of SG assembly factors reduce the toxic effects caused by exogenous expression of disease linked-proteins (Ritson et al., 2010; Sun et al., 2011a). Therefore, aberrant SG formation interferes with the normal RNA metabolism, cell signaling and growth, and is toxic to cells. Determining the underlying molecular mechanisms of SG assembly and disassembly will be crucial to decipher the roles of SG in stress and diseases.

Regulation of mRNA life cycle by DEAD-box RNA helicases

For proper gene expression, an mRNA should contain information for its export, localization, protein synthesis and stability. In addition to the coding sequence of an mRNA, the information is provided by the association of specific RNA-binding proteins. Each mRNA has a unique complement of RNA-binding proteins and this ‘mRNP code’ determines mRNA fate and function (Mitchell and Parker, 2014). The association of RNA-binding proteins is dynamic and

thus the protein composition changes during mRNA life cycle. This ensures that each step of gene expression is coupled and provides quality control (Glisovic et al., 2008; Lunde et al., 2007; Mitchell and Parker, 2014). A class of RNA-binding proteins, known as DEAD-box proteins (Dbps), plays crucial roles in maintaining a proper and specific mRNP code and thus regulates every aspect of RNA metabolism from transcription and translation to mRNA decay (Linder and Jankowsky, 2011; Rocak and Linder, 2004).

Dbps are ATP-dependent RNA helicases. They unwind inter-or intra-molecular RNA structures and/or dissociate RNA- protein complexes in an ATP dependent manner. Dbps are an evolutionary conserved family of proteins and found in animal and plant kingdom. Some viruses such as HTLV also encode for these proteins for their own replication. Dbps belong to superfamily II, the largest family of RNA helicases (Jarmoskaite and Russell, 2014; Linder and Jankowsky, 2011; Rocak and Linder, 2004). These proteins consist of a helicase core surrounded by divergent amino and carboxy-terminal regions. The core is made up of two identical domains that resemble the bacterial recombinase protein RecA. Within this core, there are at least 12 conserved motifs. Motif II is known as DEAD motif (Asp-Glu-Ala-Asp) and is conserved at similar positions in various members of this family (Caruthers and McKay, 2002; Linder et al., 1989; Singleton et al., 2007). The two Rec A domains are flexible and the ATP binding pocket is located within between both domains. This ATP binding pocket is generally available when the protein is bound to RNA. The RNA binding motif is lined by positively charged amino acids and can bind six nucleotides of single-strand RNA. The recognition of RNA is mediated by the sugar –phosphate backbone of the RNA thus explaining how Dbps usually bind RNA in a sequence-independent manner (Jarmoskaite and Russell, 2014; Linder and Jankowsky, 2011; Rocak and Linder, 2004). Discussed below are two modes of action through which Dbps function.

Non-processive RNA unwinding: Dbps usually do not translocate along the RNA but contact the RNA duplex directly, suggesting very low processivity. Recent work with DEAD-box Mss116p from *S.cerevisiae* sheds light about the molecular mechanism of RNA unwinding by Dbps. In this model, at the beginning of cycle, RNA and ATP bind to two separate RecA domains. Simultaneously binding of two ligands results in closure of the domain and hydrolysis of ATP. Domain closure also results in exclusion of one strand of RNA, bending of other, and thus local unwinding of RNA. Importantly, ATP hydrolysis is not required for RNA unwinding but release of RNA from the helicase, thus recycling of the enzyme (Mallam et al., 2012). In addition to unwinding, some Dbps can anneal two strands of RNA to form a duplex, as has been shown by Rok1 enzyme (Young et al., 2013).

Remodelling of RNP complexes: In addition to local unwinding duplexes, Dbps also remove protein from assembled mRNPs. One such example is Dbp5. Dbp5, in association with Gle1-IP_6, functions in the terminal step of mRNA export by removing selective export factors from the exporting mRNA. The selective removal of proteins ensures unidirectional export of mRNA from the nucleus. (Alcázar-Román et al., 2006; Folkmann et al., 2011; Tran et al., 2007; Weirich et al., 2006a) Similarly another Dbp, Ded1/DDX3, displaces proteins from the mRNA. Interestingly, Ded1 also shows selectivity (Bowers et al., 2006). Importantly, these rearrangements of RNA brought about by Dbps are tightly regulated.

DEAD-box proteins in stress response

As discussed above, an 'mRNP code' determines fate and function of an mRNA. Not surprisingly, extensive remodeling of mRNPs occurs during stress and various Dbps are involved this process (Linder, 2006; Linder and Jankowsky, 2011; Owttrim, 2006) . One of the best-

studied examples is DDX3 protein. DDX3 is a multifunctional Dbp that is ubiquitously expressed in metazoans. Similar to other Dbps, it exhibits ATP-dependent RNA helicase activity and can unwind long (~ 50 nucleotides) stretches of dsRNA. Notably, flanking carboxy and amino- terminal regions are required for full ATPase activity and may regulate its catalytic activity (Sharma and Jankowsky, 2014; Shih et al., 2012; Yedavalli et al., 2004). DDX3 is a nucleocytoplasmic shuttling protein and interacts with nuclear export factors such as Crm1 (a receptor for protein containing the nuclear export signal) and Tip-associated protein (TAP, an mRNA export factor) (Lai et al., 2008). Work from various laboratories shows that DDX3 is involved in various aspects of RNA metabolism such as transcription, splicing, mRNA export and translation initiation (Geissler et al., 2012; Merz et al., 2007; Soto-Rifo and Ohlmann, 2013; Soto-Rifo et al., 2012; Yedavalli et al., 2004).

Under stress conditions, DDX3 is recruited to SGs and regulates the stress response. DDX3 is believed to be a core assembly factor of SGs. Depletion of DDX3 inhibits SG assembly under heat stress (Shih et al., 2012). However, one study found that DDX3 depletion did not affect SG assembly under arsenite condition (Lai et al., 2008). This difference suggests that DDX3 may function in a stress-specific manner. In addition, overexpression of DDX3 in mammalian cells causes constitutive SG formation similar to the yeast orthologue Ded1 ((Hilliker et al., 2011; Lai et al., 2008), suggesting that DDX3 could nucleate SG assembly. Surprisingly, alterations in DDX3 protein that impair its ATPase or helicase activity do not affect SG assembly. Instead the amino-terminal region containing an eIF4E-binding motif is required for its incorporation into the SGs. Furthermore, an eIF4E-binding-defective DDX3 protein is impaired in SG assembly, suggesting that interactions with eIF4E are critical for its functions in SGs (Shih et al., 2012). Notably, Ded1 modulates translation by assembling and remodeling the

eIF4F-mRNA complex in translation initiation. Similar to DDX3, Ded1 assembles the eIF4F-mRNA complex in an ATP-dependent manner in yeast. However, ATPase activity is needed during the disassembly of the mRNPs and initiation of translation (Hilliker et al., 2011). Thus, it will be interesting to know if DDX3 disassembles SGs in an ATP-dependent manner. Depletion of DDX3 from human cells also results in reduced survival demonstrating the critical role of DDX3 in coordinating protein synthesis, SG formation and cell survival.

Considering regulation of RNA processing is a critical step during gene expression, DEAD-box helicases have emerged as key players in coordinating the various steps of RNA metabolism under stress. We have just begun to understand the role of these proteins in stress response and future research will be focused on identifying the biological targets of these proteins and how their activities are regulated in response to stress. With the advent of new technology in RNA biology, this will shed light on the diverse functions of these helicases in growth and survival.

Regulation of DEAD-box proteins by Gle1

Dbps play critical roles in most, if not all, aspects of RNA metabolism and regulation. Since they use energy derived from ATP hydrolysis to mediate RNA rearrangements, their ATPase activity must be spatially and temporally regulated to ensure proper execution of gene expression. Various co-factors have been identified that modulate the ATPase activity of Dbps (Feoktistova et al., 2013; Gustafson and Wessel, 2010; Linder and Jankowsky, 2011; Young et al., 2013). Gle1 is one such co-factor.

Gle1 is an essential multifunctional protein first identified as an mRNA export factor in *S. cerevisiae* (Murphy and Wente, 1996). It is a highly conserved protein in eukaryotic

organisms. Over the years, extensive work in the yeast and human model systems has provided important insight into the functions of Gle1 in regulation of gene expression. Specifically, these studies reveal that Gle1 functions in mRNA export and translation processes by regulating distinct Dbps (Alcázar-Román et al., 2006; Bolger and Wente, 2011; Bolger et al., 2008; Folkmann et al., 2011, 2013, 2014; Noble et al., 2011; Tran et al., 2007).

In yeast (y), Gle1 functions in the terminal step of mRNA export by modulating the ATPase activity of Dbp5. Work from our laboratory and several others has provided important insights into the molecular mechanisms of how Gle1 regulates the ATPase cycle of Dbp5 at the NPC (Montpetit et al., 2011; Noble et al., 2011; Tran et al., 2007; Weirich et al., 2006a). Both yGle1 and Dbp5 are targeted to the cytoplasmic face of the NPC through specific interactions with Nup42 and Nup159, respectively. It is proposed that yGle1 together with IP_6 promotes ATP binding to Dbp5. ATP-bound Dbp5 has the highest affinity towards RNA. mRNP binding stimulates both the release of yGle1-IP_6 and ATP hydrolysis. The ATP-ADP conversion drives a conformational change in Dbp5 facilitating mRNP remodeling and release of RNA from Dbp5. Finally, Nup159 promotes ADP release from Dbp5, thus recycling the enzyme (Folkmann et al., 2011).

Following mRNA export, yGle1-IP_6 also positively regulates Dbp5 during translation termination. yGle1 and Dbp5 physically interact with the eukaryotic release factor eRF1, and *dbp5* and *gle1* mutants show defects in translation termination (Alcázar-Román et al., 2010; Bolger et al., 2008). Intriguingly, yGle1 also plays a role in the initiation step of translation by physically interacting with eukaryotic initiation factor eIF3 and negatively regulating Ded1. Ded1 is proposed to function in translation initiation by unwinding long structured 5'UTR regions of mRNA and by promoting start site recognition. Interestingly, yGle1 inhibits ATPase

activity of Ded1 in an IP_6-independent manner and this regulation is required for proper translation initiation (Bolger and Wente, 2011). Together, these studies in yeast demonstrate that Gle1 regulates key stages of gene expression by differentially modulating Dbps (Dbp5 and Ded1).

In human (h) cells, the *GLE1* gene is alternatively spliced to generate at least two isoforms – hGle1A and hGle1B (Kendirgi et al., 2003; Watkins et al., 1998). hGle1B is the predominant isoform in HeLa cells and is mainly localized at the nuclear envelope through its interactions with Nup155 and hCG1 nucleoporins. hGle1A is similar in sequence to hGle1B except at the carboxy-terminus where it lacks the hCG1-binding site. At steady state, hGle1A is mainly localized at the cytoplasm (Figure 1.6A and 1.6B). However, both isoforms shuttle in and out of the nucleus and this shuttling activity is dependent upon an internal 39 amino acid residues common to both isoforms (Figure 1.6A) (Watkins et al., 1998; Kendirgi et al., 2003, 2005; Rayala et al., 2004). Similar to yGle1, hGle1 functions in the export of poly (A^+) mRNAs and Hsp70 mRNAs. Importantly, inhibition of the shuttling activity of hGle1 results in accumulation of poly (A^+) mRNAs in the nucleus suggesting that shuttling of hGle1 is essential for its role in mRNA export (Watkins et al., 1998; Kendirgi et al., 2003, 2005). Using biochemical and cell culture model systems, our recent work also shows that hGle1 self-associates through its conserved coiled-coil domain. Notably, this self-association is required for hGle1 function in mRNA export. Furthermore, using siRNA-add back experiments, hGle1B isoform is sufficient and necessary for mRNA export (Folkmann et al., 2013). However, function of the hGle1A isoform is unclear.

In addition to mRNA export, hGle1 also physically interacts with the eIF3f subunit (Bolger et al., 2008). Although a direct role of hGle1 translation has not been shown, this data

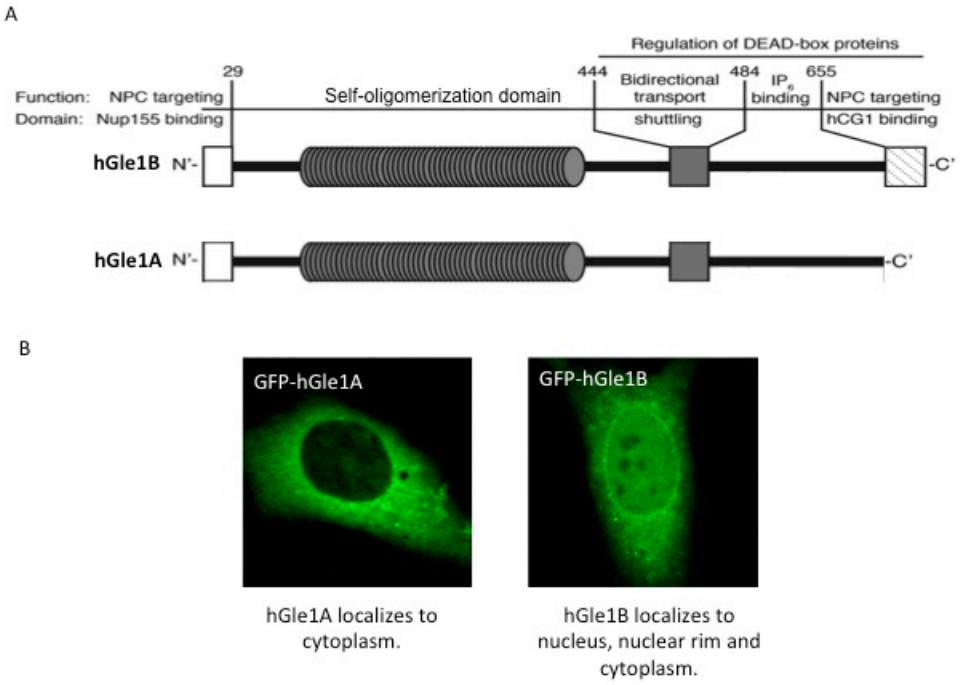


Figure 1.6: *GLE1* is alternatively spliced to generate two isoforms in human cells.

(A) Domain map of hGle1 isoforms. Both isoforms are highly similar in sequence except hGle1A isoform lacks hCG1-binding domain. Figure is adapted from (Kendirgi et al., 2003; Folkmann et al., 2013). (B) Steady-state localization of hGle1 isoforms. *GFP-hGLE1A* and *GFP-hGLE1B* plasmids were expressed in HeLa cells and localization of GFP-tagged hGle1A and hGle1B proteins was visualized by live cell microscopy.

suggests that hGle1 might have a conserved role in translation. Taken together, these studies suggest that the Gle1 protein across different kingdom performs similar functions.

hGle1 dysfunction in neurological diseases

RNA-binding proteins are involved in various aspects of the mRNA life cycle and influence its fate and function. Not surprisingly, mutations in genes encoding for various RNA-binding proteins are linked with various diseases including cancer and neurodegeneration (Lukong and Fatimy, 2012; Ramaswami et al., 2013). Interestingly, mutations in *GLE1* are linked with amyotrophic lateral sclerosis (ALS) and lethal congenital contracture syndrome 1 (LCCS-1)(Kaneb et al., 2015; Nousiainen et al., 2008).

LCCS1 is an autosomal recessive disorder characterized by lack of mobility of the fetus. LCCS1 invariably leads to prenatal death by 32 weeks of gestation. The major mutation Fin_{major} is a single nucleotide substitution that generates an illegitimate splice acceptor site in *GLE1*. This aberrant splicing results in an in-frame insertion of three amino acids in hGle1 protein (Nousiainen et al., 2008). It was believed that LCCS1 disease is defects in motor neuron development. However, modeling of this disease in zebrafish showed that defects are both neurogenic and non-neurogenic in nature (Jao et al., 2012). Moreover, recent work from our laboratory shows that hGle1-self association and mRNA export are perturbed with Fin_{major} protein (Folkmann et al., 2013). Taken together, this study provided the evidence for altered hGle1 function at the mRNA export as an underlying cause of LCCS1 pathology.

Apart from the hgle1-Fin_{major} mutation, Kaneb and colleagues found two deleterious mutations in *GLE1* that are linked with ALS pathology (Figure 1.7) (Kaneb et al., 2015). ALS is an adult onset, progressive neurodegenerative disease that affects motor neurons in brain and

spinal cord. Gradual degeneration of motor neurons results in muscular atrophy, and eventually respiratory paralysis. There is no known cure for this disease and patients usually die within 3-5 years of diagnosis. Although most cases of ALS are sporadic (sALS), 5-10% are inherited (familial ALS) (Kiernan et al., 2011). The first ALS mutation identified is a non-sense mutation (c. 209C>A) that introduces a premature stop codon in exon 2 of *GLE1*. This results in a truncated version of hGle1 protein (hGle1-S70X) (Figure 1.7). Furthermore, the c. 209C>A mRNA is degraded via the non-sense mediated decay mechanism thus leading to lower levels of hGle1 protein at the NPC and in the cytoplasm. The second mutation is recognized as a splice site mutation in intron 14 of *GLE1* (c.1965-2A>C). This mutation is predicted to result in loss of an acceptor splice site and use of an alternative acceptor site. This results in a shift in the coding sequence and replacement of the last 44 amino acids in hGle1B WT protein with a different 88 amino acid in the protein product (hGle1-IVS14-2A>C) (Figure 1.7) . Moreover, yeast two hybrid data confirms the loss of interaction of hGle1-IVS14-2A>C with hCG1 protein. Interestingly, this ALS-linked hGle1 protein is localized mainly at the cytoplasm similar to hGle1A (Kaneb et al., 2015). Using zebrafish model system, our laboratory further showed that depletion of zGle1 using anti-sense morpholinos results in motor neuron defects. These defects can be rescued by injection of h*GLE1B* WT mRNA but not with mRNA containing c.1965-2A>C and c. 209C>A mutations (Kaneb et al., 2015). Although the underlying mechanism of how *GLE1* mutations lead to ALS is unclear, this study suggests that ALS-linked mutations might alter the levels or cellular pools of hGle1 isoforms.

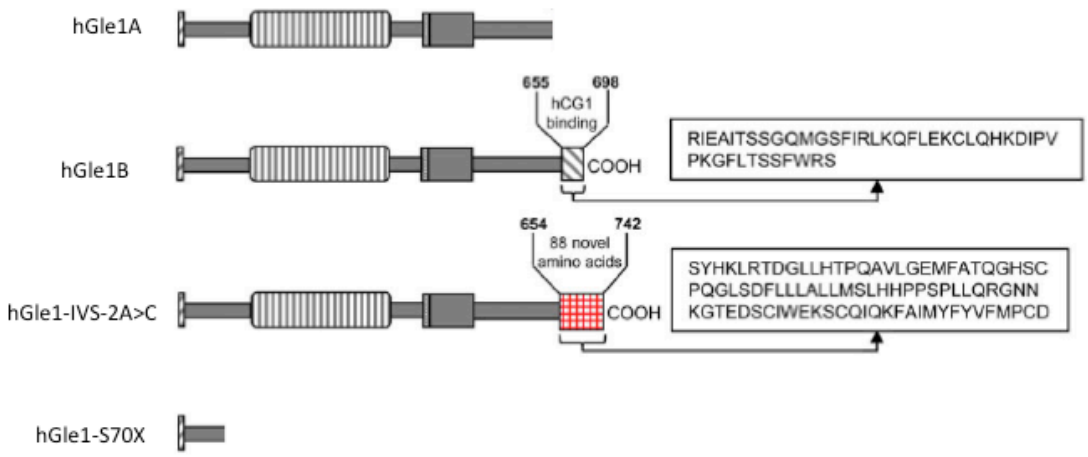


Figure 1.7: Mutations in *GLE1* have been linked with ALS.

Schematic comparisons of hGle1A, hGle1B and ALS-linked hGle1variants. hGle1-IVS-2A>C protein is generated by splice site mutation in intron 14 of *GLE1* gene resulting in shift in reading frame of protein and replacement of hCG1 binding site with an additional novel 88 amino-acid residues. hGle1-S70X protein is a result of mutation in exon 2 of *GLE1* gene that introduces a premature stop codon in the reading sequence and protein product is truncated (Kaneb et al., 2015).

Concluding remarks

Reprogramming of gene expression is central for mounting an appropriate stress response and ensures cell survival. Regulation of gene expression not only provides plasticity and long-term adaptation to stress but also contributes towards adjustment to unforeseen challenges not encountered previously. Not surprisingly, mutations in various genes that regulate distinct steps of gene expression have been linked with diseases including cancer, viral infections and neurological disorders. However, there exists a large gap in our understanding about the molecular mechanisms of stress response. Selective message sorting into distinct fates such as storage, degradation and translation initiation is crucial for cell survival during a stress response. However, how these sorting and remodeling events take place and how the balance between active and stalled translation is maintained are unclear. The work presented here provides insight into the mechanisms by which Gle1, which is thought to be a crucial regulator of the mRNA lifecycle, is involved in assembly of SGs by mediating the transition of mRNPs between SG and translation, and how mutations in *GLE1* alter the cellular pools of isoforms and contribute towards ALS.

CHAPTER 2

Cytoplasmic hGle1A regulates stress granules by modulation of translation

Abstract

When eukaryotic cells respond to stress, gene expression pathways change to selectively export and translate subsets of mRNAs. Translationally-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. Here we document that human (h) Gle1 is a critical regulator of translation during stress. hGle1 is recruited to SGs, and *hGLE1* siRNA-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 wherein SG function requires hGle1A versus 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 ALS patients, these results uncover a paradigm for hGle1A modulating the balance between translation and SGs during stress and disease.

This chapter is adapted from "Cytoplasmic hGle1A regulates stress granules by modulation of translation. Aditi, Andrew W. Folkmann, and Susan R. Wente. Mol Biol Cell. 2015, 26(8):1476-90" and "Gle1 functions during mRNA export in an oligomeric complex that is altered in human disease. Andrew W. Folkmann, Scott E. Collier, Xiaoyan Zhan, Aditi, Melanie D. Ohi, Susan R. Wente. Cell, 2013 155:582-593."

Introduction

Eukaryotic cells modulate gene expression in order to mount optimal stress responses and ensure cell survival. 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 (eIF2 α), which results in stalled translation initiation (Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2009; Spriggs et al., 2010). Moreover, translationally-stalled, mRNA-protein complexes (mRNPs) 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 mRNPs from active translation machinery, with specific mechanisms controlling storage, decay, or re-intiation of translation (Buchan and Parker, 2009; Kedersha and Anderson, 2002; Kedersha et al., 2005; 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 mRNPs 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; Vanderweyde et al.,

2013; Wolozin, 2012). In addition, diverse viruses hijack SG machinery to effectively bias active translation for their own protein synthesis (Bosco et al., 2010; Khaperskyy et al., 2012; Lloyd, 2012, 2013). Further work is required to better understand how interplay between translation and SG dynamics are regulated at the molecular level.

Importantly, the molecular composition of SGs is variable and is dependent upon the type of stress (Balagopal and Parker, 2009; Cuesta et al., 2000; Kedersha et al., 1999; Stoecklin et al., 2004). 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, 2009; 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 Dbps. However, the molecular mechanisms of how Dbps are regulated to modulate SG exchange of mRNPs require 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) (Alcázar-Román et al., 2006; Montpetit et al., 2011; Tran et al., 2007; Weirich et al., 2006b). In the cytoplasm, yGle1 interacts with translation initiation factor eIF3 and modulates the function of a different Dbp, Ded1 (for which the human orthologue is DDX3) (Bolger and Wente, 2011; Bolger et al., 2008; Yedavalli et al., 2004). In contrast, during translation termination, yGle1-inositol hexakisphosphate

interacts with Sup45 (eRF1) to again regulate Dbp5 (Bolger and Wente, 2011; Bolger et al., 2008). Given its regulation of multiple Dbps at distinct steps in the gene expression pathway, we speculated that Gle1 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 nucleus and cytoplasm and differ by only a 39 amino acid carboxy-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 (Folkmann et al., 2013; Kendirgi et al., 2003, 2005; Rayala et al., 2004). 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.

Here we define a specific role for hGle1A during SG formation and translation regulation during environmental stress responses. hGle1 localizes to SGs and hGle1 depletion causes SG assembly and 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., 2015), 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

hGle1B functions in mRNA export

Our work in *S. cerevisiae* has shown that Gle1 play a role in mRNA export. To test if hGle1 function is conserved, we established a siRNA knockdown-add back system in HeLa cells. To deplete endogenous hGle1, siRNAs targeting *GLE1* were employed and Gle1 depletion was confirmed at the protein level by western blot (Figure 2.1A). As a control, siRNA that lack homology to any known mammalian gene were used (CTRL). We next measured the bulk poly $(A)^+$ mRNA accumulation by *in situ* hybridization using Cy3-labeled oligo-dT probe. Control cells showed a widespread distribution of poly $(A)^+$ mRNA in the nucleus and cytoplasm. In contrast, h*GLE1* siRNA-treated cells showed robust nuclear accumulation of poly $(A)^+$ mRNA (Figure 2.1B and 2.1C). Interestingly, expression of siRNA resistant *GFP-hGLE1B*^{R} but not *GFP* alone (mean N/C ratio~ 1.6) rescued the mRNA export defect of hGle1-depleted cells (mean N/C ratio ~ 1.2) (Figure 2.1B and 2.1C). These results argue that mRNA export defect observed in h*GLE1* siRNA-treated cells is due to specific knockdown of hGle1 and further support the conserved role of hGle1 in mRNA export.

Having established the mRNA export assay, we next determined the ability of LCCS1-linked GFP-hgle1B- Fin_{Major} protein to rescue the mRNA export defect in hGle1-depleted cells. Interestingly, addition of siRNA resistant GFP-hgle1B- Fin_{Major} (*GFP-hgle1B- Fin*$_{Major}$ ^{R}) construct failed to rescue mRNA export defects as evident by higher N/C ratio of poly $(A)^+$ mRNA (1.44) compared to *GFP-hGLE1B*^{R} (1.21) in hGle1-depleted cells (Figure 2.1B and 2.1C). Taken together, these results suggested that hGle1B is required for mRNA export but Fin_{Major} perturbs its essential function in mRNA export.

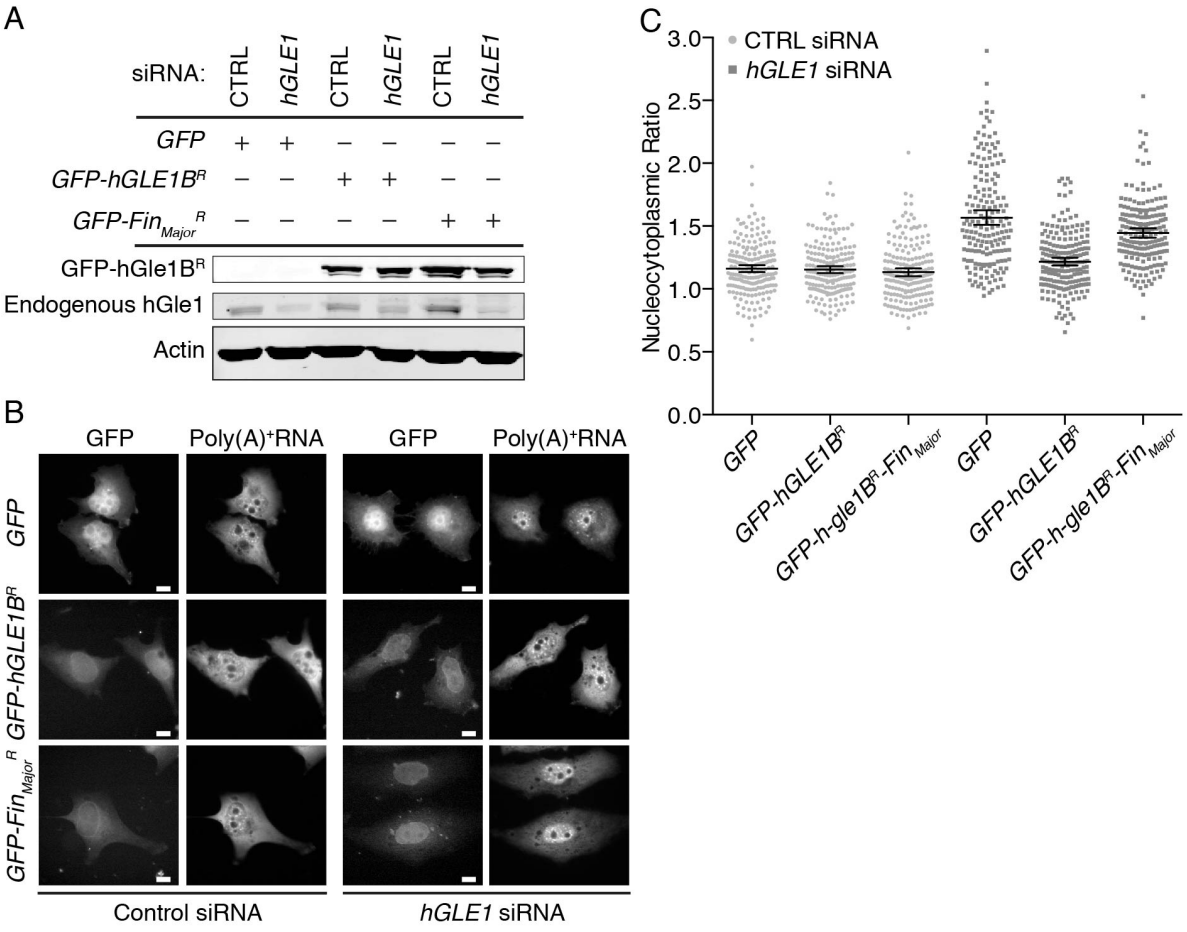


Figure 2.1: hGle1B is required for mRNA export.

(A) *hGLE1* siRNA treatment depletes endogenous hGle1 protein levels. Immunoblot analysis of hGle1 and actin protein levels in control or *hGLE1* siRNA-treated HeLa cells transfected with the indicated GFP-tagged proteins. (B) Nuclear poly(A)$^+$ RNA accumulation in *hGLE1* siRNA-treated cells expressing the indicated GFP-tagged proteins, detected by in situ oligo-dT hybridization and direct fluorescence microscopy. (C) Quantification of the nucleocytoplasmic distribution of poly(A)$^+$RNA in CTRL and *hGLE1* siRNA treated cells transfected with plasmids expressing *GFP*, *GFP-hGLE1B*^{R}, or *GFP-Fin*$_{Major}^R$. Total poly(A)$^+$RNA was detected by in situ oligo (dT) hybridization. The mean Cy3 intensity was determined for the nuclear and cytoplasmic compartment of individual GFP positive cells. Nuclear/cytoplasmic (N/C) ratios were calculated. Error bars represent mean ± 95% confidence interval with n ≥ 175 cells from three independent experiments.

hGle1A and hGle1B isoforms are differentially required during mRNA export

To follow up on our work showing hGle1B rescues mRNA export defects in hGle1-depleted cells (Folkmann et al., 2013), we investigated if hGle1A is sufficient. Immunoblotting confirmed the hGle1 depletion and respective expression of EGFP-hGle1A or EGFP-hGle1B (Figure 2.2A). To 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 *hGLE1* siRNA-treated cells (Figure 2.2B and 2.2C). Expression of *EGFP* alone, EGFP-tagged siRNA-resistant (R) *hGLE1A* (*EGFP-hGLE1A*^{R}), or EGFP-tagged siRNA-resistant *hGLE1B* (*EGFP-hGLE1B*^{R}) plasmids in control 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 2.2B and 2.2C). As reported (Folkmann et al., 2013), expression of *EGFP- hGLE1B*^{R} in *hGLE1* siRNA-treated cells rescued the nuclear poly $(A)^+$ mRNA 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 *hGLE1* siRNA cells (Figure 2.2B and 2.2C).

The steady-state enrichment of hGle1 at the NPC requires hGle1 self-association and interaction with both hNup155 and hCG1 (Folkmann et al., 2013; Kendirgi et al., 2003; Kendirgi et al., 2005; Rayala et al., 2004). 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; Kendirgi et al., 2005). To test this hypothesis, the localization of *EGFP-hGle1A*^{R} and *EGFP-hGle1B*^{R} in CTRL and *hGLE1* siRNA-treated cells was monitored using 3D-structural illumination microscopy in living cells co-expressing the NPC protein Pom121-mCherry.

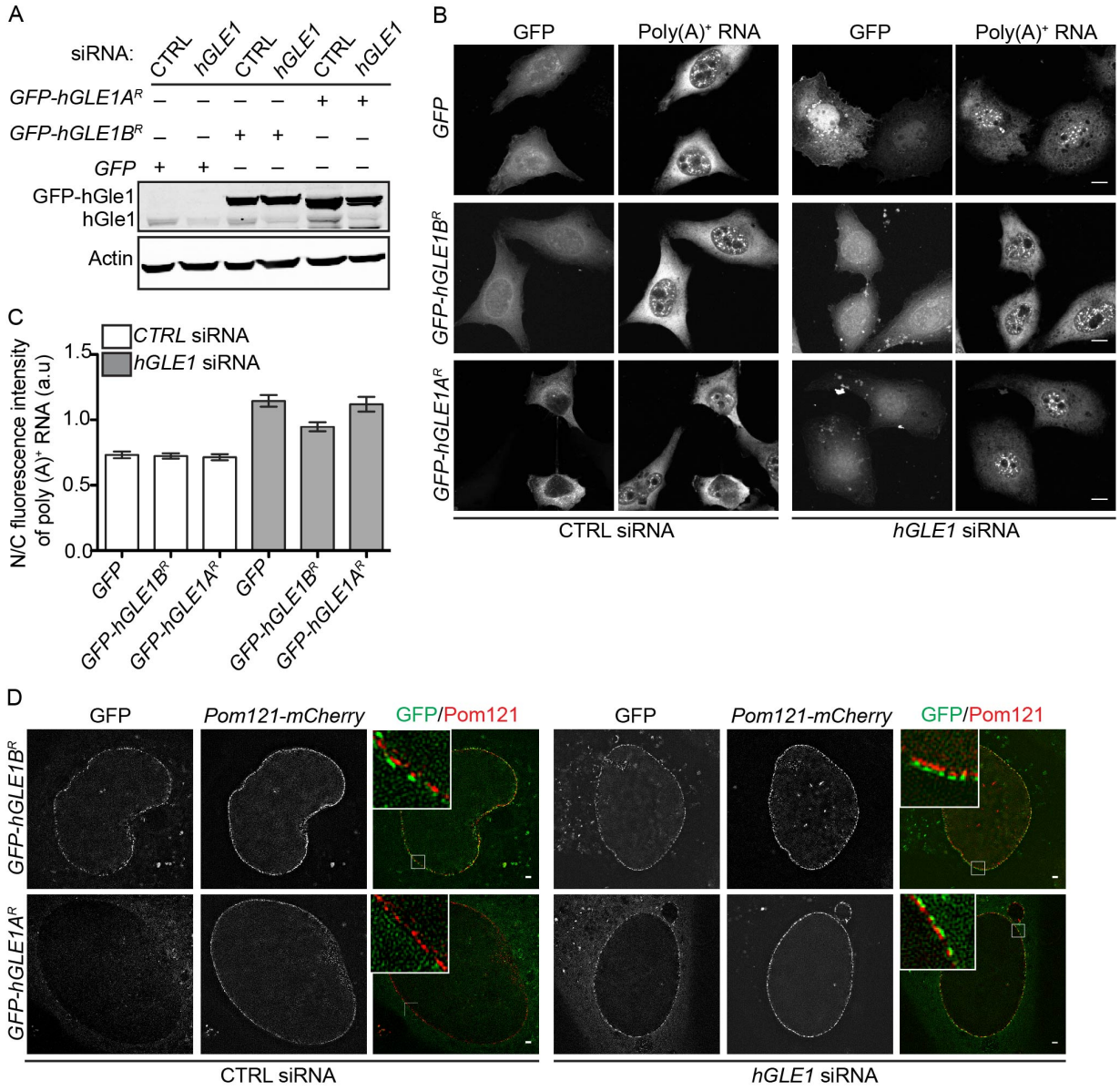

A
siRNA:
CTRL
hGLE1
CTRL
hGLE1
CTRL
hGLE1
GFP-hGLE1A^R
GFP-hGLE1B^R
GFP
GFP-hGle1
hGle1
Actin
B
GFP
Poly(A)+ RNA
GFP
Poly(A)+ RNA
GFP
GFP-hGLE1B^R
GFP-hGLE1A^R
CTRL siRNA
hGLE1 siRNA
C
CTRL siRNA
hGLE1 siRNA
N/C fluorescence intensity of poly (A)+ RNA (a.u)
1.5
1.0
0.5
0.0
GFP
GFP-hGLE1B^R
GFP-hGLE1A^R
GFP
GFP-hGLE1B^R
GFP-hGLE1A^R
D
GFP
Pom121-mCherry
GFP/Pom121
GFP
Pom121-mCherry
GFP/Pom121
GFP-hGLE1B^R
GFP-hGLE1A^R
CTRL siRNA
hGLE1 siRNA

Figure 2.2: hGle1A is not required for mRNA export.

(A) Endogenous hGle1 protein levels are reduced upon *hGLE1* depletion in HeLa cells. *hGLE1* or scrambled CTRL siRNA-treated cells were transfected with indicated EGFP-tagged plasmids and cell lysates were analyzed by immunoblot using anti-hGle1, GFP and actin antibodies. (B) Expression of EGFP-hGle1B but not EGFP-hGle1A rescues mRNA export defects in hGle1-depleted HeLa cells. Nuclear poly $(A)^+$ mRNA accumulation was detected by Cy3 labeled oligo-dT *in situ* hybridization in the CTRL or *hGLE1* siRNA-treated samples expressing *EGFP, EGFP-hGLE1A*^{R}, or *EGFP- hGLE1B*^{R} plasmids. Scale bar =10μm. (C) Quantification of N/C ratio of poly $(A)^+$ RNA in CTRL and *hGLE1* siRNA-treated samples expressing indicated plasmids. Error bars represent mean +/- 95% confidence interval. (D) Both hGle1A and hGle1B localize to the cytoplasmic face of NPC in hGle1-depleted cells. *hGLE1* or CTRL siRNA-treated HeLa cells were transfected with indicated EGFP-tagged plasmids and cells were imaged live using super resolution structural microscopy with Pom121-mCherry. Scale bar =1μm.

Interestingly, in CTRL siRNA cells, EGFP-hGle1B localized at the cytoplasmic face of nuclear envelope, whereas EGFP-hGle1A was not enriched at nuclear envelope (Figure 2.2D). However, in *hGLE1* siRNA cells, both the EGFP-hGle1A and EGFP-hGle1B isoforms were localized at the cytoplasmic face of nuclear envelope (Figure 2.2D). 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 the yGle1 roles in translation initiation and termination, we speculated that cytoplasmically-localized hGle1A was involved in regulating translation. Since SGs and translation are in dynamic equilibrium, and Dbps such as DDX3, DDX1 and eIF4A that play a role in translation are recruited to SGs (Lai et al., 2008; Low et al., 2005; Mazroui et al., 2006; Onishi et al., 2008; Shih et al., 2012), we asked if hGle1A and hGle1B are recruited to SGs upon heat shock stress. To test this, HeLa cells co-expressing *mCherry-G3BP* and either exogenous *EGFP*, *EGFP-hGLE1A*^{R}, or *EGFP-hGLE1B*^{R} were subjected to heat shock at 45°C for 60 min and imaged by live cell microscopy. EGFP-hGle1A and EGFP-hGle1B, but not EGFP, were co-localized to cytoplasmic foci with SG marker mCherry-G3BP (Tourrière et al., 2003) (Figure 2.3A). 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 2.4A). Upon heat shock, hGle1 was detected at

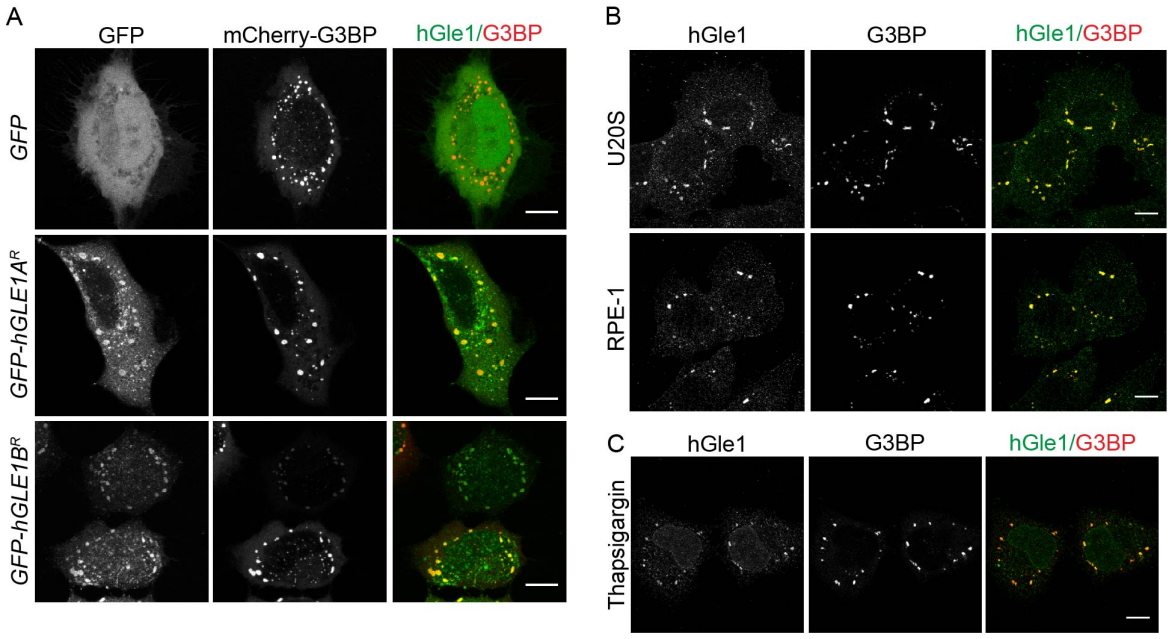


Figure 2.3: Endogenous hGle1 and exogenously expressed hGle1A and hGle1B are recruited to SGs upon stress.

(A) Both hGle1A and hGle1B are recruited to SGs upon heat shock. Plasmids expressing EGFP, EGFP-hGle1A^R or EGFP-hGle1B^R and mCherry-G3BP proteins were co-expressed in HeLa cells. Cells were heat shocked at 45°C for 45 min and imaged live by confocal microscopy. Scale bar =10µm. (B) hGle1 localization in SGs is not cell type or stress specific. U2OS and RPE-1 cells were heat shocked at 45°C for 60 min and processed for immunofluorescence using anti-hGle1 and G3BP antibodies. Scale bar =10µm. (C) hGle1 localization in SGs is not stress type specific. HeLa cells were treated with 10 µM thapsigargin for 60 min at 37°C and processed for immunofluorescence using anti-hGle1 and G3BP antibodies. Scale bar =10µm.

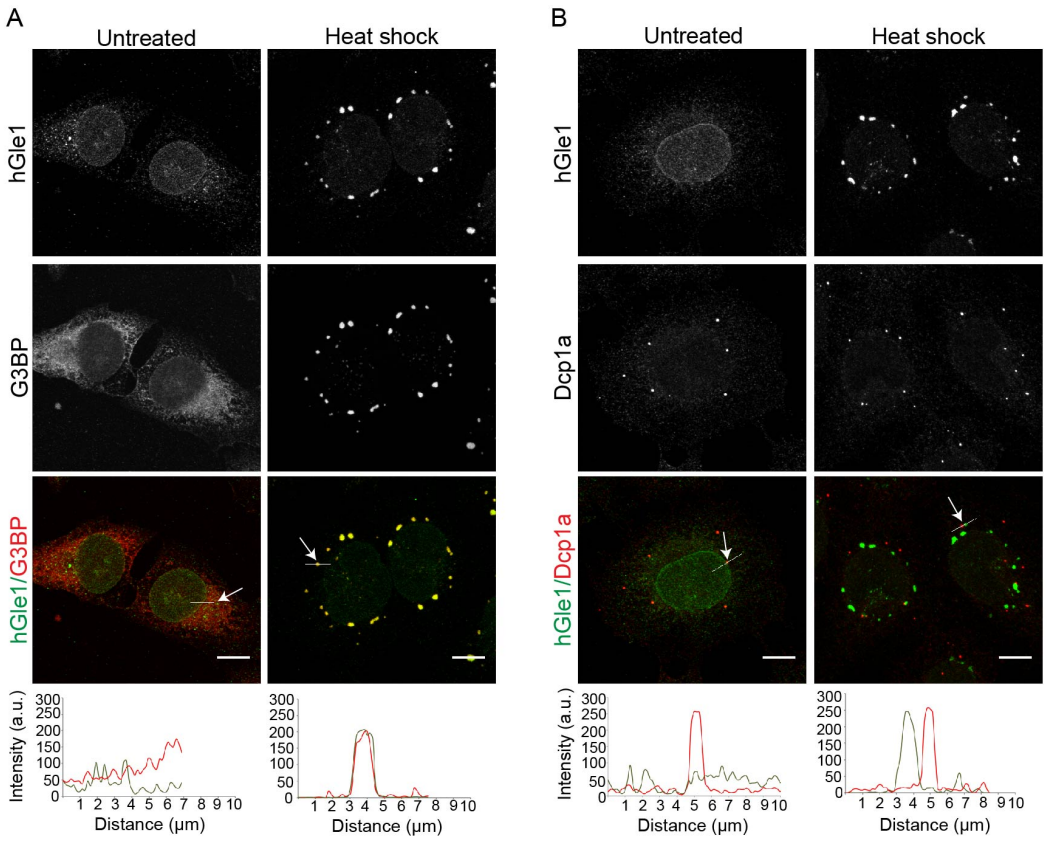


Figure 2.4: hGle1 is recruited specifically to SGs upon heat shock.

(A) Endogenous hGle1 is localized to SGs. HeLa cells were either left untreated or exposed to heat shock at 45°C for 60 min. Cells were processed for immunofluorescence using anti-hGle1 and G3BP antibodies. Scale bar =10μm. (B) hGle1 is not a component of P bodies. Immunofluorescence of hGle1 and P body marker, Dcp1a in HeLa cells treated with either heat shock or left untreated. The white line in Figure 2A and B indicates the position of the line-scan to assess co- localization in merged images using ImageJ. Arrow points toward the position of white line showing co-localization. Scale bar =10μm.

cytoplasmic foci that co-labeled with G3BP (Figure 2.4A). Line-scan analysis of individual SGs indicated that anti-hGle1 staining overlapped with anti-G3BP (Figure 2.4A). Moreover, the hGle1 cytoplasmic foci also co-localized with other SG components including DDX3, HuR and FMRP (Gallouzi et al., 2000; Lai et al., 2008; Mazroui et al., 2002; Shih et al., 2012) (Figure 2.6A). HeLa cells were treated with thapsigargin for 60 min at 37°C which induces SGs by causing endoplasmic reticulum stress, and hGle1 localization to SGs was observed (Figure 2.3C). Furthermore, hGle1 was also recruited to SGs in U2OS and RPE-1 cells upon heat shock (Figure 2.3B). Thus, hGle1 localization in SGs was a general phenomenon.

Processing bodies (P bodies) are another type of RNA granule found in cells under normal and stress conditions (Anderson and Kedersha, 2009; Balagopal and Parker, 2009). Although P bodies share many components with SGs, (Kedersha and Anderson, 2009; Kedersha et al., 2005), P bodies are linked with mRNA decay (Decker and Parker, 2012; Parker and Sheth, 2007). To investigate if hGle1 is also a component of P bodies, HeLa cells were subjected to heat shock and indirect immunofluorescence with anti-hGle1 and anti-Dcp1a (a P body marker) was performed. P bodies were detected under normal growth conditions in HeLa cells (Figure 2.4B), and upon heat shock, P bodies were localized near SGs. (Figure 2.4B). However, anti-hGle1 staining did not overlap with anti-Dcp1a, indicating that hGle1 is not a component of P bodies (Figure 2.4B). Together, these data suggested that hGle1 is a novel component of SGs.

hGle1A specifically functions in SG assembly in response to cellular stress

To determine 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 (Arimoto et al., 2008; Kim et al., 2005)). 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 to CTRL cells (Figure 2.5A). Other hGle1-depleted cells showed either diffuse cytoplasmic distribution of G3BP or localization to a few disorganized foci (Figure 2.5A). Similar phenotypes were observed when other SG markers were analyzed (DDX3, HuR and FMRP) (Figure 2.6A). As controls for off-targets effects, two independent siRNA sets were employed that target different regions of the *hGLE1* gene. A similar increase perturbation of SGs was observed with both, indicating that the phenotype is specific to hGle1 depletion (Figure 2.6D). SG changes were also observed in hGle1-depleted HeLa cells treated with thapsigargin and in hGle1-depleted U20S and RPE-1 cells upon heat shock. (Figure 2.6B and 2.6C). Thus, hGle1 depletion altered SG assembly and the effects were not limited to stress or cell types.

To analyze if the SG defects observed with hGle1 depletion were due to altered mRNA export, knockdown experiments were conducted for two other NPC-associated essential mRNA export factors: DDX19B (yDbp5 homolog) and NXF1 (Grüter et al., 1998; Herold et al., 2000; Hodge et al., 2011; Kang and Cullen, 1999). siRNA-mediated knockdown of *NXF1* or *DDX19B* resulted in nuclear accumulation of bulk poly $(A)^+$ RNA indicative of an mRNA export defect (Figure 2.7A). However, neither *NXF1* siRNA nor *DDX19B* siRNA-treated cells showed perturbations of SGs like that in hGle1-depleted cells (Figure 2.7A and 2.7B). 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.

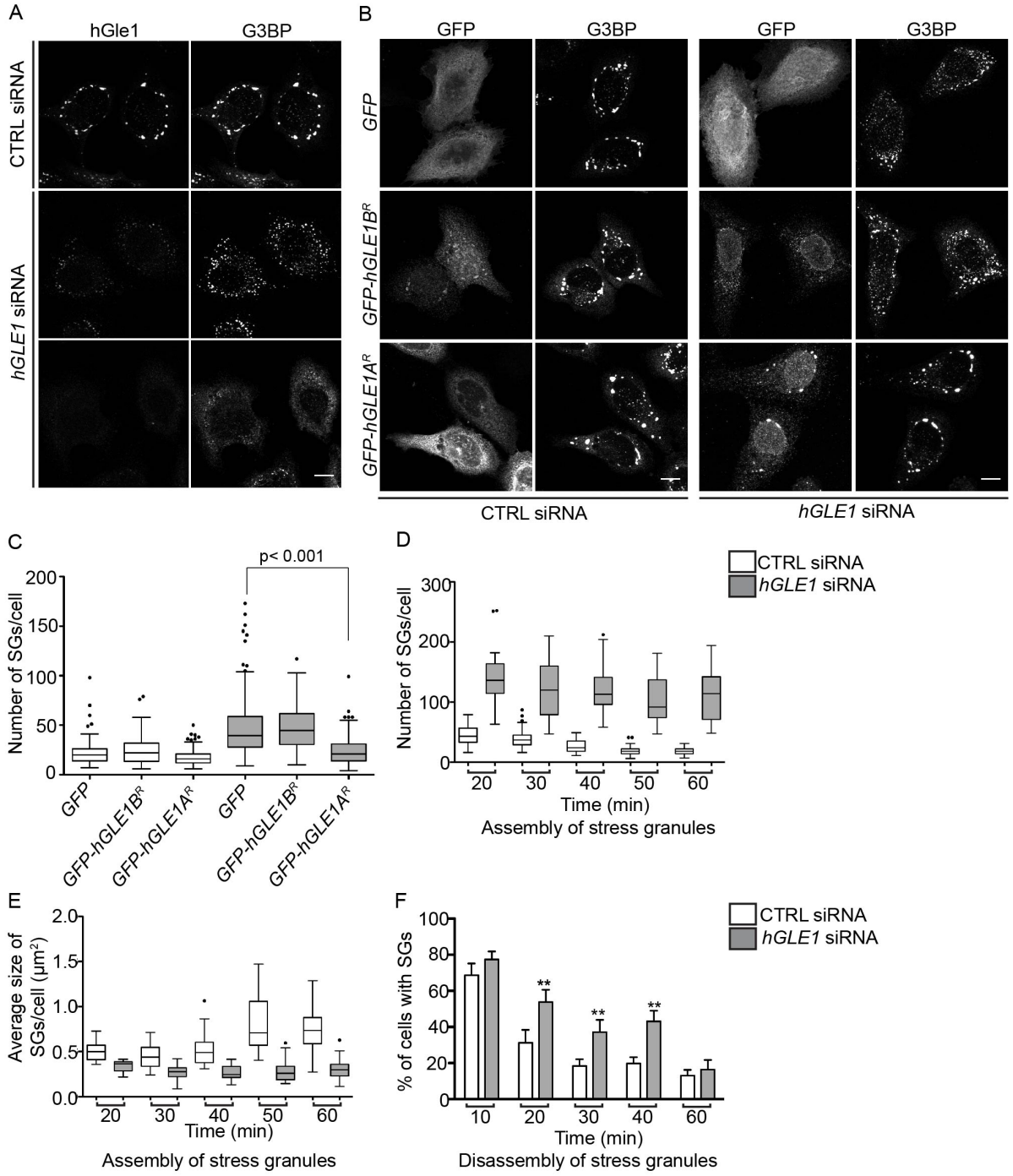

A
hGle1
G3BP
CTRL siRNA
hGLE1 siRNA
B
GFP
G3BP
GFP
G3BP
GFP
GFP-hGLE1B^R
GFP-hGLE1A^R
CTRL siRNA
hGLE1 siRNA
C
p< 0.001
Number of SGs/cell
200
150
100
50
0
GFP
GFP-hGLE1B^R
GFP-hGLE1A^R
GFP
GFP-hGLE1B^R
GFP-hGLE1A^R
D
CTRL siRNA
hGLE1 siRNA
Number of SGs/cell
300
200
100
0
20
30
40
50
60
Time (min)
Assembly of stress granules
E
Average size of SGs/cell (µm²)
2.0
1.5
1.0
0.5
0.0
20
30
40
50
60
Time (min)
Assembly of stress granules
F
CTRL siRNA
hGLE1 siRNA
% of cells with SGs
100
80
60
40
20
0
**
**
**
10
20
30
40
60
Time (min)
Disassembly of stress granules

Figure 2.5: 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 =10μm. (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*^{R}, or *EGFP-hGLE1B*^{R} plasmids, heat shocked and processed for immunofluorescence detection of G3BP and hGle1. Scale bar =10μm**.** (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 to 60 min, and processed for immunofluorescence detection of G3BP and hGle1. $p < 0.000001$ for each data pair. (F) SG disassembly is delayed in hGle1-depleted cells. Following heat shock at 45°C for 60 min, cells were incubated at 37°C for the indicated time and processed for immunofluorescence using anti-G3BP and hGle1 antibodies. Error bar represents mean +/- standard error from three independent experiments. ** indicates $p < 0.001$.

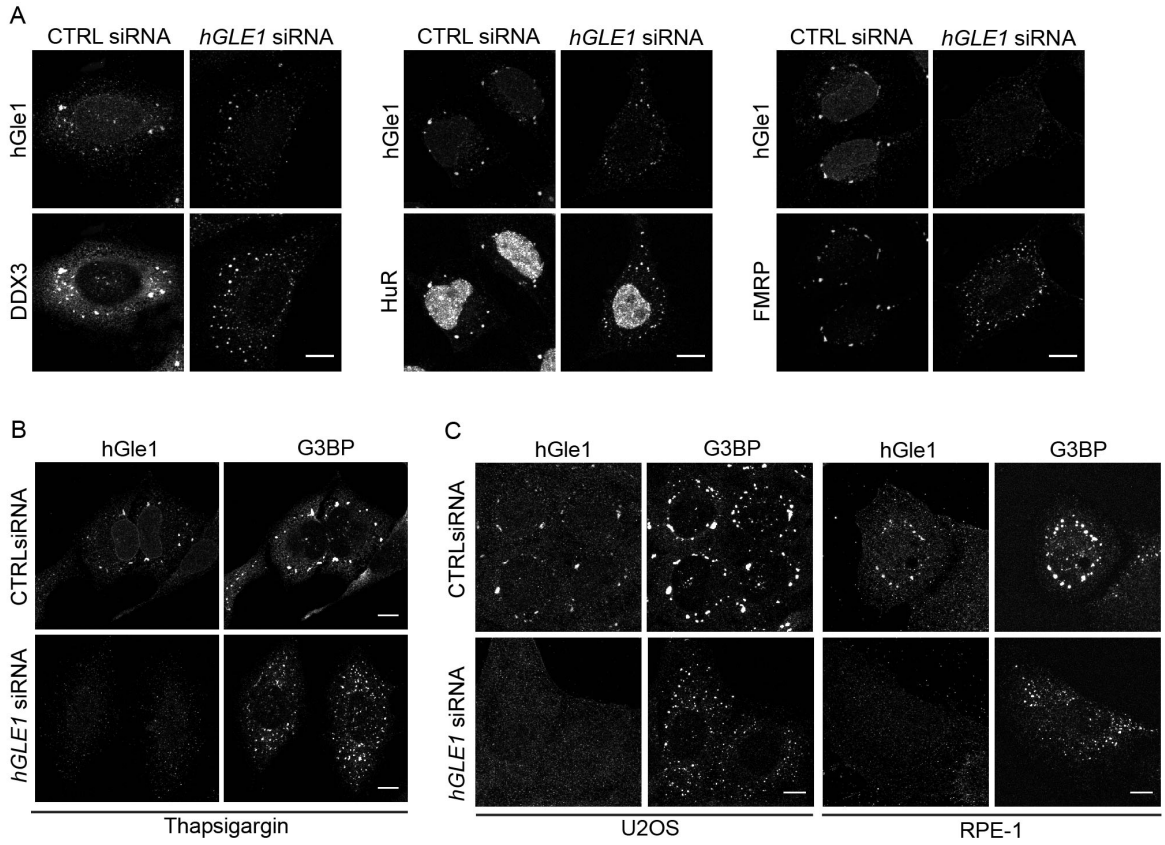

A
CTRL siRNA
hGLE1 siRNA
hGle1
DDX3
HuR
FMRP
B
hGle1
G3BP
CTRLsiRNA
hGLE1 siRNA
Thapsigargin
C
U2OS
RPE-1
D
hGLE1 siRNA No .4
hGLE1 siRNA No .6

Figure 2.6: hGle1-dependent SG defects are not stress or cell type specific.

(A) CTRL or *hGLE1* transfected HeLa cells were heat shocked at 45°C for 60 min and processed for immunofluorescence with anti-hGle1 and DDX3, HuR, or FMRP. Scale bar =10μm. (B) hGle1-depleted cells exhibit SG defects in response to thapsigargin treatment. CTRL or *hGLE1* siRNA-treated HeLa cells were incubated with 10 μM thapsigargin for 60 min at 37°C. Following treatment, cells were processed for immunofluorescence using anti-hGle1 and G3BP antibodies. Scale bar =10μm. (C) hGle1-dependent SG defects are not limited to HeLa cells. U20S and RPE-1 cells were transfected with *hGLE1* and CTRL siRNAs. After 72 h siRNA transfection, cells were heat shocked at 45°C for 60 min and processed for immunofluorescence with anti-hGle1 and G3BP antibodies. Scale bar =10μm. (D) SG defects in hGle1-depleted cells are not due to off targets effects of siRNAs. HeLa cells transfected with either CTRL, *hGLE1* siRNA No.4 or *hGLE1* siRNA No.6. Cells were heat shocked at 45°C for 60 min and processed for immunofluorescence with anti-hGle1 and G3BP antibodies. Scale bar=10μm.

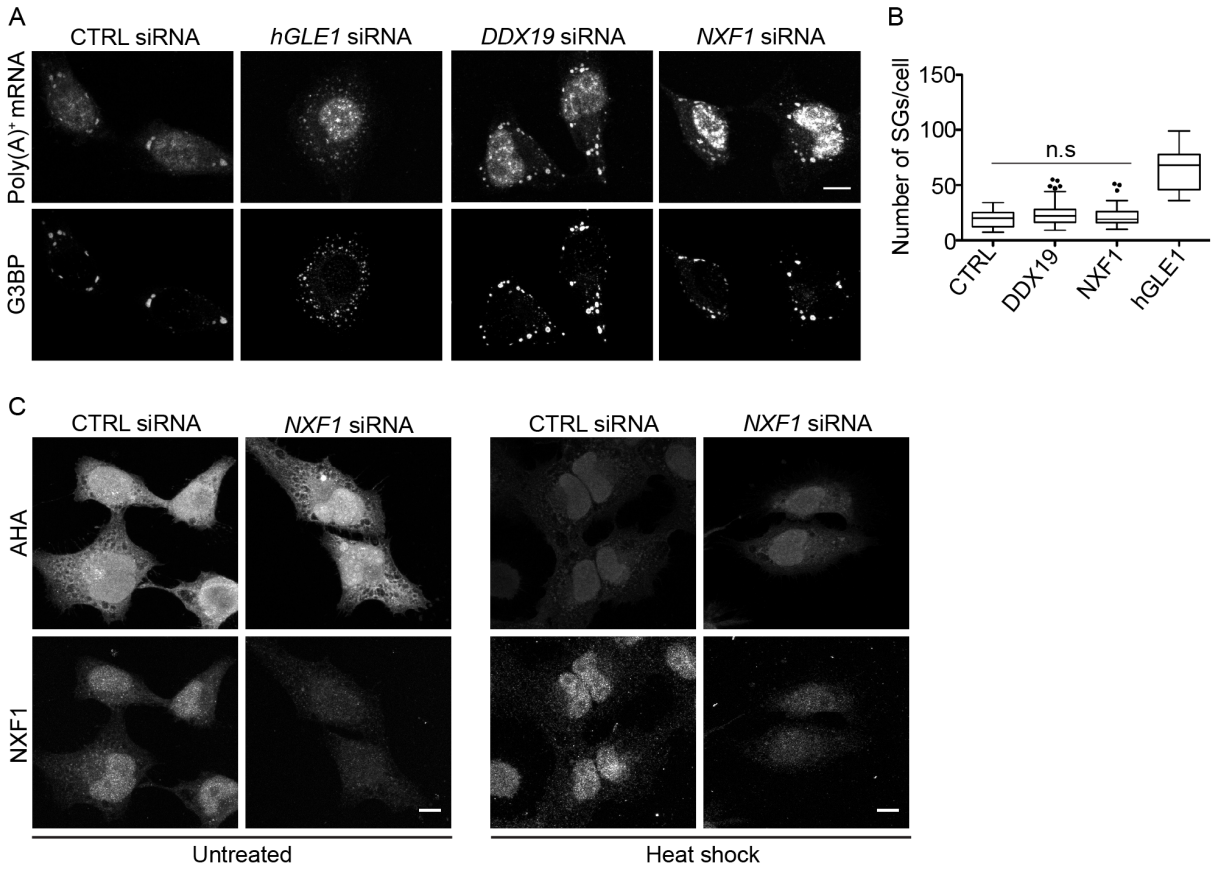


Figure 2.7: SG defects or translation defects in hGle1-depleted cells are not due to mRNA export defects.

(A) HeLa cells were treated with CTRL, *hGLE1*, *NXF1,* or *DDX19B* siRNAs for 48 h. After siRNA treatments, cells were heat shocked at 45°C for 60 min and processed for immunofluorescence with anti-G3BP antibody followed by *in situ* hybridization using Cy3 oligo-dT probe. Scale bar =10µm. (B) NXF1 knockdown does not cause deregulation in translation similar to hGle1-depleted cells. HeLa cells were transfected with either CTRL or *NXF1* siRNAs. Cells were either left untreated or heat shocked at 45°C for 15 min followed by metabolically labeling with AHA at either 37°C or 45°C for 30 min. After fixation, CTRL and *NXF1* siRNA-treated cells were processed for detection of AHA–labeled proteins using Alkyne-488 followed by immunofluorescence with anti- NXF1 antibodies. Scale bar =10µm.

CTRL siRNA or *hGLE1* siRNA cells were transfected with either *EGFP*, *EGFP-hGLE1A^R,* or *EGFP-hGLE1B^R* plasmids. After 24 h, cells were subjected to heat shock and G3BP localization was assessed as an indicator of SG formation. Expression of *EGFP*, *EGFP-hGLE1A^R,* or *EGFP-hGLE1B^R* plasmids had no impact on the number of SGs formed in CTRL siRNA-treated cells (Figure 2.5B and 2.5C), and the number of SGs formed was significantly higher in *hGLE1* siRNA-treated cells expressing *EGFP* (Figure 2.5B and 2.5C). Strikingly, the expression of *EGFP-hGLE1A^R* but not *EGFP-hGLE1B^R* led to a significant rescue of the SGs defect induced upon hGle1 depletion (Figure 2.5B and 2.5C). 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). To pinpoint 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 2.5D and 2.5E). 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 were significantly higher compared to CTRL siRNA-treated cells at all time points during heat shock (Figure 2.5D). Second, the average SG size was significantly smaller in hGle1-depleted cells compared to CTRL cells at all time points (Figure 2.5E). Finally, SG numbers or size did not change much over the entire course of heat shock for the hGle1-depleted cells compared to CTRL cells (where SGs became bigger and fewer over time) (Figure 2.5D and 2.5E). 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 2.5F). 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 disruptions by drugs that depolymerize (nocodazole) or stabilize (taxol) MTs result in numerous smaller SGs (Chernov et al., 2009; Ivanov et al., 2003; Kolobova et al., 2009; Nadezhdina et al., 2010). Given the similar phenotype, we tested if 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-alpha tubulin antibodies confirmed that MT networks were disrupted after treatments with taxol or nocodazole compared to vehicle alone (Figure 2.8A, 2.8B and 2.8C). 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 number of SGs (Figure 2.8A, 2.8B and 2.8C). Interestingly, quantification of SG numbers revealed that a significantly higher level in *hGLE1* siRNA cells compared to CTRL cells in the presence of either taxol or nocodazole (Figure 2.8D). 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 non-translated 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 if hGle1 modulates translation and if this function is linked with SG formation. To measure translation in CTRL and *hGLE1* siRNA-treated HeLa cells under non-stress 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 alkyne-488 using click chemistry (Dieterich et al., 2006, 2007).

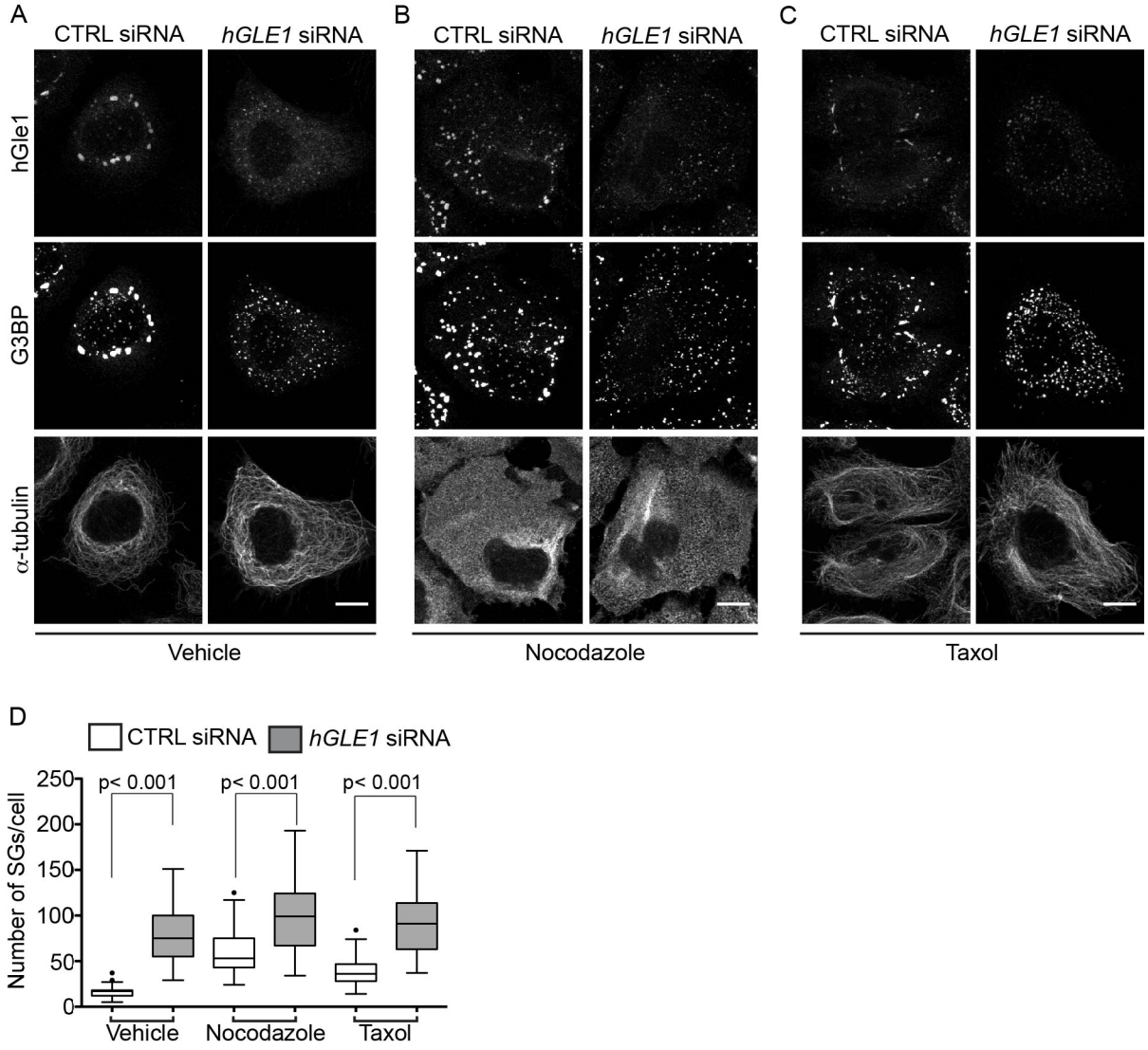


Figure 2.8: hGle1-dependent SG defects are not linked with microtubules.

(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 =10µm.(D) Quantification of SG numbers in *hGLE1* and CTRL siRNA cells treated with indicated drugs after heat shock.

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 non-stress conditions (Figure 2.9A). Strikingly, *hGLE1* siRNA-treated cells exhibited reduced AHA-488 signal as compared to CTRL siRNA-treated cells. A ^{35}S 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 to CTRL siRNA cells (Figure 2.9D). Reduced protein synthesis under non-stress conditions was expected due to the role of hGle1 in mRNA export. Therefore, we tested if depletion of NXF1 also led to reduced protein synthesis under non-stress conditions using AHA labeling. However, we did not observe significant reduction in AHA signal in *NXF1 siRNA* -treated cells compared to control cells (Figure 2.7C). To further test if hGle1 has a role in translation, polysome profiles were performed in hGle1-depleted cells. Strikingly, we observed increased monosome (80S) peak and reduced polysomes compared to CTRL siRNA cells (Figure 2.9E). This result was consistent with yGle1 suggesting that hGle1 plays a conserved role in translation initiation (Bolger et al., 2008).

We next examined the effect of hGle1 depletion on translation during heat shock. Following heat shock of CTRL siRNA cells, the AHA-488 labeling decreased (Figure 2.9B) in agreement with previous reports of global translation downregulation upon heat shock (Panniers, 1994). In comparison, the AHA-488 signal dramatically increased in heat-shocked hGle1-depleted cells (Figure 2.9B and 2.9C). Moreover, cycloheximide treatment led to a loss of AHA-488 signal in *hGLE1*-siRNA cells (Figure 2.10). 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 to CTRL siRNA-treated cells upon heat shock (Fig 2.7C).

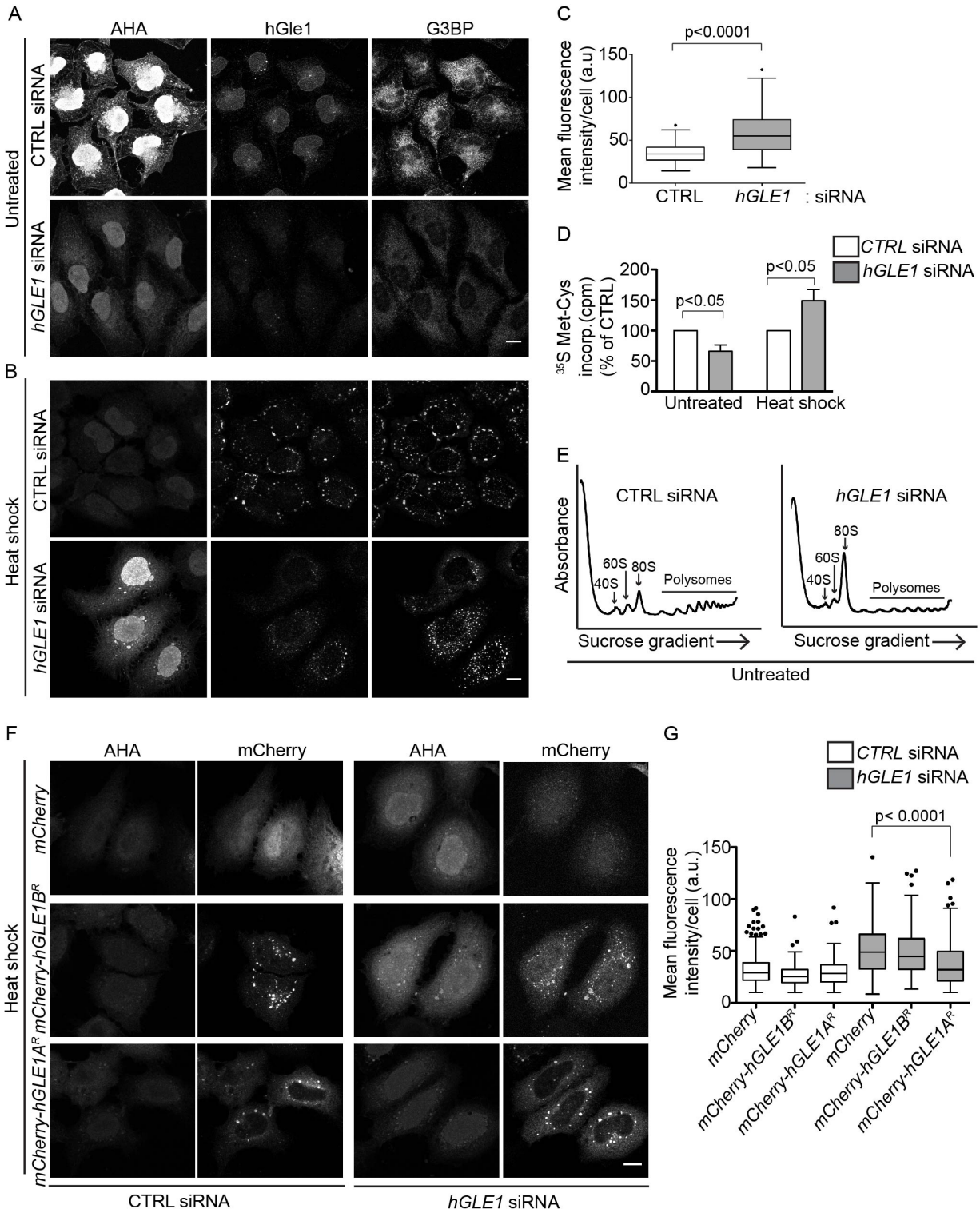

A
AHA
hGle1
G3BP
Untreated
CTRL siRNA
hGLE1 siRNA
B
Heat shock
CTRL siRNA
hGLE1 siRNA
C
p<0.0001
Mean fluorescence intensity/cell (a.u)
150
100
50
0
CTRL
hGLE1
: siRNA
D
CTRL siRNA
hGLE1 siRNA
p<0.05
p<0.05
35S Met-Cys incorp.(cpm) (% of CTRL)
200
150
100
50
0
Untreated
Heat shock
E
CTRL siRNA
hGLE1 siRNA
Absorbance
60S
80S
40S
Polysomes
Sucrose gradient
Untreated
F
AHA
mCherry
AHA
mCherry
Heat shock
mCherry
mCherry-hGLE1B^R
mCherry-hGLE1A^R
CTRL siRNA
hGLE1 siRNA
G
CTRL siRNA
hGLE1 siRNA
p< 0.0001
Mean fluorescence intensity/cell (a.u.)
150
100
50
0
mCherry
mCherry-hGLE1B^R
mCherry-hGLE1A^R
mCherry
mCherry-hGLE1B^R
mCherry-hGLE1A^R

Figure 2.9: 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 is added to the incubations and heat shock treatment was continued for additional 30 min. Samples were processed Alkyne-488 staining followed by immunofluorescence with anti-G3BP and hGle1 antibodies. Scale bar =10µm**.** (C) Quantification of AHA-488 staining. Mean fluorescent 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 100µCi/ml ^{35}S methionine/cysteine for 30 min at either 37°C or 45°C. Cells were lysed and ^{35}S 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 7%-47% sucrose gradient centrifugation. The 40S, 60S, 80s and polysome peaks are labeled. (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*^{R}, or *mCherry- hGLE1B*^{R} plasmids, heat shocked and processed for metabolic labeling using AHA. AHA incorporation was detected with alkyne-488 using click chemistry. Scale bar =10µm**.** (G) Quantification of AHA-488 staining in CTRL and *hGLE1* siRNA cells expressing indicated plasmids.

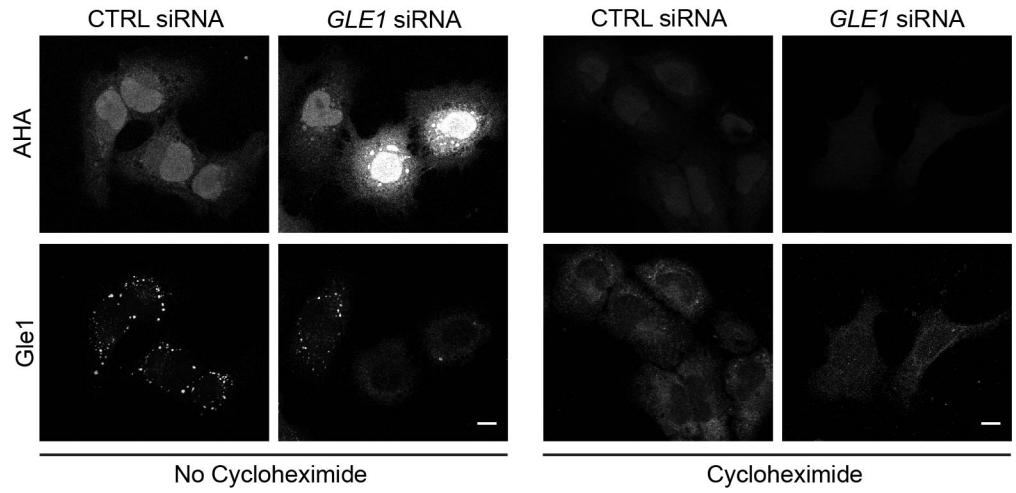


Figure 2.10: AHA incorporation in hGle1-depleted cells is due to nascent protein synthesis.

HeLa cells were transfected with either CTRL or *hGLE1* siRNAs. Cycloheximide was added to cells and cells were heat shocked at 45°C for 15 min followed by metabolically labeling with AHA for 30 min at 45°C. After fixation, *hGLE1* and CTRL siRNA-treated cells were processed for detection of AHA –labeled proteins using Alkyne-488 followed by immunofluorescence with anti-hGle1 antibodies. Scale bar =10μm.

Therefore, the de-regulation of translation during heat shock in hGle1-depleted cells was not due to global perturbations in mRNA export. To confirm AHA labeling results by alternative method, we measured nascent protein synthesis upon heat shock in CTRL and *hGLE1* siRNA cells by a ^{35}S metabolic assay. Consistent with AHA labeling results, we observed increased incorporation of ^{35}S-methionine/cysteine in hGle1-depleted cells (Figure 2.9D).

Finally, by evaluating the SG defects in the hGle1-depleted cells, the de-regulation in translation strongly correlated with the SG defects. Approximately 70% of hGle1-depleted cells with increased AHA-488 labeling either did not assemble SGs or had an increased number of SGs (Figure 2.9B). Since hGle1 depletion resulted in defects in translation, we next investigated if the hGle1A and/or hGle1B isoform rescues translation defects in hGle1-depleted cells. CTRL or *hGLE1* siRNA-treated cells were transfected with *mCherry* alone, *mCherry-hGle1A^R,or mCherry-hGLE1B^R* plasmids. 24h after plasmid transfection, cells were heat shocked and processed for AHA labeling. Interestingly, expression of mCherry-hGle1A but not mCherry-hGle1B or mCherry rescued translation defects in hGle1-depleted cells upon heat shock (Figure 2.9F and 2.9G). Together, these results suggested that hGle1A modulates SG formation by regulating translation upon heat shock.

During cell stress conditions, various signaling pathways converge to result in the phosphorylation of eIF2α at serine 51, which inhibits eIF2 recycling and results in a global reduction in translation (Spriggs et al., 2010; Wek et al., 2006). To gain insight into the mechanisms for how hGle1 depletion alters translation, we analyzed the phosphorylation levels of eIF2α. Immunoblotting with anti-eIF2α P [Ser51] antibody revealed an increase in eIF2α phosphorylation in CTRL siRNA-treated cells after 30 min of heat shock, which persisted to the 60 min time point (Figure 2.11A and 2.12B). Strikingly, the relative eIF2α phosphorylation level

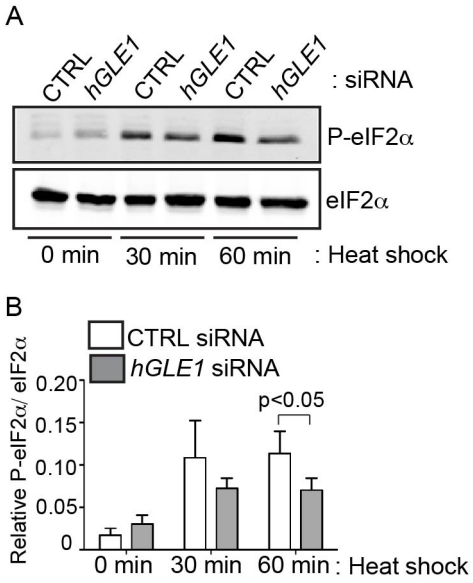


Figure 2.11: Phosphorylation of eIF2α is reduced in hGle1-depleted cells.

(A-B) HeLa cells transfected with either CTRL or *hGLE1* siRNAs were left untreated or heat shocked at 45°C for 30 min or 60 min. Lysates were analyzed by immunoblotting using anti-eIF2α and anti-Phospho-eIF2α (Ser51) antibodies. Phospho- eIF2α levels were quantified by densitometry and normalized to total eIF2α protein levels. Error bar represents standard deviation from mean from four independent experiments.

was reduced modestly (~36%) but significantly in hGle1-depleted cells compared to CTRL siRNA cells (Figure 2.11A and 2.11B). These results indicated that reduced eIF2α phosphorylation might be linked to translation deregulation 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 dynamic equilibrium between SGs and translation. For example, cycloheximide treatment prevents SG formation whereas puromycin 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 towards active translation, 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 towards SG formation by addition 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 2.12A and 2.12B). Notably, treatment of hGle1-depleted cells with either 0.1mg/ml puromycin or 0.5mg/ ml puromycin significantly reduced the number of SGs (Figure 2.12A and 2.12B). Thus, puromycin partially rescued the SG defects in hGle1-depleted cells upon heat shock. As a control, we tested if puromycin rescues the increase in SGs observed in nocodazole-treated cells and found it did not (Figure 2.13A and 2.13B). Since puromycin failed to rescue MT-dependent SG defects, puromycin and nocodazole were affecting SG formation at distinct steps. We concluded that puromycin rescues the SG phenotype in hGle1- depleted cells by increasing the free mRNP pool.

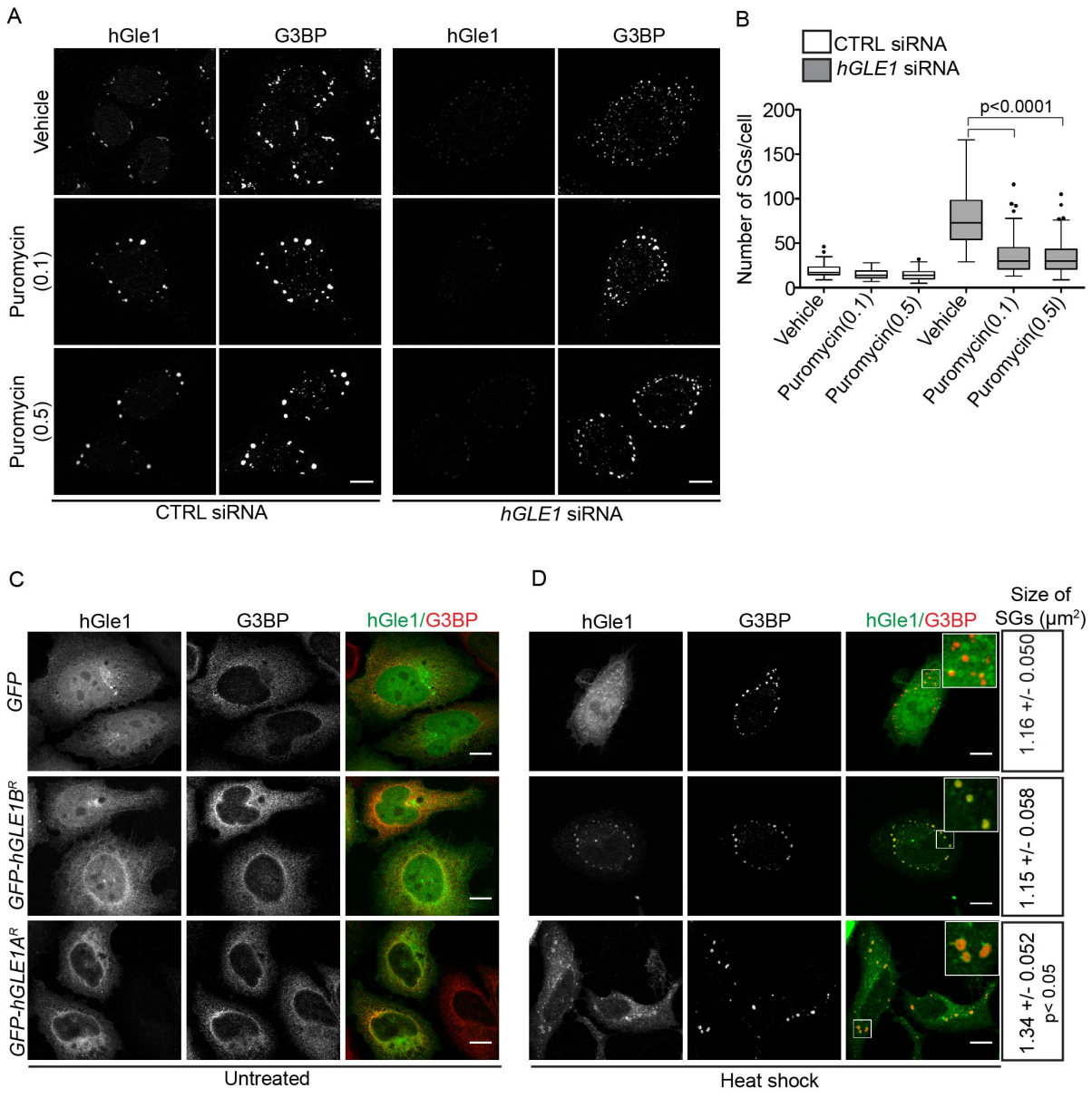


Figure 2.12: hGle1 regulates balance between active and stalled translation upon stress.

(A-B) Puromycin rescues SG assembly defects in hGle1-depleted cells. CTRL or *hGLE1* siRNA HeLa cells were treated with vehicle alone, 0.1 mg/ml, or 0.5 mg/ml puromycin for 60 min at 45°C. Cells were processed for immunofluorescence using anti-hGle1 and G3BP antibodies. Scale bar =10µm. (C-D) EGFP-hGle1A but not EGFP-hGle1B overexpression results in bigger stress granules: HeLa cells were transfected with either *EGFP, EGFP*-h*GLE1A*^{R}, or *EGFP-hGLE1B*^{R} and either C) left untreated or D) heat shocked for 60 min at 45°C. After heat shock, cells were processed for immunofluorescence using anti-hGle1 and G3BP antibodies. Data represent mean +/- standard error from three independent experiments. Scale bar =10µm.

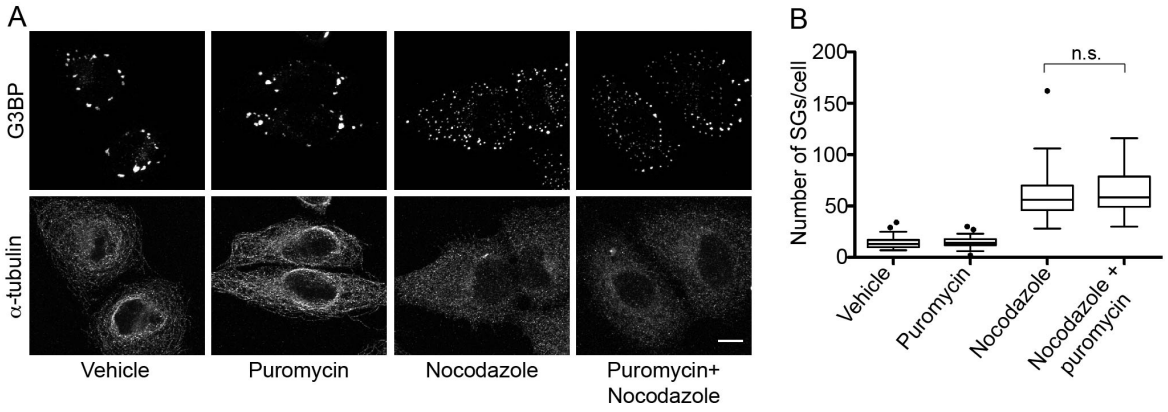


Figure 2.13: Puromycin does not rescue microtubule-dependent SG defects.

(A-B) HeLa cells were pre-incubated with 5 μM nocodazole for 120 min at 37°C. Cells were treated with vehicle alone, 0.5 mg/ml puromycin, 5 μM nocodazole, or 0.5 mg/ml puromycin and 5 μM nocodazole for 60 min at 45°C. Cells were processed for immunofluorescence using anti α-tubulin and G3BP antibodies. Scale bar =10μm.

If hGle1 levels impact the free mRNP pool by changing the distribution of mRNPs between active translation and SGs, then overexpression 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^R,* or *EGFP-hGLE1B^R* plasmids were heat shocked and analyzed for SG formation. G3BP-positive SGs assembled in cells expressing either *EGFP*, *EGFP-hGLE1A^R,* or *EGFP-hGLE1B^R* plasmids (Figure 2.12D). Overexpression of hGle1A caused a modest but significant increase in SG size (mean=1.34μm^2) compared to EGFP (mean=1.16μm^2) or hGle1B (mean=1.15μm^2) (Figure 2.12D). Thus, hGle1A but not hGle1B overexpression resulted in larger SG formation. TTP, TIA-1 and G3BP proteins play a role in early assembly of SGs and their overexpression drives constitutive SG formation under non-stress conditions(Gilks et al., 2004; Tourrière et al., 2003). However, in non-stress conditions, expression of either EGFP, *EGFP-hGLE1A^R,* or *EGFP-hGLE1B^R* plasmids in HeLa cells showed pan cellular cytoplasmic staining for G3BP suggesting that hGle1 cannot nucleate SG formation in the absence of stress (Figure 2.12C).

DDX3 suppresses loss of hGle1 function

Based on the published connections between yGle1 and Ded1 during translation initiation (Alcázar-Román et al., 2006; Bolger and Wente, 2011; Bolger et al., 2008), 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 (Hilliker et al., 2011; Lai et al., 2008; Shih et al., 2012), we evaluated whether hGle1 function in SGs is mediated through interaction with DDX3. First, co-immunoprecipitation experiments were conducted with HeLa cell lysates from untreated or heat shocked cultures. hGle1 and DDX3 were co-isolated in the presence or absence

of stress (Figure 2.14A). We also examined if expression of DDX3 rescues hGle1-dependent SG defects. CTRL or *hGLE1* siRNA-treated cells were transfected with *EGFP* alone, *EGFP-DDX19B,* or *DDX3-EGFP*. DDX3-GFP was expressed at low levels so that the cells did not form constitutive stress granules (Figure 2.15). Cells were subjected to heat shock for 60 min at 45°C. Notably, only *DDX3-EGFP* expression partially rescued SG defects in hGle1-depleted cells (Figure 2.14B and 2.14C). Next, we tested if DDX3 also rescues translation defects in hGle1-depleted cells upon heat shock. CTRL or *hGLE1* siRNA cells were transfected with *mCherry* alone or *mCherry-DDX3* plasmids and heat shocked 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 2.14D and 2.14E). 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 towards 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.

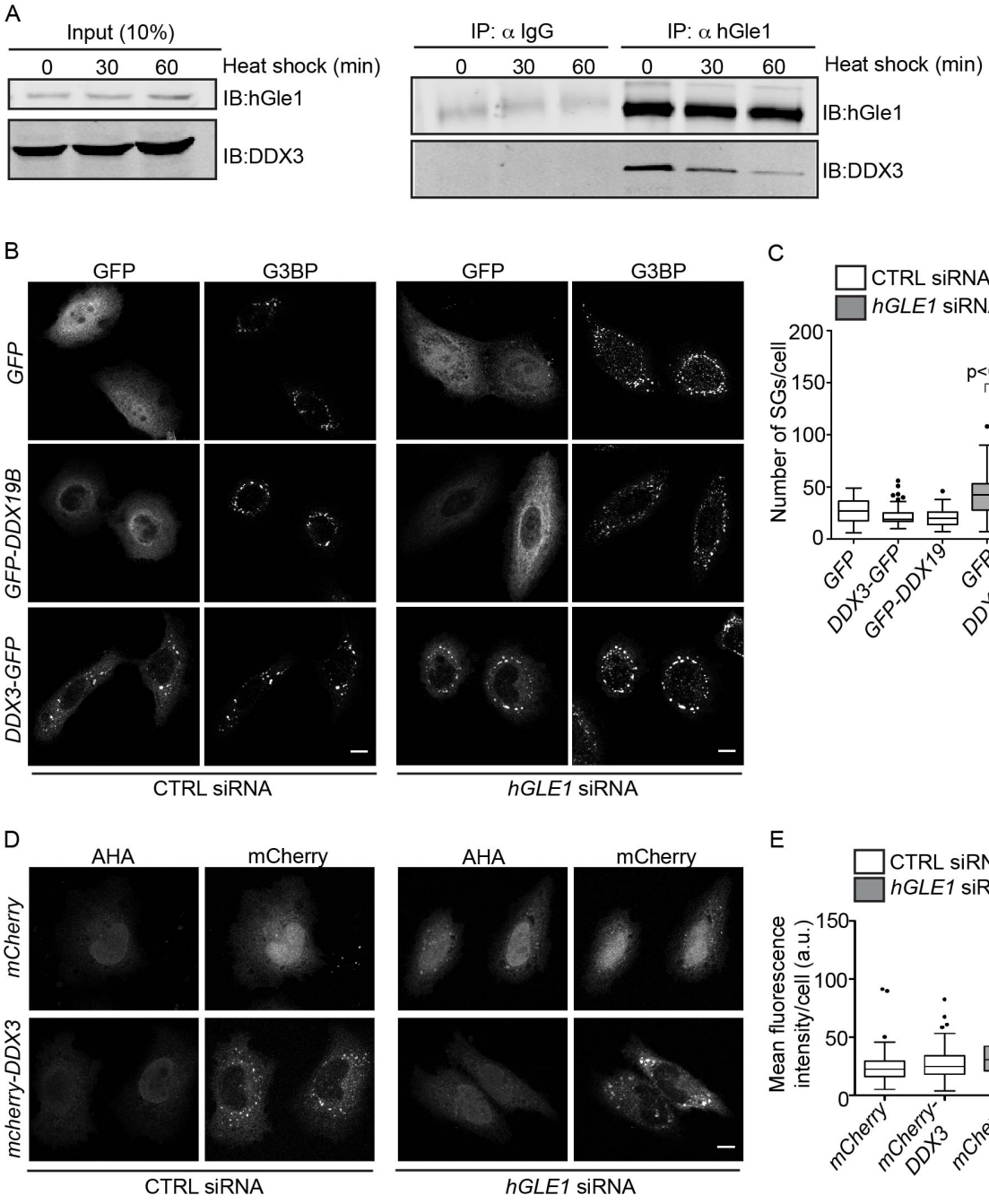

A
Input (10%)
0 30 60
Heat shock (min)
IB:hGle1
IB:DDX3
IP: α IgG
IP: α hGle1
0 30 60 0 30 60
Heat shock (min)
IB:hGle1
IB:DDX3
B
GFP
G3BP
GFP
G3BP
GFP
GFP-DDX19B
DDX3-GFP
CTRL siRNA
hGLE1 siRNA
C
CTRL siRNA
hGLE1 siRNA
Number of SGs/cell
200
150
100
50
0
p<0.0001
GFP
DDX3-GFP
GFP-DDX19
GFP
DDX3-GFP
GFP-DDX19
D
AHA
mCherry
AHA
mCherry
mCherry
mcherry-DDX3
CTRL siRNA
hGLE1 siRNA
E
CTRL siRNA
hGLE1 siRNA
Mean fluorescence intensity/cell (a.u.)
150
100
50
0
p<0.001
mCherry
mCherry-DDX3
mCherry
mCherry-DDX3

Figure 2.14: DDX3 rescues hGle1-dependent SG and translation defects.

(A) DDX3 co-immunoprecipitates with hGle1. HeLa cells were either left untreated or heat shocked for 30 min or 60 min and cell lysates were immunoprecipitated using anti-hGle1 or IgG control antibodies and immunoblotted with anti-hGle1 or DDX3 antibodies. (B) DDX3 partially rescues SG defects in hGle1-depleted cells. CTRL or *hGLE1* siRNA-treated cells were transfected with *EGFP, EGFP*-DDX19B, or *DDX3-EGFP* plasmids and heat shocked. Cells were processed for immunofluorescence detection of G3BP and hGle1. Scale bar =10μm. (C) SG numbers in CTRL and *hGLE1* siRNA cells expressing indicated plasmids were quantified. (D) DDX3 partially rescues translation defects in hGle1-depleted cells. CTRL or *hGLE1* siRNA-treated HeLa cells transfected with *mCherry* or *mCherry-DDX3* plasmids were heat shocked at 45°C. After 15 min of heat shock, AHA was added to the incubations and heat shock treatment was continued for additional 30 min. Samples were processed for detection of AHA labeled proteins with alkyne 488 using click chemistry. Scale bar =10μm**.** (E) Quantification of AHA-488 staining. Mean fluorescent intensity of AHA-488 staining in individual cells was calculated in CTRL and *hGLE1* siRNA cells expressing indicated plasmids.

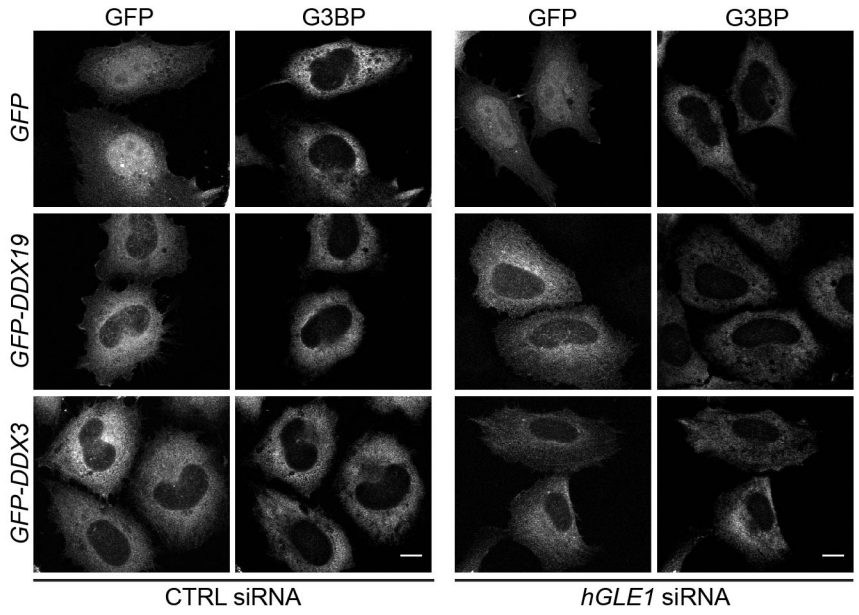


Figure 2.15: Expression of DDX3 in hGle1-depleted cells under non-stress conditions.

CTRL or *hGLE1* siRNA-treated cells were transfected with *EGFP, EGFP-DDX19B*, or *DDX3-EGFP* plasmids. Cells were processed for immunofluorescence detection of G3BP and hGle1. Scale bar =10μm.

Similarly, puromycin is not sufficient alone to nucleate SGs but induces larger granule formation in the presence of 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.

Previously, we defined functions for Gle1 as a regulator of Dbps during mRNA export and translation (Bolger and Wente, 2011; Bolger et al., 2008; Murphy and Wente, 1996; Noble et al., 2011; Tran et al., 2007). Given the effects here on translation in the hGle1-depleted 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 NXF1 does not result in SG defects. Moreover, hGle1A rescues SG defects yet fails to rescue the mRNA export defects. It is clear that the hGleA 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 strictly localization at NPCs. Indeed, our very recent studies support this conclusion as interactions between the carboxy-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 long standing question in the field (Anderson and Kedersha, 2008; Kedersha et al., 2005; 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 DEAD-box protein 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, eIF2α phosphorylation at Ser51 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 non-phosphorylatable serine to alanine mutant at residue 51 of eIF2α allows continued protein synthesis upon heat shock in CHO cells (Murtha-Riel et al.,

1993), eIF2α phosphorylation is likely one of the primary mechanisms to inhibit global protein synthesis upon stress (Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2009; Wek et al., 2006). It is intriguing that hGle1 depletion affects eIF2α phosphorylation and cells maintain translation under stress. The eiF2 kinase, PKR is often activated by its association with dsRNAs, 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 eIF2α phosphorylation. Additionally, translation perturbations observed in hGle1-depleted cells could be due to other regulatory pathways that may or may not be linked with eIF2α. Further studies are required to determine precisely how hGle1 levels influence eIF2α 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 stable SGs under normal stress conditions (Buchan et al., 2013; Dewey et al., 2011; Parker et al., 2012; Vance et al., 2013; Wolozin, 2012)(Buchan et al., 2013; Dewey et al., 2011; Parker et al., 2012; Vance et al., 2013; Wolozin, 2012). 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., 2015). 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 depleting specific hGle1 isoforms differentially affect mRNA export and

translation. Additionally, mutations in *hGLE1* linked with inherited human diseases LCCS1 and LAAHD (Nousiainen et al., 2008) are due to disregulated mRNA export (Folkmann et al., 2013, 2014).

Taken 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 specifically hGle1A function 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 media or DMEM-F12 (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals) at 37°C in 5% CO2. To induce heat stress, cells were incubated at 45°C in a non-$C0_2$ air incubator for 60 min unless indicated otherwise. For ER induced stress, cells were exposed to 10 μM thapsigargin (Sigma) for 60 min at 37°C. Puromycin (Sigma) was used at either 0.1 mg/ml or 0.5 mg/ml for 60 min. Cycloheximide (Sigma) was used at 10ug/ml. For MT perturbations, cells were incubated with 5 μM nocodazole (Sigma) or 500 nM Taxol (Sigma) for 120 min at 37°C followed by heat shock at 45°C for 60 min. Plasmids expressing EGFP-C1, EGFP-hGle1B^R *(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-hGLE1A^R, mCherry-hGLE1A^R and mCherry-hGLE1B^R* 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:20129) purchased from GeneCopoeia Inc. and cloned into pEGFP-N3 vector using XhoI 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. *NXF1* and *DDX19B* Smart pool siRNAs were purchased from Dharmacon. Cells were reverse transfected with indicated 20 nM siRNAs using HiPerFect (Qiagen) following manufacturer's instructions. Plasmid transfections were performed using Fugene 6(Promega) following manufacturer's instructions. For rescue of SG and translation phenotype, siRNA resistant ("R") *EGFP, EGFP-hGLE1B*^{R} and *EGFP-hGLE1A*^{R} expression constructs were transfected after 48 h of siRNA transfections.

Co-immunoprecipitation

HeLa cells were plated in 100 mm dishes (Fisher Scientific) and either left untreated or heat shocked for indicated time at 45°C. After treatments, cells were washed with 1X 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 PMSF and protease inhibitors cocktail (Roche). Cell lysates were spun at 13000 rpm for 10 min at 4°C and the supernatants were incubated with control IgG or affinity-purified guinea pig polyclonal antibodies raised against recombinant amino- terminus of hGle1 amino acids 1-360 (ASW48, Covance laboratory) for 5 h at 4°C. Protein A-sepharose (GE Healthcare)

beads were added and lysates were further incubated for additional 1 h at 4°C. Beads were washed with lysis buffer five times and immune complexes were eluted with 50 µl 2X SDS-laemmli buffer. The eluted complexes were resolved on 7.5% SDS PAGE and subjected to immunoblotting using anti-hGle1, 1:1000 (ASW48) and anti-DDX3,1:1000 (Bethyl Laboratories) 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, 50mM 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 (Molecular probes), anti-actin,1:5000 (Sigma), anti- eIF2α, 1:1000(Cell Signaling Technology), and anti-Phospho-eIF2α (Ser51),1:1000 (Cell Signaling Technology). Far-red dyes-conjugated secondary antibodies were used for detection and blots were scanned using LI-COR Odyssey scanner. For immunofluorescence, cells were plated on 1.5 mm round coverslips in a 24 well plate (Fisher Scientific). Following treatments, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% triton X-100 for 5 min. Coverslips were blocked with 10%FBS/PBS for 1 h at room temperature. Following primary antibodies were used: anti-hGle1, 1:300 (ASW48), anti-G3BP, 1:300 (BD Transduction), anti-DDX3, 1:300 (Bethyl Laboratories), anti-HuR,1:100 (Santa Cruz), anti- FMRP, 1:300 (Milipore) and anti-α tubulin, 1:500 (Abcam) for approximately 3 h at RT. Alexa-fluor conjugated secondary antibodies (Life Technologies) were

used and slides were mounted using Prolong Gold AntiFade (Life Technologies). Cells were imaged using 63X (1.4 NA) oil-immersion objective on confocal microscope (Leica TCS SP5).

In Situ hybridization

HeLa cells were fixed with 4% paraformaldehyde for 15 min. Cells were washed with 1XPBS followed by permeablization with 0.2% tritonX100/1XPBS for 5 minutes. Cells were incubated with 1ng/ul of Cy3-conjugated oligo dT in hybridization buffer containing 125 µg/ml tRNA, 1 mg/ml ssDNA and 1%BSA, 10% dextran sulfate, 50% formamide, 5X SSC, for 1h 30 min at 37°C. After staining, coverslips were washed with 2X SSC two times followed by washes with 1X SSC. Coverslips were rinsed finally with 1X PBS and slides were mounted using Prolong Gold AntiFade. When combined with immunofluroscence, *in situ* hybridization was performed following secondary antibody incubations. Images were acquired using 63X (1.4 NA) oil-immersion objective on Leica TCS SP5 confocal microscope. Images were processed using ImageJ. For each EGFP positive cell, mean Cy3 intensity was determined for cytoplasmic and nuclear compartment, and N/C ratios were calculated.

Live cell microscopy

HeLa cells were co-transfected with plasmids encoding for mCherry-G3BP and either EGFP, EGFP-hGle1B^{R}, and EGFP-hGle1A^{R} using Fugene 6. Before imaging, culture medium was replaced with phenol free DMEM supplemented with 25 mM HEPES and 10% FBS. Cells were imaged live on a heated stage at 45°C using 63X (1.4NA) oil objective lens on Leica SP5 microscope. For 3D structural illumination microscopy, CTRL or *hGLE1* siRNAs cells were co transfected with *Pom121-mCherry* and either *EGFP, EGFP-hGLE1B^{R},* or *EGFP-hGLE1A^{R}*

plasmids. Images were acquired in 3D structural illumination microscopy mode on a heated stage at 37°C using a Delta Vision OMX microscope (Applied Precision).

Metabolic labeling for protein synthesis

F and *hGLE1* siRNAs transfected cells were washed with DMEM media without cysteine and methionine and incubated in the same medium for 45 min at 37°C. Cells were either left untreated or subjected to heat shock at 45°C for 15 min. 50 μM methionine analogue AHA (Life Technologies) was added to incubations and cells were either left untreated or heat shocked at 45°C for additional 30 min. Following treatments, 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 ^{35}S metabolic labeling, CTRL and *hGLE1* siRNA-treated cells were washed with DMEM media without cysteine and methionine and incubated in the same medium for 45 min at 37°C. 100μCi/ml radioactive ^{35}S methionine and cysteine (Perkin Elmer) was added to the medium and cells were incubated for additional 30 min at 37°C. Cells were lysed in RIPA buffer and protease and phosphatase inhibitor cocktail. Protein concentrations were determined using DC protein assay kit (Bio-Rad). For liquid scintillation counts, equal amounts of labeled cell lysates were precipitated with 1ml of 10% TCA and BSA as a carrier protein for 30 min on ice. TCA precipitates were filtered onto GC/F filter disks 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 liquid scintillation counter (Beckman Coulter).

Measurement of SG size and number

Post-image processing was done using ImageJ software (NIH). ImageJ plug-in "3D objects counter" was used to calculate size and numbers of SGs of individual cell. 3D objects counter measurement parameters were set to 'surface' and minimum size filter was set to 3. Surface measurements were used to calculate size of SGs. SG size and numbers were represented using box plot generated in Prism6 (GraphPad). The percentages of cells with SGs were calculated as 100 X [(No. of cells with G3BP positive foci)/ (Total No. of cells)]. All data are result of at least three independent experiments. Statistical significance was determined using Student's t test (Excel) and Fisher exact test (GraphPad) where appropriate.

Polysome profiles

Cells were grown in 10cm dishes and lysed in 10mM Tris-Cl pH 7.5, 100mM NaCl, 30mM $MgCl_2$, 200µg heparin/ml, cycloheximide 100µg/ml and 1% tritonX 100. The lysates were cleared by centrifugation at 5000rpm for 5 min. Cleared lysates were layered on 7%-47% sucrose gradients cast in 50mM Tris pH7.5, 50mM NH_4Cl, 12mM $MgCl_2$, 100ug/ml cycloheximide in ultracentrifuge tubes (Beckman). Gradients were centrifuged in a Beckman SW-41 rotor for 3h at 28800 rpm. An absorbance profile was collected from the gradients at 254 nm.

CHAPTER 3

An amyotrophic lateral sclerosis-linked mutation in *GLE1* alters the cellular pool of human Gle1 functional isoforms

Abstract

Amyotrophic lateral sclerosis (ALS) is a lethal late onset motor neuron disease with underlying cellular defects in RNA metabolism. In prior studies, two deleterious heterozygous mutations in the gene encoding human (h)Gle1 were identified in ALS patients. hGle1 is an mRNA processing modulator that requires inositol hexakisphosphate (IP_6) binding for function. Interestingly, one *hGLE1* mutation (c.*1965-2A>C*) results in a novel 88 amino acid carboxy-terminal insertion, generating an altered protein. Like hGle1A, at steady state, the altered protein termed hGle1-IVS14-2A>C is absent from the nuclear envelope rim and localizes to the cytoplasm. hGle1A performs essential cytoplasmic functions in translation and stress granule regulation. Therefore, we speculated that the ALS disease pathology results from altered cellular pools of hGle1 and increased cytoplasmic hGle1 activity. GFP-hGle1-IVS14-2A>C localized to stress granules comparably to GFP-hGle1A, and rescued stress granule defects following siRNA-mediated hGle1 depletion. As described for hGle1A, overexpression of the hGle1-IVS14-2A>C protein also induced formation of larger SGs. Interestingly, hGle1A and the disease associated hGle1-IVS14-2A>C overexpression induced the formation of distinct cytoplasmic protein aggregates that appear similar to those found in neurodegenerative diseases.

This chapter is adapted from "An amyotrophic lateral sclerosis-linked mutation in *GLE1* alters the cellular pool of human Gle1 functional isoforms. Aditi, Laura Glass, T. Renee Dawson and Susan R. Wente. Adv Biol Regul. 2015 Nov 11 pii: S2212-4926 (15) 30031-2".

Strikingly, the ALS-linked hGle1-IVS14-2A>C protein also rescued mRNA export defects upon depletion of endogenous hGle1, acting in a potentially novel bi-functional manner. We conclude that the ALS-linked *hGle1-c.1965-2A>C* mutation generates a protein isoform capable of both hGle1A- and hGle1B-ascribed functions, and thereby uncoupled from normal mechanisms of hGle1 regulation.

Introduction

The regulation of RNA metabolism is a critical facet of the eukaryotic cell's capacity to react to its environment and modulate gene expression. At each stage in the mRNA lifecycle, RNA-binding proteins associate and disassociate with a transcript, forming messenger ribonucleoprotein complexes (mRNPs) with specific protein compositions that direct association of the message with the proper cellular machinery. This tightly orchestrated association of transcripts with specific RNA-binding proteins is a primary mediator of an mRNA's fate (Mitchell and Parker, 2014). For example, in *Saccharomyces cerevisiae*, association of the RNA-binding proteins Nab2 with an mRNP in the nucleus mediates binding of the mRNA export receptor Mex67-Mtr2 (vertebrate NXF1/TAP) for transport through the nuclear pore complex (NPC, (Carmody et al., 2010; Iglesias et al., 2010) ; then release of Nab2 and Mex67 at the cytoplasmic face of the NPC confers directionality to the export process and primes the mRNP for its cytoplasmic fate (Brockmann et al., 2012; Lund and Guthrie, 2005; Tran et al., 2007). Neurons are particularly vulnerable to misregulation of mRNP composition given the additional hurdle of transporting mRNPs to the synapse for localized translation (Alami et al., 2014; Hutten et al., 2014; Liu-Yesucevitz et al., 2014). Indeed, a recent surge in genetic studies has firmly implicated the disregulation of mRNP metabolism in numerous neurodegenerative diseases,

including Alzheimer's disease, Parkinson's disease, and the late onset motor neuron disease amyotrophic lateral sclerosis (ALS; (Fox and Tibbetts, 2015; Nussbacher et al., 2015; Philips and Rothstein, 2015; Ramaswami et al., 2013).

ALS is a devastating disease characterized by rapid degeneration of the motor neurons. The most prevalent form of motor neuron disease, diagnosis with ALS brings with it a prognosis of paralysis and fatality within two to five years. The list of ALS-linked mutations in genes encoding mRNP components and modulators is rapidly expanding, and includes TAR DNA-binding protein 43 (TDP-43; (Sreedharan et al., 2008)), Fused in Sarcoma (FUS; (Kwiatkowski et al., 2009; Vance et al., 2009)), hnRNP1 and hnRNPA2 (Kim et al., 2013), and, more recently, Gle1 (Kaneb et al., 2015). Gle1 is a highly conserved and essential modulator of RNA-dependent DEAD-box ATPases during mRNA export (Alcázar-Román et al., 2006; Folkmann et al., 2013; Murphy and Wente, 1996) translation (Alcázar-Román et al., 2010; Bolger and Wente, 2011; Bolger et al., 2008) and stress granule dynamics (Aditi et al., 2015). By inducing the release of select RNA-binding proteins, DEAD-box proteins facilitate changes in the mRNP protein composition through a process termed mRNP remodeling, and these mRNP changes play critical roles in an mRNA's processing (Jarmoskaite and Russell, 2014). At least two isoforms of human (h)Gle1 result from the *hGLE1* pre-mRNA transcript (Kendirgi et al., 2003). The more highly expressed isoform, hGle1B, contains a 39 amino acid extension at its carboxy-terminus that binds to the NPC component hCG1 and localizes at steady-state primarily to the nuclear rim (Folkmann et al., 2013; Kendirgi et al., 2003, 2005; Rayala et al., 2004). The molecular mechanism of Gle1-stimulated DEAD-box activation at the NPC is largely based on the *S. cerevisiae* model system (Folkmann et al., 2011); however, our recent work shows that only the highly conserved Gle1B isoform, presumably in complex with the cofactor inositol

hexakisphosphate (IP_6), is capable of activating the DEAD-box protein for mRNP remodeling during mRNA export (Folkmann et al., 2013). Conversely, the shorter hGle1A isoform lacking the hCG1-binding domain localizes primarily to the cytoplasm at steady state and is required for proper stress granule (SG) function (Aditi et al., 2015). Thus, the hGle1A and hGle1B isoforms exist in separate cellular pools and play multiple independent roles in regulating mRNPs, any one of which might be impacted in hGle1-associated ALS pathogenesis.

A *c.1965-2A>C* ALS-linked mutation in intron 14 of *hGLE1* destroys a splice acceptor site and results in the expression of a protein hGle1-IVS14-2A>C, in which the 44 amino acid hCG1 binding site is replaced with a novel 88 amino acid carboxy-terminal domain (Kaneb et al., 2015). Similar to hGle1A, the hGle1-IVS14-2A>C isoform localizes at steady state in the presence of endogenous hGle1 to primarily the cytoplasm, and, by two-hybrid assays, does not bind hCG1 (Kaneb et al., 2015). These properties suggest that the ALS hGle1-IVS14-2A>C protein may function like hGle1A in SG biology, yet perhaps in a disregulated fashion. Considering that a defining hallmark of ALS motor neuron pathology is cytoplasmic inclusions of aggregated RNA-binding proteins (Leigh et al., 1991), aberrant SG function is plausible as a potential molecular mechanism in ALS pathogenesis.

The most prevalent neuronal inclusions reported in ALS patient samples are those containing aggregates of mutant TDP-43 or FUS (Arai et al., 2006; Neumann et al., 2006). TDP-43 and FUS are highly conserved DNA and RNA-binding proteins with roles in transcriptional repression, pre-mRNA splicing and localized translation (Bentmann et al., 2013); and like hGle1, TDP-43 also facilitates stress granule assembly (McDonald et al., 2011). Many of the mutations catalogued for such genes encode proteins with low complexity glycine-rich, prion-like domains that promote their cytoplasmic aggregation and loss of nuclear functions (Dormann and Haass,

2011). Although these aggregates are distinct from normal SGs in that they are not reversible (Bentmann et al., 2013), it has been suggested that SGs may play a role in forming the inclusions by sequestering mutant TDP-43 or FUS and 'seeding' the initial aggregation step (King et al., 2012; Parker et al., 2012). Reports differ on whether stress granule markers colocalize with neuronal inclusions (Colombrita et al., 2009; Dormann et al., 2010; Liu-Yesucevitz et al., 2010); however, several lines of evidence support the hypothesis. Repeated episodes of severe stress can induce the formation of wild-type TDP-43 aggregates in SGs (Parker et al., 2012), and mutant forms of FUS increase the size and number of SGs (Baron et al., 2013). Furthermore, expression of mutant stress granule components alters the formation of FUS aggregates in *Saccharomyces cerevisiae* (Sun et al., 2011b) and an inhibitor of stress granule formation reduces TDP-43 cytotoxicity in *Drosophila* and mammalian neurons (Kim et al., 2014). These observations suggest that SGs might function in an early step of ALS progression by promoting the formation of insoluble protein aggregates.

With regard to ALS pathogenesis, it remains unclear whether the loss of TDP-43 and FUS nuclear functions or the accumulation of cytoplasmic inclusions are causative for neurodegeneration. Similar obstacles exist for discerning the pathogenicity of hGle1 in ALS since it functions both in mRNA export across the nuclear envelope and in cytoplasmic functions of translation and stress granule biology. Thus, in this study, we sought to gain insight by identifying the functional consequences of the *c.1965-2A>C* mutation on hGle1 biology.

Results

The ALS-linked hGle1-IVS14-2A>C protein is recruited to stress granules.

The emerging model that neuronal inclusions are related to SGs suggests that ALS pathogenicity may arise from defects in the formation or clearance of these granules. Previously, we reported that hGle1 is a component of SGs and is required for SG assembly and disassembly (Aditi et al., 2015). Interestingly, hIPK1, the kinase required for producing hGle1's cofactor IP_6, also relocalizes to SGs upon stress (Brehm et al., 2007). We speculated that the carboxy-terminal alterations in the ALS-linked hGle1-IVS14-2A>C protein might perturb its recruitment to SGs and/or functional capacity upon stress. To address this, subcellular localization experiments were conducted with HeLa cells coexpressing mCherry-G3BP and either GFP, GFP-hIPK1, or GFP-hGle1-IVS14-2A>C (the protein encoded by *GFP-hGLE1-c.1965-2A>C*^{R}). The cells were subjected to heat shock at 45^{o}C for 45 minutes or left untreated at 37^{o}C, and imaged by live cell microscopy. At 37^{o}C, both GFP-hGle1-IVS14-2A>C and GFP-hIPK1 exhibited cytoplasmic steady state localization, with no apparent localization at the nuclear envelope or nucleoplasm (Figure 3.1). The cytoplasmic distribution of GFP-hGle1-IVS14-2A>C with exclusion from the nucleus was indistinguishable from previously reported hGle1A localization (Aditi et al., 2015; Kaneb et al., 2015; Kendirgi et al., 2003), and had no similarity to hGle1B localization (Folkmann et al., 2013; Kendirgi et al., 2003). Interestingly, as previously observed for both hGle1A and hGle1B, GFP-hGle1-IVS14-2A>C was recruited to SGs upon heat shock at 45^{o}C (Figure 3.1). GFP-hIPK1 also colocalized to SGs with mCherry-G3BP, confirming the previously published report (Brehm et al., 2007). Thus, the ALS hGle1 variant exhibits steady state localization and SG recruitment comparable to hGle1A.

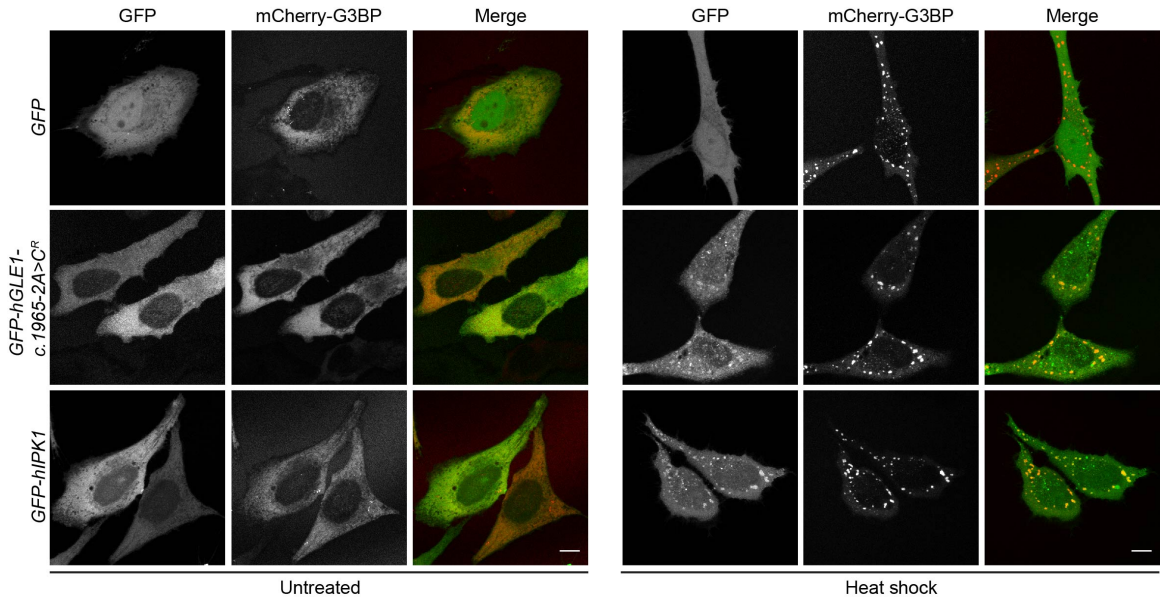


Figure 3.1: ALS-linked hGle1-IVS14-2A>C is localized to SGs upon heat shock.

The *mCHERRY-G3BP* plasmid and either *GFP*, *hGLE1* siRNA resistant *GFP-hGle1-c.1965-2A>C*^{R}, or *GFP-hIPK1* plasmids were co-transfected in HeLa cells. After 24h post-transfection, HeLa cells were heat shocked at 45°C for 45 min and imaged live by confocal microscopy. Both hGle1-IVS14-2A>C and hIPK1 are localized to SGs upon heat shock. Scale bar represents 10 µm.

The ALS-linked hGle1-IVS14-2A>C is capable of SG function

We recently found that hGle1A is required for proper SG assembly, disassembly and translational modulation (Aditi et al., 2015). The depletion of endogenous hGle1 from HeLa cells results in a striking decrease in the size and increase in the total population of SGs formed upon heat shock, as compared to control siRNA-treated cells. This defect is rescued by expression of hGle1A, but not hGle1B (Aditi et al., 2015). Since GFP-hGle1-IVS14-2A>C localized to SGs, we next investigated whether this variant was functionally competent to rescue SG defects in hGle1-depleted cells. To test this, CTRL siRNA or *hGLE1* siRNA-treated cells were transfected with either *GFP* or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids. After 24h, cells were heat shocked at 45^{o}C for 60 minutes, fixed and SG formation was assessed by indirect immunofluorescence of the SG protein G3BP (Tourrière et al., 2003). In CTRL siRNA-treated cells, no change in SG numbers was observed in cells expressing either *GFP* or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids (Figure 3.2A). Consistent with our previous report (Aditi et al., 2015), in *hGLE1* siRNA-treated cells transfected with *GFP*, there was a significant increase in the number of SGs and apparent decrease in individual size (Figure 3.2A and 3.2B; Aditi et al., 2015). However, strikingly, expression of *GFP-hGLE1-c.1965-2A>C*^{R} led to a significant reduction in SG numbers in hGle1-depleted cells (Figure 3.2B). This data suggested that the ALS-linked hGle1 variant is capable of functioning in SGs similarly to hGle1A, at least at a gross morphological level. Future studies are required to determine whether assembly and disassembly rates are impacted by the *hGLE1-c.1965-2A>C* mutation.

Overexpression of the ALS-linked hGle1-IVS14-2A>C alters stress granule morphology

The mRNPs in SGs and those engaged by translation machinery are in dynamic

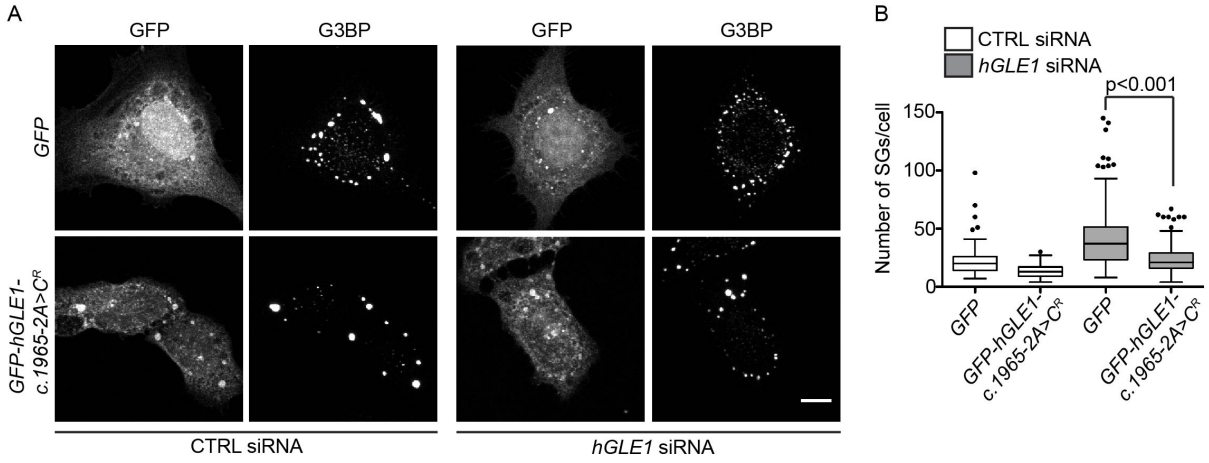


Figure 3.2: ALS-linked hGle1-IVS14-2A>C rescues SG assembly defects in hGle1-depleted cells similar to that of hGle1A.

(A) CTRL or hGLE1 siRNA-treated cells were transfected with either GFP or GFP-hGLE1-c.1965-2A>CR plasmids. Cells were heat shocked at 45°C for 60 minutes and processed for indirect immunofluorescence using anti-G3BP antibodies. Scale bar represents 10 μm. (B) Quantification of SG numbers in CTRL and hGLE1 siRNA cells expressing indicated plasmids. ImageJ plug-in "3D objects counter" was used to count SG numbers of an individual cell. Statistical significance was determined using Student's t test (Excel).

equilibrium (Aditi et al., 2015; Kedersha et al., 2000). Previously, we demonstrated that hGle1A modulates this equilibrium and thus influences SG formation. Overexpression of hGle1A but not hGle1B alters the cell's translation activity and increases the pool of free mRNPs, resulting in the formation of larger SGs (Aditi et al., 2015). Since the ALS-linked hGle1 variant rescues an SG defect in hGle1-depleted cells, we hypothesized that it might also impact SG size in a fashion similar to hGle1A overexpression. To test this, SG morphology was examined in cells transfected with *GFP, GFP-hGle1A* or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids and subjected to heat shock stress. G3BP staining was used to assess SG formation. As reported previously, expression of GFP-hGle1A resulted in a modest but significant increase in mean SG size compared to GFP alone (Figure 3.3; Aditi et al., 2015). Interestingly, we observed that expression of GFP-hGle1-IVS14-2A>C also led to an increase in size of SGs (Figure 3.3). In fact, no significant difference was detected for SG morphology in cells expressing hGle1A versus hGle1-IVS14-2A>C (Figure 3.3). This data further argued that the general capacity for hGle1 functions in SG assembly are unaffected by the ALS-linked hGle1 variant.

Overexpression of hGle1A or the ALS-linked hGle1-IVS14-2A>C results in cytoplasmic aggregates that do not co-localize with SGs components

Our analysis of SG morphology following overexpression of hGle1 isoforms also revealed the presence of cytoplasmic GFP-hGle1A or GFP-hGle1-IVS14-2A>C aggregates in approximately 10% of the transfected cell population. We categorized these aggregates into two groups. In one group, the cells formed small "foci" aggregates reminiscent of SGs (Figure 3.4, center column). In the second group, the cells exhibited large "fibrous" aggregates that appeared to form from long extended fibers of hGle1 protein (Figure 3.4, right column).

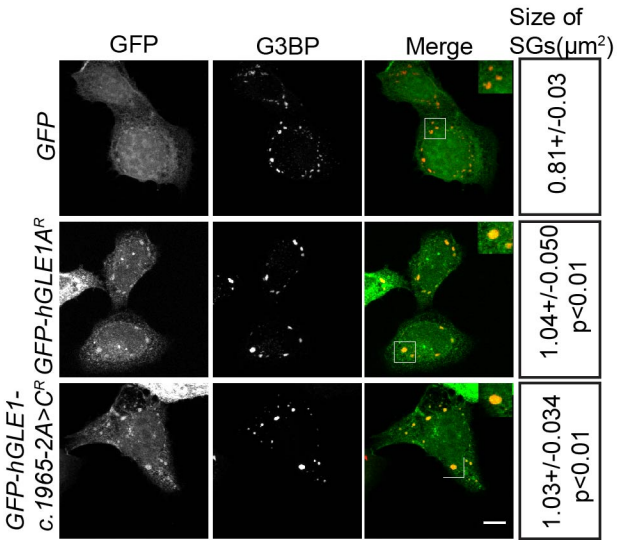


Figure 3.3: Expression of ALS-linked hGle1-IVS14-2A>C results in larger SGs similar to that of hGle1A.

HeLa cells were transfected with GFP, hGLE1 siRNA resistant GFP-hGLE1AR, or GFP-hGLE1-c.1965-2A>CR plasmids. Cells were heat shocked for 60 minutes at 45°C. After heat shock, cells were processed for indirect immunofluorescence using anti-G3BP antibodies. Size of SGs was determined using ImageJ plug-in "3D objects counter". Data are mean +/- standard error from three independent experiments. Statistical significance was determined using Student's t test (Excel); whiskers represent 10-90 percentile. Scale bar represents 10 μm.

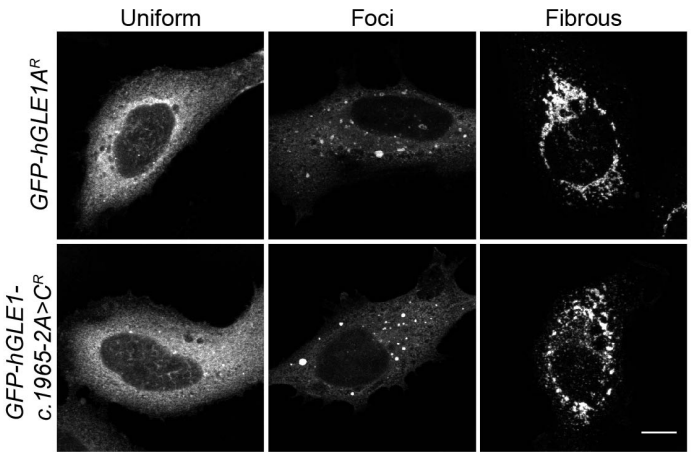


Figure 3.4: Overexpression of ALS-linked hGle1-IVS14-2A>C and hGle1A result in formation of cytoplasmic aggregates.

GFP, *GFP-hGle1A*^{R} or *GFP-hGle1-c.1965-2A>C*^{R} plasmids were transfected in HeLa cells. After 24h, cells were fixed and processed for indirect immunofluorescence. Approximately 90% of cells showed uniform cytoplasmic localization of GFP-hGle1A and GFP-hGle1-IVS14-2A>C. However, ~10% of cells formed either "foci" or "fiber-like" aggregates. Scale bar represents 10 μm.

To examine whether these aggregates contain SG components, indirect immunofluorescence was performed for G3BP and DDX3 in cells transfected with *GFP*, *GFP-hGLE1A*^{R} or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids. No co-localization was observed between G3BP or DDX3 and the hGle1A or hGle1-IVS14-2A>C aggregates (Figure 3.5A and 3.5B). On the contrary, in cells exhibiting "fibrous" hGle1 aggregates, G3BP formed independent cytoplasmic foci resembling SGs. This data suggests that the stress response pathway was provoked by the presence of hGle1 aggregates and likely does not initiate the aggregation. However, it cannot be ruled out that the SG components might be co-recruited in a sequential or temporal manner, or that other SG components might be preferentially recruited to these foci.

hGle1A and ALS-linked hGle1-IVS14-2A>C aggregates co-localize with TDP-43

It is proposed that the aggregation of ALS-linked TDP-43 and FUS altered proteins sequester several other RNA-binding proteins to promote pathological formation of the neuronal inclusions (Bentmann et al., 2013). Therefore, indirect immunofluorescence microscopy was used to test whether the hGle1A or hGle1-IVS14-2A>C positive aggregates sequester wild type TDP-43 or FUS. "Foci-like" hGle1 aggregates did not co-localize with either FUS or TDP-43 proteins (Figure 3.6A and 3.6B). However, TDP-43 did colocalize with the "fibrous" GFP-Gle1A and GFP-hGle1-IVS14-2A>C aggregates (Figure 3.6A and 3.6B). This data indicated that overexpression of hGle1A or ALS-linked hGle1 variant can sequester wild type TDP-43 protein in this cell model system.

Hsp90 colocalizes with hGle1A and hGle1-IVS14-2A>C aggregates

Hsp90 is a molecular chaperone and master regulator of the stress response program,

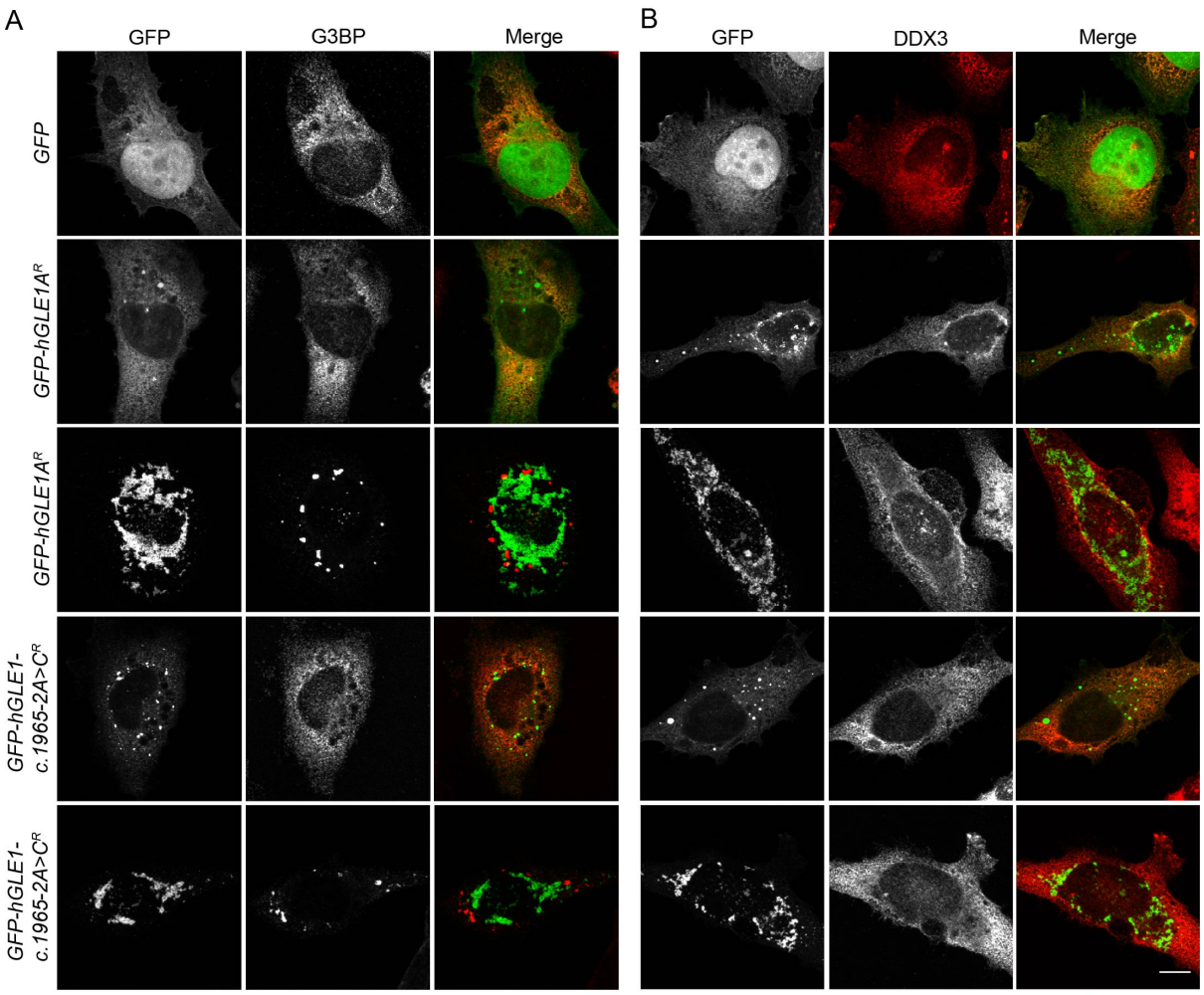


Figure 3.5: Overexpression of ALS-linked hGle1-IVS14-2A>C and hGle1A result in formation of cytoplasmic aggregates that do not co-stain with SG components, DDX3 and G3BP.

HeLa cells were transfected with *GFP, GFP-hGLE1A*^{R}, or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids. 24 hours after transfection, cells were fixed and processed for indirect immunofluorescence using (A) anti-DDX3 and (B) anti-G3BP antibodies. Scale bar represents 10 µm.

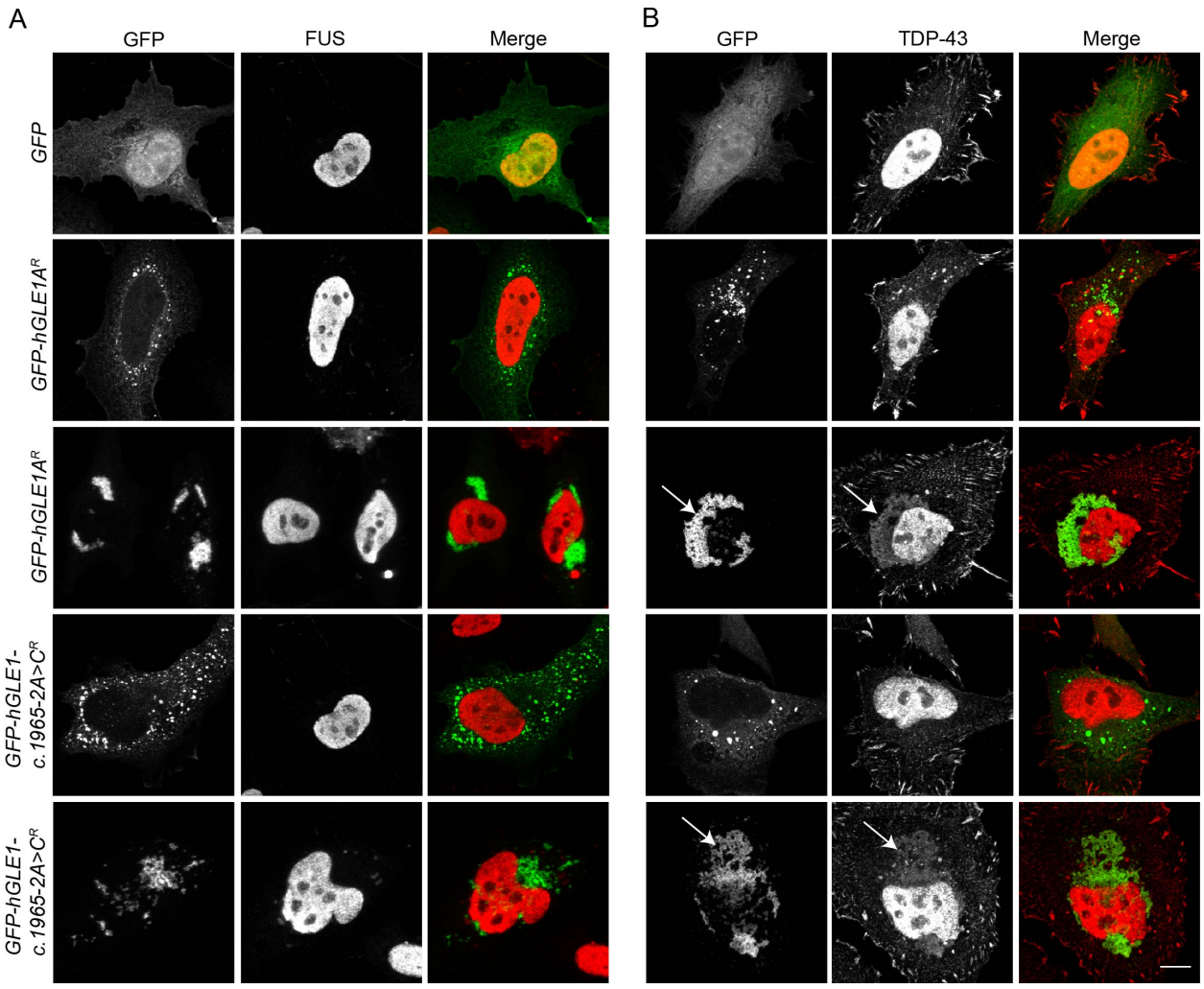


Figure 3.6: Overexpression of ALS-linked hGle1-IVS14-2A>C and hGle1A result in formation of cytoplasmic aggregates that co-stain with TDP-43.

HeLa cells were transfected with *GFP, GFP-hGLE1A*^{R}, or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids. After 24h, cells were fixed and processed for indirect immunofluorescence using anti-TDP-43 and anti-FUS antibodies. Scale bar represents 10 μm.

which has been suggested to modulate the stability and degradation of mutant protein aggregates in neuronal inclusions (Luo et al., 2010). To determine whether Hsp90 might be involved in regulating hGle1 inclusions, we tested whether hGle1A or hGle1-IVS14-2A>C aggregates co-localize with Hsp90 protein. As described, HeLa cells expressing GFP, GFP-hGle1A or GFP-hGle1-IVS14-2A>C were fixed and processed for indirect immunofluorescence of Hsp90. Although no colocalization was observed in "foci-like" hGle1 aggregates, Hsp90 co-localized with "fibrous" hGle1 aggregates in both GFP-Gle1A and GFP-hGle1-IVS14-2A>C expressing cells (Figure 3.7A). This data suggests that Hsp90 might be recruited to hGle1-IVS14-2A>C cytoplasmic aggregates in ALS motor neurons. Since deregulation of RNA metabolism is also linked to ALS (Ramaswami et al., 2013), *in situ* hybridization was performed to determine if these aggregates also contain poly(A)$^+$ RNA. No nuclear accumulation of poly(A)$^+$ RNA was observed in GFP-Gle1A or GFP-hGle1-IVS14-2A>C aggregates (Figure 3.7B), however, we cannot exclude the possibility that specific types of RNA are present in these aggregates.

The ALS-linked hGle1-IVS14-2A>C protein rescues mRNA export defects of hGle1-depleted cells

Efficient and expedient mRNA export from the nucleus is essential for proper gene expression and cellular function. In complex with IP_6, hGle1B plays an integral role in this process by regulating mRNP remodeling at the NPC cytoplasmic face (Folkmann et al., 2011). Based on the lack of nuclear rim localization for the ALS-linked hGle1-IVS14-2A>C and its functional similarity with hGle1A in SG biology, we predicted that hGle1-IVS14-2A>C would

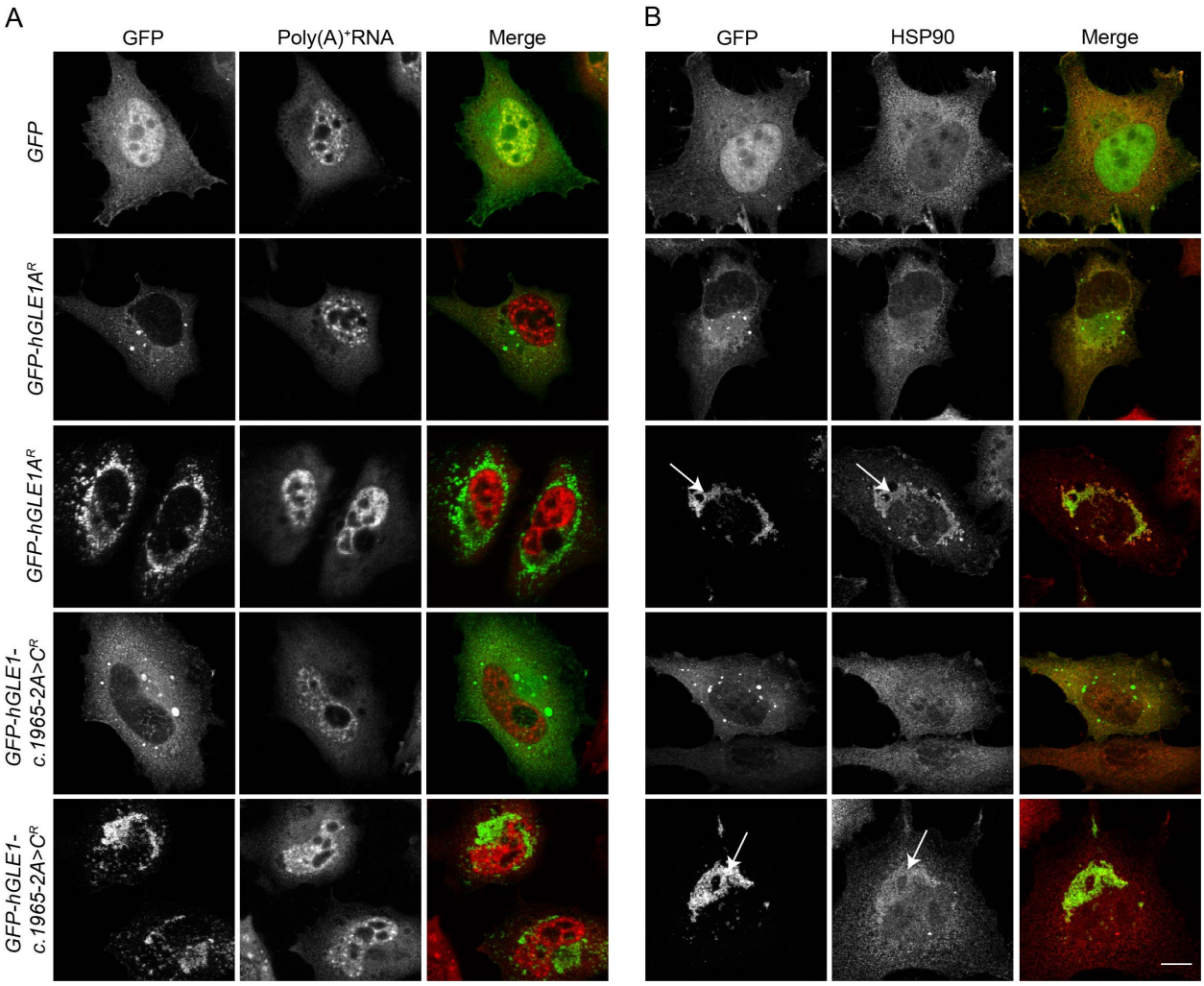


Figure 3.7: Overexpression of ALS-linked hGle1-IVS14-2A>C and hGle1A result in formation of cytoplasmic aggregates that co-stain with Hsp90.

HeLa cells were transfected with *GFP, GFP-hGLE1A*^{R}, or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids. Cells were fixed and processed for either indirect immunofluorescence using anti-Hsp90 antibodies or Cy3-conjugated oligo-dT *in situ* hybridization for poly(A)$^+$ RNA. Scale bar represents 10 μm.

not support mRNA export. Using our previously validated knockdown:add-back approach of hGle1 depletion and exogenous expression (Folkmann et al., 2013; Aditi et al., 2015), we investigated whether the hGle1-IVS14-2A>C protein was capable of mediating mRNA export. Following CTRL siRNA or hGle1 siRNA treatment, HeLa cells were transfected with GFP, GFP-*hGLE1B*^{R}, GFP-*hGLE1A*^{R} or GFP-*hGLE1-c.1965-2A>C*^{R} plasmids. A small, yet statistically significant, increase in the nucleocytoplasmic ratio of poly(A)$^{+}$ RNA was observed in hGle1-depleted cells expressing GFP, as compared to CTRL siRNA-treated cells (Figure 3.8). The mRNA export defect was even more pronounced in hGle1-depleted cells expressing exogenous hGle1A. However, expression of exogenous hGle1B or, surprisingly, exogenous ALS-linked hGle1-IVS14-2A>C rescued the mRNA export defect caused by endogenous hGle1 depletion (Figure 3.8). Thus, hGle1-IVS14-2A>C supports some level of poly(A)$^{+}$ RNA export.

Discussion

hGle1 is a multifunctional regulator of DEAD-box proteins that is essential for proper mRNP dynamics at several stages in the mRNA lifecycle (Bolger and Wente, 2011; Bolger et al., 2008; Murphy and Wente, 1996). Although the use of a single factor to control multiple processes can provide the cell a mechanism for rapid and coordinated changes in gene expression, it also comes with a fair degree of risk for catastrophic effects in the event of hGle1 dysfunction. We recently uncovered one mechanism by which the cell compartmentalizes hGle1, elucidating that the two hGle1 isoforms hGle1A and hGle1B localize to different cellular pools and perform non-overlapping critical functions (Table 1; Folkmann et al., 2013; Aditi et al., 2015). Mutations that impact the balance in the functional pools of hGle1 or alter the function of both hGle1 isoforms should have far-reaching impact on mRNA metabolism.

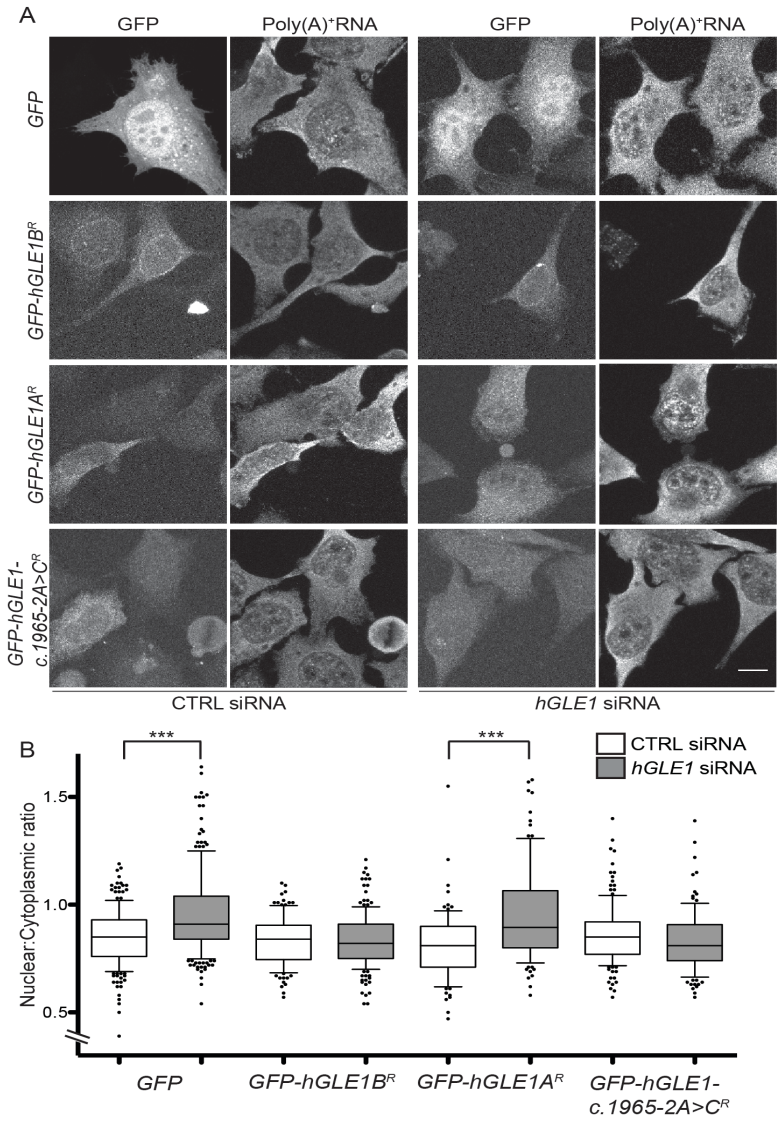


Figure 3.8: ALS-linked hGle1-IVS14-2A>C rescues mRNA export defects in hGle1-depleted cells similar to hGle1B.

Cells treated with either CTRL siRNA or *hGLE1* siRNA were co-transfected with *GFP, GFP-hGLE1A*^{R}, *GFP-hGLE1B*^{R}, or *GFP-hGLE1-c.1965-2A>C*^{R} plasmids and assessed for mRNA export defect using Cy3-conjugated oligo-dT *in situ* hybridization for poly(A)$^+$ RNA. Representative images from each condition are shown (A). Scale bar represents 10 μm. (B) Data were analyzed using mean intensity of nuclear:cytoplasmic fractions, where increase in ratio demonstrates nuclear retention of poly(A)$^+$ RNA, indicating an poly(A)$^+$ RNA export defect. Data represents 3 independent experiments; ***$p<0.0001$ using Student's t test (Excel); whiskers represent 10-90 percentile.

The lethal inherited diseases LCCS1 and LAAHD are prime examples of this, wherein mutations that decrease hGle1's ability to homo-oligomerize or destabilize its structural integrity lead to mRNA export defects that are linked to the arrest of proper development ((Folkmann et al., 2013; Nousiainen et al., 2008).

In this study, we characterized the functional capacity of a rare ALS-linked *hGLE1-c1965-2A>C* mutation that alters the carboxy-terminal domain of hGle1 to produce a protein with a novel 88 amino acid domain in place of the predicted hCG1-binding interface. Based on the loss of the NPC-anchoring hCG1 binding domain, we previously surmised that this mutation probably results in haploinsufficiency by reducing the pool of hGle1 supporting mRNA export (Kaneb et al., 2015). Surprisingly, we discovered here that the ALS-linked hGle1-IVS14-2A>C is functional for both stress granule homeostasis and mRNA export, making it the first known bi-functional hGle1 isoform capable of functions assigned to both hGle1A and hGle1B.

In Table 1, the functional capacities of the hGle1-IVS14-2A>C protein is compared to the wild type isoforms hGle1A and hGle1B. The novel hGle1-IVS14-2A>C protein functioned in all aspects of stress granule modulation that we examined and also rescued mRNA defects under the conditions tested. The only known molecular characteristic that hGle1-IVS14-2A>C appears to lack is its reported inability to bind hCG1 (Kaneb et al., 2015) and therefore weak association with the NPC (demonstrated herein). This lack of hGle1-IVS14-2A>C interaction with hCG1 has only been tested using the qualitative yeast-two-hybrid. It is possible that the hGle1-IVS14-2A>C protein has reduced affinity for hCG1 compared to hGle1B, but is not as compromised as hGle1A. Alternatively, the extended carboxy-terminal domain in hGle1-IVS14-2A>C may support another interaction that compensates for loss of hCG1 binding. In either case, such a bi-functional isoform would impact the balance between the hGle1B and hGle1A cellular pools and

Table 1: ALS-linked hGle1-IVS14-2A>C exhibits behavior mimicking both hGle1B and hGle1A isoforms.

†(Kaneb et al., 2015), *Rescues defect in hGle1-depleted cells

Isoform	Localizes to SGs	Rescues SG defects*	Forms larger SGs	Forms aggregates	Rescues mRNA export defects*	Binds hCG1†
GFP-hGle1A	+	+	+	+	-	-
GFP-hGle1B	+	-	-	Not tested	+	+
GFP-hGle1-IVS14-2A>C	+	+	+	+	+	-

could limit the cell's ability to properly regulate hGle1 activity.

Several caveats must be considered in drawing conclusions from this data with regard to mechanisms of ALS disease pathogenesis. First, the ability of hGle1-IVS14-2A>C to function in mRNA export was observed in the absence of any other endogenous hGle1 protein, whereas the ALS patients were heterozygous. Second, the expression level for the ALS-linked hGle1-IVS14-2A>C in HeLa cells is likely not at a level representative of its endogenous steady state levels in ALS patients; nor are HeLa cells a model for the neuronal disease. Moreover, hGle1-IVS14-2A>C does not localize to the nuclear rim in the presence of endogenous hGle1B. Therefore, in a heterozygous background, some reduction in mRNA export competent hGle1 function is expected. More work will be required to determine if an mRNA export defect or another nucleocytoplasmic transport defect is exhibited by the patient cell lines isolated from patients carrying the *hGLE1-c1965-2A>C* mutation. Nevertheless, the data shown here suggests that this mutation does not result in a complete loss of hGle1 function, but rather is more likely to impact ALS pathology by confounding the carefully balanced separation of hGle1 functions.

The potential disregulation of mRNP dynamics by the ALS-linked hGle1-IVS14-2A>C represents one of several recently discovered links between ALS and Gle1-associated functions. Several studies have implicated disregulation of the hGle1-cofactor IP_6 in neurodegenerative disease. First, activation of the caspase-independent apoptosis pathway is associated with familial ALS, and is provoked through phosphorylation of IP_6 following cytoplasmic translocation of IP_6 kinase (reviewed in Ghavami et al., 2014). Second, IPK1 is recruited to stress granules and increased levels of IP_6 are neuroprotective, suggesting that IP_6 production in SG might facilitate disaggregation of proteins sequestered there (Anekonda et al., 2011; Brehm et al., 2007; Lv et al., 2015; Xu et al., 2008). Importantly, general disruption of NPC function is

also highly associated with ALS. A clustering of ALS-linked mutations in sequence encoding the nuclear localization sequence (NLS) in FUS directly demonstrated the importance of nuclear import in ALS pathogenesis (Dormann et al., 2010). Furthermore, very recently, the most common form of familial, inherited ALS, *C9ORF72* repeat expansion, was shown to induce pathogenesis by disrupting nucleocytoplasmic transport through the NPC (Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015a). Finally, irregular Nup62 localization/nuclear envelope shape, loss of nuclear importins or cytoplasmic redistribution of importins are observed in the anterior horns of ALS patients and in mouse models of ALS (Kinoshita et al., 2009; Zhang et al., 2006).

Given that the ALS-linked *hGLE1-c.1965-2A>C* mutation results in an altered balance of hGle1 activity rather than a complete loss of function, we speculate that a second pathogenic trigger might induce neurodegeneration in combination with expression of hGle1-IVS14-2A>C. A complete genetic profile was not obtained for patients carrying the *hGLE1-c.1965-2A>C* mutation. Furthermore, analyses of other ALS-linked mutations as well as the frequency of sporadic ALS suggest this to be a disease of multiple contributing factors. A major contributing factor in TDP-43, FUS, and hGle1 biology is the effect of subcellular distribution on their aggregation potential (worked presented here; (Dormann and Haass, 2011; Johnson et al., 2008; Vance et al., 2013). Thus, any mutations or conditions that increase their cytoplasmic distribution, when combined with an altered state of nucleocytoplasmic transport, could shift the cellular equilibrium toward pathogenesis. Such a combinatorial pathway to the disease state is consistent with the late onset of ALS pathology. One major change in RNA metabolism that would also contribute to late onset is the detrimental effect of aging NPCs on nucleocytoplasmic transport. Numerous reports have implicated decreased efficiency of nucleocytoplasmic transport

in neurodegenerative diseases (Patel and Chu, 2011), and our laboratory recently discovered specific defects in transport pathways that occur as a result of ageing-related Nup deterioration (Lord et al., 2015). This must be accounted for in models of ALS pathogenesis, particularly with respect to sporadic ALS where aging-related transport defects might combine with another ALS risk factor such as repeated stress (Fidler et al., 2011) to induce disease progression.

In recent years, several studies have highlighted the connections between deregulation in RNA metabolism, protein aggregation, and neurodegenerative diseases including ALS. For example, FUS and TDP-43 are found in protein aggregates. These FUS and TDP-43 inclusions occur independently and are distinct from stress granules (SGs) in that they not reversible, but rather are insoluble (Bentmann et al., 2013). Here, we have demonstrated that hGle1 is capable of forming protein aggregates upon its overexpression. Strikingly, these hGle1 aggregates sequestered both endogenous TDP-43 and Hsp90 proteins. Although the precise pathological effects of hGle1 protein aggregates are unknown, we propose that hGle1 aggregates sequester native RNA-binding proteins such as TDP-43 and thereby promote ALS by the gain- and/or loss of native RNA-binding protein functions. This idea is further supported by recent studies demonstrating that the lariat debranching enzyme Dbr1 modifies TDP-43 toxicity by capturing TDP-43, preventing its aggregation and thus the sequestration of other RNA-binding proteins with it (Armakola et al., 2012).

The seeding of cytoplasmic aggregates due to delayed or nonexistent SG disassembly is another attractive model for how the characteristic insoluble neuronal inclusions of ALS motor neurons form. This model is supported by the cellular pathology of ALS-linked mutations in the gene encoding ubiquitin segregase Valosin-containing protein (VCP). VCP is required for clearance of SGs after the stress response, and mutations in VCP result in constitutive SG

formation. Strikingly, though, these mutations also result in sequestration of wild type TDP-43 within the constitutive SGs (Buchan et al., 2013; Johnson et al., 2010). We observed no functional alterations in the ability of hGle1-IVS14-2A>C to support SG homeostasis; however, there are certainly other potential aspects of SG function that might be perturbed by hGle1-IVS14-2A>C expression. Our future studies will need to examine the capacity of this hGle1-IVS14-2A>C in the kinetics of SG function, such as the rate of SG disassembly.

Taken together, this data provides evidence that the hGle1-IVS14-2A>C protein is a bi-functional isoform capable of mediating both mRNA export, like hGle1B at the NPC, and SG function, like hGle1A in the cytoplasm. This surprising discovery highlights the critical nature of the carboxy-terminal domain in regulating hGle1, and calls for further investigation to determine its mechanistic role in hGle1 function, interactions and regulation. We also found that hGle1 assembles into cytoplasmic aggregates that harbor TDP-43 and Hsp90, both of which are found in the neuronal inclusions of ALS patients. It is intriguing that hGle1, like TDP-43 and FUS, plays roles at multiple stages of the mRNA life-cycle and that all three are linked to devastating human diseases with motor neuron pathologies. Previous studies do not report any direct functional links between hGle1 and either TDP-43 or FUS; however all three play roles in regulating both SGs and translation activity. Thus, one molecular mechanism of ALS pathology might be the combined impact of their misregulation on translational activity. Alternatively, hGle1 misregulation might alter the nuclear export of specific mRNAs that interact with TDP-43 and FUS for localized translation. Future studies are needed to shed light on these molecular connections and further characterize the effect of hGle1 misregulation on motor neuron health and in ALS pathogenesis.

CHAPTER 4

Conclusions and future directions

Modulation of mRNA metabolism has a central role in the cellular adaptive response against changing conditions (de Nadal et al., 2011). This regulation occurs at both transcriptional and post-transcriptional levels and is mediated by RNA-binding proteins that determine the fate of mRNAs. Extensive rearrangements of mRNPs occur during stress conditions, altering their downstream fate during RNA processing, export, translation and decay (Keene, 2007; de Nadal et al., 2011; Nakaminami et al., 2012; Shih and Lee, 2014). However, we do not understand the mechanisms by which messages are sorted for their distinct fates. Our data suggests that an mRNA regulatory factor, hGle1, plays a critical role in the stress response by mediating mRNP rearrangements. In chapter 2, We found that hGle1 regulates SGs by modulating the level of active translation. Interestingly, only the hGle1A isoform plays a role in SG formation, whereas only hGle1B functions in mRNA export. We propose that hGle1A modulates SGs by regulating the activity of DDX3, since DDX3 suppresses hGle1-mediated SG and translation phenotypes, and interacts with hGle1 (Aditi et al., 2015). Taken together, this work defines an isoform-specific role for hGle1A in mediating the stress response by modulating the trafficking of mRNPs between an actively translating pool and a repressed pool sequestered in SGs.

In the second part of this work (chapter 3), we determined that the ALS-linked hGle1 variant protein (hGle1-IVS14-2A>C) is capable of functioning in both mRNA export and SG formation, in essence behaving as both hGle1A and hGle1B isoforms (Aditi et al., 2015). Collectively, this work suggests that an ALS-linked *GLE1* mutation leads to the loss of isoform

specific hGle1 function, leading to deregulation of normal mRNA metabolism. We speculate that the resulting loss of proper gene expression in response to stress is a major contributing factor in the pathogenesis of ALS.

In summary, this work elucidated that hGle1A and hGle1B perform distinct functions and also suggested a potential mechanism underlying ALS pathogenesis. However, these studies did not offer mechanistic insights into the distinct non-overlapping functions of hGle1 isoforms. Given that the ALS-linked hGle1-IVS14-2A>C can complement the functions of both hGle1A and hGle1B isoforms, understanding the mechanistic underpinnings of each activity will be crucial to tease out the disregulation underlying ALS pathology. In the following sections, I discuss potential future avenues.

Molecular mechanisms underlying the distinct roles of hGle1 isoforms

In human cells, the hGle1A and hGle1B isoforms are generated by alternative splicing of transcripts from a single h*GLE1* locus. These isoforms are very similar in sequence except that hGle1A lacks 39 amino acids at its carboxy-terminal (Kendirgi et al., 2003). Our work reveals that despite sharing more than 90% of amino-acid sequence, these two isoforms perform distinct functions in a cell; hGle1B is required for mRNA export (Folkmann et al., 2013), while hGle1A functions in SGs (Aditi et al., 2015). Interestingly, this functional specificity is not due solely to subcellular localization. Similar to hGle1B, hGle1A is localized at NPCs in h*GLE1* siRNA-treated cells. Likewise, hGle1A and hGle1B are recruited to SGs in response to stress. This raises the question as to how the functional specificity of each hGle1 isoforms is achieved? There are at least two possible reasons for this. The additional residues in hGle1B could allow it to bind to unique protein partners necessary for modulating mRNA export. Indeed, we previously showed

that the additional 39 residues in hGle1B are required for its interaction with the nucleoporin hCG1 (Kendirgi et al., 2005). This observation raises the possibility that hGle1B function during mRNA export is modulated by hCG1. In fact, this conclusion is supported by recent work from the Wente laboratory demonstrating that interaction between yGle1 and the carboxy-terminal domain of Nup42 (a yeast homologue of hCG1) is required for mRNP remodeling (Adams et al., 2014). Moreover, there could be additional factors that differentially influence functions of each hGle1 isoforms. Therefore, future studies could be focused on defining the unique protein interaction partners of hGle1A and hGle1B. We have generated stable HeLa cell lines expressing low levels of either GFP, GFP-hGle1A or GFP-hGle1B. These cell lines are ideal for performing immunoprecipitation since the expression of hGle1 isoforms is homogenous on a cell-to-cell basis in contrast to transient transfection. Immunoprecipitation using GFP-antibodies coupled to magnetic beads could be performed with cell lysates prepared under normal and stress conditions, followed by liquid chromatography tandem mass spectrometry to identify interacting partners. Novel interactions could be validated and studied by various methods including co-immunoprecipitation, FRET and *in vitro* binding assays. However, an important caveat of using this method is that these isoforms are not expressed from their endogenous promoter. The GFP tag might hinder the binding to some protein partners, thus biasing the identification of interactor proteins. A complementary approach could be to manipulate the levels of hGle1 isoforms endogenously. hGle1A contains a unique 4 amino acids at its carboxy-terminus. Therefore, siRNAs that only target either h*GLE1A* or h*GLE1B* isoforms could be designed followed by immunoprecipitation of isoforms using hGle1 antibodies and mass spectrometry.

An alternative explanation, but not mutually exclusive from above, for separate functions of hGle1 isoforms could be that the isoforms regulate different Dbps. Based on work in yeast,

Gle1 regulates mRNA export and translation termination by stimulating the ATPase activity of Dbp5 (human homologue DDX19) (Alcázar-Román et al., 2006, 2010; Bolger et al., 2008). In contrast, yGle1 inhibits ATPase activity of Ded1 (human homologue DDX3) during translation initiation (Bolger and Wente, 2011). Therefore, it is likely that different Dbps dictate the distinct functions of hGle1 isoforms. In human cells, DDX3 is a critical component of SGs and siRNA knockdown of *DDX3* perturbs SG assembly (Shih et al., 2012). DDX3 is also linked with the export of viral RNA but not with export of bulk poly $(A)^+$ mRNA (Yedavalli et al., 2004). On the other hand, we have observed that depletion of DDX19B in HeLa cells results in mRNA export defects, but not SG defects (Aditi et al., 2015). Therefore, I speculate that hGle1A and hGle1B specifically regulate DDX3 and DDX19 ATPase activity, respectively. Several pieces of evidence support this model. 1) Endogenous hGle1 co-immunoprecipitates with DDX19B and DDX3 proteins in HeLa cells (Aditi et al., 2015 and unpublished results). 2) mRNA export is dependent upon both hGle1B and DDX19, as their depletion results in accumulation of poly (A^+) mRNA in the nucleus (Aditi et al., 2015). Moreover, expression of the dominant-negative mutant of *DDX19B*, *ddx19-R372G* also results in mRNA export defects, by acting as a competitive inhibitor of wild-type DDX19B and sequestering hGle1 (Hodge et al., 2011). 3) hGle1A-dependent SGs and translation defects are rescued by expression of *DDX3* but not by *DDX19B* (Aditi et al., 2015). Taken together, these results suggest that, similar to yGle1, hGle1 regulates the ATPase activity of DDX3 and DDX19. However, this hypothesis needs to be directly verified with regard to the isoform specificity for each Dbp. This could be tested by investigating whether each hGle1 isoform directly interacts with DDX3 or DDX19 using an *in vitro* soluble binding assay. Next, an *in vitro* ATPase assay could be performed with the recombinant proteins to test if hGle1 modulates the ATPase activity of DDX3 and DDX19. However, the

interpretation of the experimental outcome might be limited due to the absence of post-translation modifications or additional cofactors that might dictate the endogenous specificity of hGle1 modulation of Dbps. Therefore, if purified bacterially expressed-recombinant proteins do not yield any clear results, insect cell lines could be used to purify these proteins. Alternatively, human lysates could be fractionated and tested for additional cofactors.

An important remaining aspect of this research is to examine Dbp modulation by hGle1 isoforms. In this regard, structure determination of hGle1A-DDX3 and hGle1B-DDX19 protein complexes could very helpful. It is challenging to purify full-length hGle1A and hGle1B at a high concentration, however, the Wente laboratory has shown that the carboxy-terminus of yGle1 is sufficient to modulate the ATPase activity of Dbps (Alcázar-Román et al., 2006; Bolger and Wente, 2011). In fact, the Weis laboratory has reported the crystal structure of Dbp5 in complex with the carboxy-terminus of yGle1-IP_6 (Montpetit et al., 2011). Therefore, solving the structure of carboxy-terminus of hGle1A and hGle1B proteins could be very helpful to understand the basis of distinct functions of each isoform.

In summary, elucidation of the mechanisms underlying hGle1 regulation of Dbps and the identification of additional interaction partners could provide great insights into the mechanism by which hGle1 isoforms achieve functional specificity. These investigations would aid in our understanding of misregulation of altered hGle1 protein arising from h*gle1* disease-linked mutations.

Investigating the role of hGle1 isoforms in translation

To date, we have shown that hGle1A regulates stress granules by modulating translation during stress. However, it is unclear whether hGle1A also regulates translation under basal conditions. In *S. cerevisiae*, yGle1 physically interacts with translation initiation factor eIF3

subunits, Prt1 and Ded1, and modulates the ATPase activity of Ded1 during AUG start site selection. Likewise, yGle1 physically interacts with the translation termination factor Sup45 and is required to regulate the ATPase activity of Dbp5 for efficient translation termination (Bolger and Wente, 2011; Bolger et al., 2008). Our work in human cells shows that the siRNA-knockdown of h*GLE1* also results in translation defects (Aditi et al., 2015). Using sucrose-density centrifugation, hGle1-depleted cells show an increase in the monosome 80S peak and reduction in polysomes. This phenotype is similar to the polysome profile defects observed with the *ygle1* temperature-sensitive alleles, arguing that hGle1 too plays a role in translational control. In addition, we observe reduced ^{35}S methionine incorporation in protein extracts from hGle1-depleted cells, indicative of overall reduction in protein synthesis (Aditi et al., 2015). However, these studies did not address the mechanisms behind the role of hGle1 in translation.

To investigate the roles of hGle1A and/or hGle1B in translational control, *in vitro* translation assays using ribosome enriched extracts and *in vitro*-transcribed luciferase mRNA with recombinant hGle1A and hGle1B proteins could be performed. Additionally, siRNA knockdown of endogenous h*GLE1* and add-back of either h*GLE1A* or h*GLE1B* could be performed to test if either isoform rescues polysome defects in hGle1-depleted cells. To test for a specific role in start site selection (similar to yGle1), *in vivo* ATF4 reporter based assays could be performed in control and h*GLE1* siRNA-treated cells (Bolger et al., 2008; Hinnebusch, 2011; Wagner et al., 2014). This assay measures leaky scanning, which results when the pre-initiation complex fails to recognize the start codon and continues scanning. An *ATF4* construct is designed such that it lacks uORF2, 3 and 4 but retains the stimulatory uORF1. This reporter is translationally active and serves as a control. A second reporter is designed where uORF2 of *ATF4* is followed by a sequence that encodes for luciferase. This product is only synthesized if

the start site in uORF2 is bypassed through leaky scanning, thus providing a measure of an estimate of scanning defects during start site selection. An alternative assay to assess defects in start site recognition is to test for start site selection fidelity. Start site fidelity defects are characterized by the use of a near cognate codon for initiation (Hinnebusch, 2011; Wagner et al., 2014). In this assay, a set of reporters coding for firefly-luciferase are expressed in control and hGle1-depeleted cells. One set of reporters contains a consensus AUG start codon, whereas the other set of reporters has a mutated UUG site. An increase in the ratio of UUG to AUG indicates start site fidelity defects. If hGle1-depleted cells exhibit a defect in start site selection, rescue assays could be performed by adding-back siRNA resistant h*GLE1A* and/or h*GLE1B* constructs to determine which isoforms functions in translation initiation.

Similarly, translation termination defects could be assessed by transfecting control and hGle1-depleted cells with tandem constructs expressing luciferase and renilla proteins. Three types of constructs are used- a construct encoding a stop codon between luciferase and renilla, a second construct encoding no stop codon, and a third construct encoding a stem-loop upstream of the stop codon. Following their transfection and expression, read through efficiency will be measured in control and hGle1-depleted cells. Following this analysis, constructs expressing each isoform could be added back to determine if rescue is specific.

To understand the context of hGle1's actions in translation, it would be informative to know the mRNA targets of hGle1. With the advent of ribosome profiling (Ingolia et al., 2012), it is possible to identify the mRNAs that are being actively translated. Therefore, ribosome profiling in control and hGle1-depleted cells could be used to examine whether hGle1 is required for translation of all or a specific subset of mRNAs. One could speculate that hGle1 targets of translation would overlap with the subset of mRNAs identified as DDX3 targets (Lai et al.,

2010). However, previous studies have generated conflicting results about the role of DDX3 in translation. Some studies suggest that DDX3 is involved in translation of mRNAs containing long unstructured 5' UTRs. One of the better known targets is cyclin E1 (Lai et al., 2010). Therefore, comparing the ribosome profiling results between hGle1 and DDX3-depleted cells would allow us to test more definitively if hGle1 and DDX3 share similar mRNA targets.

To further delve into the mechanism of hGle1 regulation, it would be informative to understand how hGle1 interacts with translation factors. My preliminary data suggests that hGle1 binds to eIF4E cap complex (data not shown). Since yGle1 also physically and genetically interacts with translation initiation and termination factors (Bolger and Wente, 2011; Bolger et al., 2008), future experiments could explore how hGle1 works with translation machinery to modulate protein synthesis.

Taken together, these assays would provide important clues into the mechanisms underlying the role of hGle1 in translation under normal and disease conditions.

Investigating the role of hGle1 in translation and SG formation under stress

Global translation is down regulated under stress conditions (Holcik and Sonenberg, 2005). Surprisingly, we noticed that hGle1-depleted cells fail to down-regulate their translation and exhibit SG defects. We rescued SG defects by treating cells with puromycin, which disassembles polysomes (Aditi et al., 2015). Based on these observations, we propose that hGle1 modulates the equilibrium between SGs and translation. Since reprogramming of translation is essential for survival of cells during stress conditions, this raised several important questions. For instance, why do hGle1-depleted cells failed to downregulate translation even under continuous stress conditions? Interestingly, we also noticed that eIF2α phosphorylation is reduced in hGle1-

depleted cells compared to control cells (Aditi et al., 2015). As mentioned earlier, phosphorylation of eIF2α at serine 51 is one of the primary mechanisms to inhibit global protein synthesis under stress conditions. In fact, expression of a non-phosphorylatable serine to alanine substitution at residue 51 of eIF2α results in continued protein synthesis under heat stress (Murtha-Riel et al., 1993). Therefore, the continued translation observed in hGle1-depleted cells could be due to reduced eIF2 α phosphorylation. This potential regulatory loop should be investigated by examining the regulation of eIF2α phosphorylation in hGle1-depleted cells. We also anticipate that additional mechanisms, independent of eIF2α, might be involved by which hGle1 regulates translation under stress conditions.

Another avenue of further investigation would be to identify the nature of mRNAs that are being synthesized during stress condition in hGle1-depdelted cells. It is known that the IRES-containing mRNAs such as *HSP70* and *ATF4*, but not the housekeeping genes, are preferentially translated (Blais et al., 2004; Holcik and Sonenberg, 2005; McGarry and Lindquist, 1985). In order to investigate if translation is globally altered or specific to a subset of mRNAs in hGle1-depleted cells, we could biotin label newly synthesized proteins using click-chemistry reaction (Dieterich et al., 2006, 2007). These biotin-labeled peptides can be immunoprecipitated and analyzed by mass spectrometry to identify the nature of the nascent synthesized proteins under stress conditions. The identified targets could be further validated either by western blotting or by *in vivo* reporter translation assays performed in control and h*GLE1* siRNA-treated cells. Another complementary approach would be to perform ribosome profiling with control and hGle1-depleted cells under stress conditions (Ingolia et al., 2012). It is possible that hGle1 have different mRNA targets during basal and stress conditions and this may explain why hGle1-depletion have an opposing effects on translation under stress and non-stress conditions.

SGs formation is intimately linked with translation. Our work reveals that hGle1 modulates the distribution of mRNPs between those sequestered in SGs and those associated with the active translational machinery. Interestingly, in yeast, Ded1 is shown to regulate release of mRNA from SGs, promoting translation (Hilliker et al., 2011). Our work also suggests that DDX3 plays similar role as Ded1, however, the mechanistic details are not clear. Therefore, future work could be focused on determining the steps by which hGle1, through its regulation of DDX3, influences the movement of mRNPs between SG and translation. For this purpose, it is important to know which mRNAs reside in SGs. Roy Parker's laboratory has recently published a protocol to purify SGs from cells (Jain et al., 2016). Based on this protocol, we could purify the SGs from control, hGle1 and DDX3-depleted cells. Following SGs purification, the identity of proteins and mRNAs could be investigated by mass spectrometry and RNA-Seq, respectively. If hGle1 and DDX3 have common targets, further validation could be performed by sucrose density centrifugation followed by isolation of mRNAs in the free mRNP fraction versus mRNPs-bound in the polysome fractions. If hGle1 and DDX3 are involved in exchange of mRNPs between SGs and translation, we would expect to see a change in the distribution pattern of mRNAs in control versus hGle1 and DDX3-depleted cells.

The reprogramming of translation is important during stress conditions. However, we do not fully understand the molecular mechanism by which stress redirects the translation machinery. Since hGle1 depletion has a dramatic effect on translation and SGs, these studies are designed to understand the mechanisms by which hGle1 regulates translation and SG formation would be informative in deciphering the steps involved in translation regulation under stress conditions.

Role of hGle1 self-association in SG formation and translation

hGle1 self-associates in human cells. This self-association is mediated through interaction between the coiled-coil domains and is required during mRNA export (Folkmann et al., 2013). Notably, mutations that disrupt the sequence encoding the coiled-coil domain of hGle1 (hgle1-Fin_{major}) alter hGle1 self-association and result in mRNA export defects. Interestingly, Fin_{major} mimic insertions in *ygle1* also exhibit genetic interactions with mRNA export mutants but not with translation mutants (Folkmann et al., 2013). These data suggest that yGle1-self association is not required during translation. However, since hGle1-depleted cells show opposite effects on translation under basal and stress conditions, it is possible that there might be differential requirements for hGle1 self-association under normal and stress conditions. Future experiments could be focused on testing the requirement of hGle1 self-association during SG formation and translation. It is known that hGle1B homo-oligomerizes at the NPC and in the cytoplasm in living cells. But, we do not know if hGle1A also self-associates. As a starting point, Fluorescence Resonance Energy Transfer (FRET) experiments could be performed with mCerulean-hGLE1A and mVenus-hGle1A expressing FRET-pair constructs. In addition, *in vitro* binding experiments could be performed with recombinant MBP-hGle1A and ^{35}S labeled-hGle1A. To test for the requirement for hGle1 self-association during SG formation, deletion constructs lacking the coiled-coil region (ΔCC) could be tested for their ability to rescue SG defects in hGle1-depleted cells. An important caveat of this experiment is that a major portion of protein is missing and thus lack of rescue could be due to other reasons. Alternatively, the GFP-hgle1-Fin_{major}, which is compromised for self-association, could be tested for its ability to rescue SG defects in hGle1-depeleted cells. Similarly for translation, the *GFP-hgle1-*Fin_{major}^{R} and *GFP-hGLE1ΔCC* constructs could be tested for their ability to rescue translation defects in hGle1-

depeleted cells. It is unknown if a hGle1A-hGle1B heterodimer complex exists in a cell. Therefore, FRET experiments could be performed to test this hypothesis. Additionally, immunoprecipitation of hGle1A followed by immunoblotting with hGle1B could give information if this pair exists. Given that ALS-linked hGle1-IVS14-2A>C functions as both hGle1A and hGle1B (Aditi et al., 2015), it is possible that this variant may have greater tendency to form a heterodimer and thus could serve as a bifunctional isoform. Therefore, if hGle1 isoforms form heterodimers, it will be important to decipher the functional consequence of this heterodimer association.

Regulation of hGle1 function by post translational modification

hGle1 is an essential mRNA regulatory factor that controls various steps of gene expression. Importantly, mutations in *GLE1* have been linked with various diseases and thus hGle1 function must be tightly regulated (Kaneb et al., 2015; Nousiainen et al., 2008). However, we do not know how hGle1 activity is regulated inside a cell. One possibility already discussed is that its protein partners/localization modulates its function. A second mechanism could be regulation by post-translation modifications. Indeed, several large-scale mass spectrometry studies have identified phosphorylated hGle1 peptides (Mayya et al., 2009; Olsen et al., 2010; Sharma et al., 2014; Stuart et al., 2015). Furthermore, a recent study also showed that hGle1 is ADP-ribosylated at the NPC (Carter-O'Connell et al., 2016). Interestingly, we have also observed that hGle1 is phosphorylated at various residues upon stress (unpublished work; discussed in appendix A section, Figure A.2). We noticed that the cytosolic but not the nuclear pool of hGle1 is phosphorylated. Moreover, inhibition of SG formation did not affect hGle1 phosphorylation suggesting that hGle1 phosphorylation is not dependent upon SGs (unpublished work; discussed in appendix A section). Thus, phosphorylation could be one of the possible

mechanisms to regulate hGle1 function. Future efforts will be focused on understanding the role of hGle1 phosphorylation. To test if phosphorylation of hGle1A plays any role in SGs and translation, the siRNA-knockdown add-back assay could be performed with phospho-dead or phospho-mimetic h*gle1* constructs and assayed for their effect on SG assembly, disassembly and translation. Similarly, constructs expressing for phospho-dead or phospho-mimetic hGle1B proteins could be tested for the rescue of mRNA export in hGle1-depleted cells. These assays would give insights into the requirement of phosphorylation at various steps of gene expression.

Interestingly, we have identified four phosphorylated residues of hGle1 that are clustered together and lie in a low complexity region near to the coil-coil domain (unpublished work; discussed in appendix A section). This observation raises the possibility that hGle1 phosphorylation might affect its self-association. FRET experiments along with soluble binding assay with recombinant phospho-dead and phospho-mimetic hGle1 proteins could be performed to test if phosphorylation has any impact on hGle1 self-association. Recent studies have also highlighted the importance of low complexity domains to help phase-separate proteins into liquid-like droplets. Importantly, SG formation is also thought to be mediated by a phase-separation process (Hyman and Simons, 2012; Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). Therefore, it would be interesting to test whether hGle1 phase-separates *in vitro* using recombinant proteins, and moreover if phosphorylation influences this property.

Finally, it will be informative to know how hGle1 phosphorylation influences its association with Dbps and their activity. To address this, ATPase assays performed with recombinant phospho-dead or phospho-mimetic hGle1 could be done. The Roy Parker laboratory has shown that Ded1 ATPase activity is required during stress recovery for disassembly of SGs

and resumption of translation. Therefore, it would be informative to know if phosphorylated hGle1 modulates the ATPase activity of Dbps differently than the non-phosphorylated hGle1.

In summary, the proposed studies would give an indication how phosphorylation of hGle1 regulates its activity and impacts various steps of gene expression.

Exploring hGle1 connections with ALS

A recent surge of papers has linked altered nucleocytoplasmic transport as an underlying cellular mechanism in neurological diseases, especially ALS. Although 90% of ALS cases are spontaneous, 5-10% of cases are familial in nature (Kiernan et al., 2011). A GGGCC hexanucleotide repeat expansion (HRE) in *C9ORF72* gene has been associated with about half of the fALS cases (Van Blitterswijk et al., 2012). Three laboratories independently showed that nuclear export and import pathways are affected as a result of HRE expansion in *C9ORF72* gene in yeast, fly and iPSC-derived neuron model systems (Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015). Although, there is not a clear overlap in the protein players identified by these studies, they all converge on defective NPC transport as a contributing factor in neurodegenerative diseases.

Notably, mutations in *GLE1* have been linked with ALS disease (Kaneb et al., 2015). Therefore, an exciting area of research would be to understand if mutations in *GLE1* also result in altered nucleocytoplasmic transport defects similar to ALS-linked *C9ORF72* mutations. Interestingly, siRNA knockdown-add back experiments performed with the construct expressing ALS-linked hGle1-IVS14-2A>C in HeLa cells suggest that this protein variant is capable of function in mRNA export similar to wild-type hGle1B protein (Aditi et al., 2015). Further supporting this conclusion, patient derived lymphoblastoid cells also showed no obvious defects

in mRNA export (unpublished work). This is reasonable given that patients develop disease at later stages of their life. However, we do not know if export of a specific subset of mRNAs might still be affected. Intriguingly, expression array data comparing the neural tissues derived from LCCS1 post mortem fetuses and control tissues show deregulation of specific mRNA involved in synapses and neuron development (Pakkasjärvi et al., 2007). Thus, it is likely that neuronal cells have differential requirements for hGle1 and mutations in *GLE1* lead to perturbation in the fate of specific mRNAs.

Recently, several studies have shown that patient-derived induced-pluripotent stem cells can be differentiated into motor neuron cell types for use as a model system for ALS (Lee and Huang., 2015). Therefore, RNA-Seq experiments could be performed using patient-derived iPSC motor neurons to identify if export of a specific set of mRNAs is inhibited/ promoted. Similar to mRNA export, it is likely that translation of certain transcripts is inhibited or promoted. The translational landscape could also be tested by ribosome profiling of these control and patient-derived iPSC motor neurons. Taken together, these studies would suggest if mRNA export/translation of a specific subset of mRNAs is perturbed in patient cells.

A second possibility is that mutations in *GLE1* might impact transport of molecules between nucleus and cytoplasm indirectly. Intriguingly, a recent study showed that cytoplasmic aggregates observed in neurodegenerative diseases interfere with the nucleocytoplasmic transport of RNA and protein in the cells by sequestering nuclear transport factors (Woerner et al., 2016). Interestingly we also notice that over-expression of ALS-linked hGle1-IVS14-2A>C protein results in protein aggregates that differentially sequester wild-type TDP-43 and Hsp90 (Aditi et al., 2015). We did not look at the co-localization of various transport factors in the hGle1 aggregates. Thus, it is possible that mutations in *GLE1* do not affect its function in mRNA

export but may promote cytoplasmic aggregation of hGle1 protein in neurons over time, which sequester various proteins including, transport factors, and ultimately impact nucleocytoplasmic transport pathway. Therefore, it would be interesting to test if ALS-linked hGle1-IVS14-2A>C aggregates-containing HeLa cells also exhibit nculeocytoplasmic defects. Furthermore, iPSC neuronal model system could be use to test if patient cells show any cytoplasmic aggregates and if these aggregates sequester proteins including, transport factors. Also, it would be informative to know if aggregates found in other type of ALS models sequester wild type hGle1.

Finally, it would be interesting to investigate how ALS-linked hGle1-IVS14-2A>C protein behaves as bifunctional hGle1 isoform. It is shown that ALS-linked hGle1-IVS14-2A>C does not interact with hCG1 using yeast two hybrid assay (Kaneb et al., 2015). However, the expression levels of yeast two-hybrid constructs were not determined. Therefore, the lack of yeast two-hybrid interaction could be due to low expression of ALS-linked hGle1-IVS14-2A>C protein compared to wild-type hGle1B. In fact, ALS-linked hGle1-IVS14-2A>C protein is expressed at lower levels in human cells compared to hGle1B (Kaneb et al., 2015). Therefore, soluble binding assays with recombinant proteins could be done to test the interactions of ALS-linked hGle1-IVS14-2A>C with hCG1. Furthermore, determining the structure of ALS-linked hGle1-IVS14-2A>C protein and examining its impact on Dbps ATPase activity would be insightful towards understanding its function.

Global questions related to SG biology

SGs are highly dynamic structures that play important roles in RNA metabolism and cell survival. Although more than 200 proteins are identified as SG components, we still do not fully understand the rules that govern their assembly and disassembly process (Buchan, 2014; Buchan

and Parker, 2009; Kedersha et al., 2013). Below, two key unresolved questions associated with SG biology are discussed.

1) What is the fate of mRNA in SGs?

SGs are reversible complexes whose components move in and out of SGs. This observation has led to a model where SGs serve as a site to sort mRNPs for storage, decay and re-initiation for translation. However, this model has never been tested due to limitation of available technologies. In recent years, various tools have been developed to either track single mRNA species or visualize translation at a specific location. Approaches to test this model by using recently developed tools are discussed below.

The Doudna laboratory has recently described the RCas9 system where Cas9 protein can be specifically directed to bind or cut RNA targets (O'Connell et al., 2014). Using this system, nuclease-insensitive fluorescently tagged-RCas9 system could be used to track single or multiple mRNAs under stress. An important advantage of this method is that multiple endogenous mRNA species can be tracked without introducing any additional sequences to mRNAs. A recent study has identified several mRNA species that reside in SGs (Jain et al., 2016). Therefore, we could label multiple mRNA species that reside in SGs and track them live, in cells that co-express SG and P body markers, during a stress response. This way, it would be possible to get an idea if mRNAs are exchanged between SG and P bodies during a stress response. To further test if mRNAs that reside in SGs could also enter into translation, a modified version of proximity specific ribosome profiling protocol developed by the Weissman laboratory, could be used (Jan et al., 2014). Using this protocol, the Weissman laboratory biotinylated ER ribosomes in intact cells by co-expressing an ER restricted biotin ligase fusion protein (BirA) along with ribosomes containing an AviTag, which makes ribosomes substrate for BirA. The *in vivo* biotinylation of

ER-bound ribosomes followed by ribosome profiling provided information regarding the messages that were translated specifically at the ER (Jan et al., 2014). In the modified approach to test for translation targets at SGs, BirA will be redirected to translation machinery by expressing it as a fusion protein with a translation factor such as 60S subunit protein (This translation factor should not localize to SGs). Nuclease-insensitive RCas9 with an Avi tag will be expressed in cells that can be targeted to a specific transcript. A short biotin pulse will be applied during stress and biotin-streptavidin pull down protocol will be followed. If an mRNA is translated, RCas9 will be biotinylated. Thus, we could test if an mRNA enters into translation or follows a different path during stress. Although this method offers labeling of endogenous mRNAs, RCas9 system has not been tested extensively in mammalian cells and it may have off-targets effects.

2) Is SG disassembly required for translation-reinitiation?

The SGs are proposed to function in global translation repression by storing translationally-repressed mRNAs (Buchan and Parker, 2009; Kedersha and Anderson, 2009). However, there are conflicting reports that suggest that SG formation is not needed for translation repression (Buchan, 2014; Kedersha et al., 2013). Furthermore, it is unclear if SG disassembly is prerequisite for translation re-initiation after stress. There are several pharmacological reagents that interfere with SG assembly and disassembly. For example, K252a addition prevents SG disassembly, while ISRIB promotes disassembly (Sidrauski et al., 2015; Wippich et al., 2013). Using these drugs we could investigate the effects of SG assembly/disassembly on translation. Cells will be treated with either K252a, ISRIB and vehicle alone under stress and non-stress conditions. Following treatments, cells will be processed for

ribosome profiling to determine the translational landscape in control and drug-treated cells under normal and stress conditions. This analysis would give us some clues whether translation is affected if cells fail to assemble or disassemble granules. However, it is possible that stalled polysome-bound mRNAs are enriched rather than active polysomes involved in translation.

In conclusion, to investigate the involvement of SGs in diseases, it is imperative to decipher the nature and function of these granules. The proposed studies would give us some clues regarding the formation and function of these organelles and their deregulation in diseases.

APPENDIX A

Analysis of hGle1 phosphorylation in response to stress

Introduction

The human genome consists of approximately 20,000 protein-coding genes. However, the proteome of humans is very complex with additional diversity and complexity generated by post-translational modifications (PTMs), in addition to changes during the transcription, splicing and translation levels (Di Giammartino et al., 2011; Nilsen and Graveley, 2010; Shabalina et al., 2014; Walsh et al., 2005). PTMs usually refer to the covalent addition of a functional group to proteins or the proteolytic processing of protein subunits. A variety of PTMs occur in cells, including phosphorylation, methylation, acetylation, ubiquitylation, and glycosylation. PTMs often influence the function of a protein by modulating its activity, localization or interaction with other binding partners, and thus are capable of regulating various aspects of biological processes (Beltrao et al., 2013; Lothrop et al., 2013; Prabakaran et al., 2012; Seo and Lee, 2004).

Under stress conditions, PTMs also provide a rapid and reversible response, as no new protein synthesis is required. Interestingly, PTMs of some SG components regulate the interaction with other proteins and affect SG functions (De Leeuw et al., 2007; Kwon et al., 2007; Stoecklin et al., 2004; Tourrière et al., 2003). For example, phosphorylation of growth factor receptor-bound protein 7 (Grb7) by focal adhesion kinases influences SG assembly and disassembly. Under stress conditions, hypo-phosphorylated Grb7 is recruited to SGs through its interaction with the RNA-binding protein HuR. Grb7 stabilizes TIA-1 aggregation and promotes SG assembly. During recovery phase, Grb7 is phosphorylated by FAK1 kinases, resulting in reduced interaction with HuR and SG components, thus promoting SG assembly (Tsai et al.,

2008). Similarly, several SG components including ribosomal proteins are O-linked N-acetylglucosamine (O-GlcNac) modified and depletion of O-GlcNac transferases inhibits SG assembly (Ohn et al., 2008). Additionally, PTMs can determine the partitioning of a protein into an aggregated or a soluble state and thereby influence its localization in granules. For instance, phosphorylation of FUS protein by DNA-PK enzyme impedes its recruitment to *in vitro* hydrogels compared to hypo-phosphorylated FUS (Han et al., 2012) . Thus, PTMs of proteins have a major influence on RNA granule dynamics.

Interestingly, our preliminary results show that hGle1 is post-translationally modified under stress conditions. Therefore, we sought to determine if posttranslational modification of hGle1 regulates its activity during the stress response. Below a summary of these findings is presented.

Results and discussion

hGle1 is phosphorylated during stress

Under stress conditions, we discovered that hGle1 protein exhibited a reduced electrophoretic mobility compared with untreated control samples on SDS-PAGE. This was detected by immunoblotting with antibody raised against the amino-terminal region of hGle1. To test if this slower migration is due to phosphorylation, cell lysates from control, heat shocked and sodium arsenite-treated samples were incubated with lambda phosphatase in the presence or absence of phosphatase inhibitors. Lambda phosphatase treatment resulted in the collapse of the higher migrating band of hGle1 in samples prepared from stressed cells, while no change was observed in the presence of phosphatase inhibitors **(**Figure A.1A**)**. This data suggested that the altered

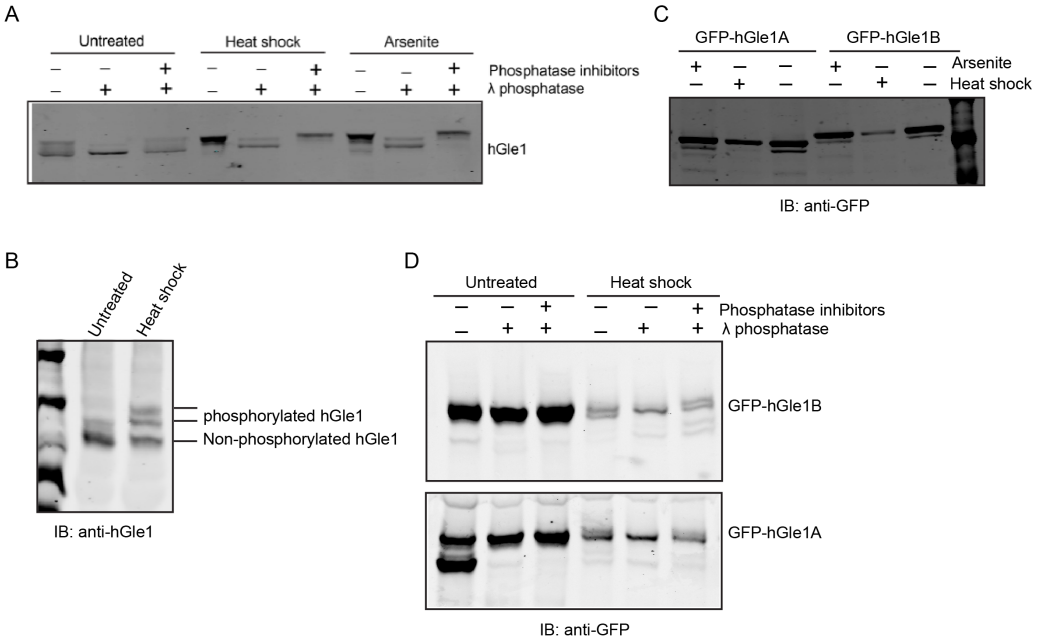


Figure A.1: hGle1 is phosphorylated in response to stress.

(A) HeLa cells were either left untreated, exposed to heat shock at 45°C or treated with sodium aresenite for 60 min. After treatments, cell lysates were prepared and lysates were either incubated with phosphatase buffer alone, lambda phosphatase (500UI) or lambda phosphatase (500UI) and phosphatase inhibitors together for 30 min. Samples were resolved on SDS-PAGE and immunoblotted with anti-hGle1 antibodies. (B) Untreated or heat shocked cell lysates were resolved on a phos-tag gel and immunoblotted with anti-Gle1 antibody**.** (C and D) HeLa cells exressing *GFP-hGLE1A* or *GFP-hGLE1B* plasmids were either left untreated at 37°C, heat shocked at 45°C or treated with 0.5mM sodium arsenite for 60 min at 37°C. Following treatments, cell lysates were resolved by (C) SDS-PAGE, or (D) phos-tag SDS-PAGE, and immunoblotted with anti-GFP antibodies.

mobility of hGle1 protein occurred as a result of hGle1 phosphorylation under stress. To examine hGle1 phosphorylation by an alternative method, we utilized the phos-tag gel method. Phos-tag is a functional molecule that binds specifically to phosphorylated ions. Phosphorylated proteins migrate more slowly within phos-tag SDS-PAGE due to their interaction with the phos-tag molecule. This results in a separation of phosphorylated proteins from non-phosphorylated proteins (Kinoshita et al., 2006). We observed three distinct bands for hGle1 on phos-tag SDS PAGE in heat shocked samples **(**Figure A.1B**)**. The lowermost band corresponded to non-phosphorylated hGle1 (Figure A.1B; lane 2), while middle and upper bands represented phosphorylated hGle1 (Figure A.1B; lane 2). This data suggested that hGle1 is phosphorylated to varying degrees upon stress. Importantly, we also observed a basal level of phosphorylation of hGle1 in lysates prepared from control cells (Figure A.1B; lane 1). However, the proportion of phosphorylated hGle1 was much higher in heat shock-treated samples compared to controls. Thus, hGle1 is basally phosphorylated under normal conditions and this phosphorylation is enhanced during stress.

We next sought to determine if both hGle1A and hGle1B isoforms are phosphorylated under stress conditions. We did not observe any electrophoretic mobility shift for exogenously expressed GFP-hGle1A and GFP-hGle1B proteins by a SDS-PAGE gel and immunoblotting of lysates (Figure A.1C). This was not surprising since the addition of a GFP tag changes the molecular weight of hGle1 protein by ~27 kD, potentially masking the addition of phosphorylated group's mass. Therefore, we turned to the phos tag gel system to examine the phosphorylated status of hGle1 isoforms. HeLa cells expressing GFP-hGle1A or GFP-hGle1B proteins were either left untreated or heat shocked at 45^{o}C for 60 min and cell lysates were prepared in RIPA buffer. The proteins were resolved on phos-tag SDS-PAGE. Both GFP-

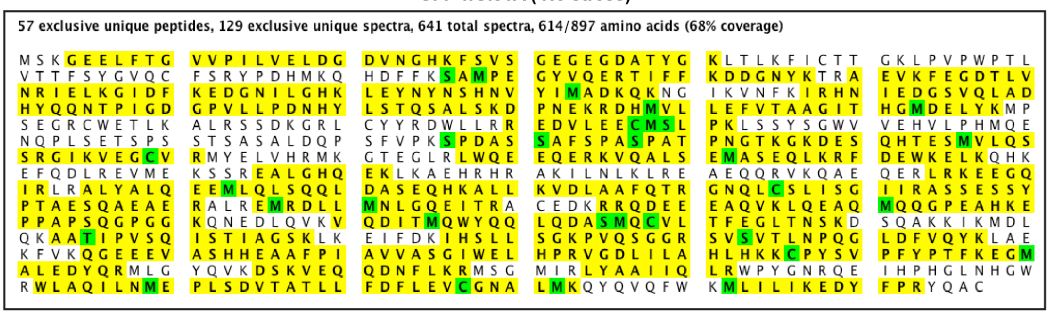
hGle1A and GFP-hGle1B showed two distinct bands in phos-tag SDS-PAGE for the heat shocked sample (Figure A.1D; lane 4). To test if the higher migrating band represented phosphorylated hGle1A or hGle1B, cell lysates were treated with lambda phosphatase. The higher migrating band collapsed into a single band upon lambda phosphatase treatment in both GFP-hGle1A and GFP-hGle1B samples under stress conditions, while there was no change in the migration pattern in the presence of lambda phosphatase and phosphatase inhibitors (Figure A.1D; lane 5 and lane 6). Thus, both GFP-hGle1A and GFP-hGle1B isoforms are phosphorylated. Taken together, these results demonstrated that hGle1 is phosphorylated.

Identification of phosphorylated residues by mass spectrometry

In order to identify phosphorylated amino-acid residues of hGle1, we took a proteomic approach. Hela cells transfected with *GFP-hGLE1A*^{R} plasmid were either left untreated or treated with 0.5mM sodium arsenite for 60 min. Following treatment, cell lysates were prepared in RIPA buffer and GFP-hGle1A was immunoprecipitated and proteins were separated on a SDS-PAGE gel. We treated cells with sodium arsenite instead of heat shock because GFP-hGle1A and GFP-hGle1B protein levels were reduced upon heat shock (Figure A.1C and A.1D). There were also technical difficulties in immunoprecipitating enough material from heat-shocked cells to identify phosphorylation sites by mass spectrometry. A band corresponding to GFP-hGle1A was excised from the gel, trypsin digested and samples were processed for mass spectrometry. The peptide spectra data was aligned with a theoretical peptide database, identifying a total of thirteen putative phosphorylation sites under stress conditions compared to six phosphorylation sites under normal conditions (Figure A.2A, A.2B and A.2D). Interestingly,

the six phosphorylation sites identified in GFP-hGle1A under normal conditions were also identified during stress

A



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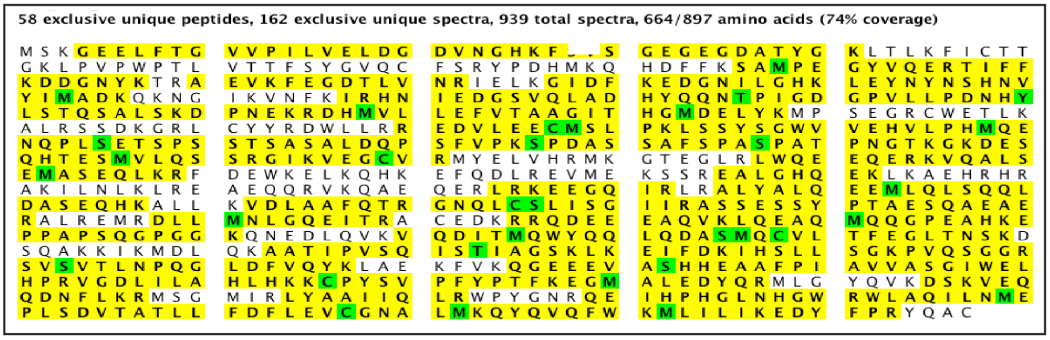

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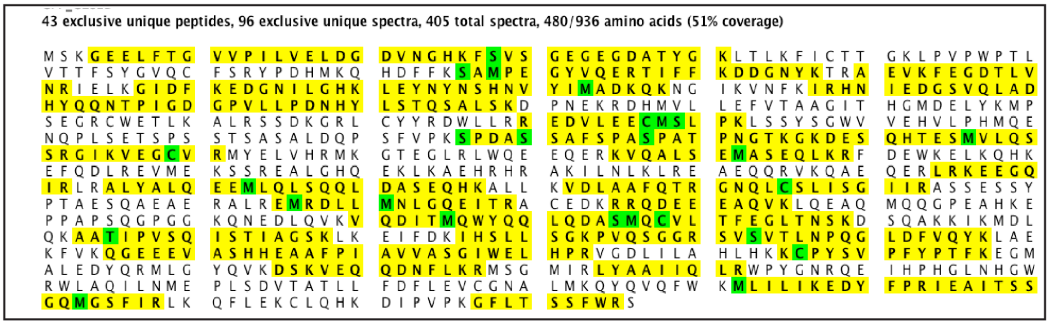

D A summary of phosphorylated amino acids identified by mass spectrometry

GFP-hGle1A (no stress)	GFP-hGle1A (Arsenite)	GFP-hGle1B (Arsenite)
S41	S67	S41
S88	S88	S88
S93	S92	S92
S99	S93	S99
T427	S99	S397
S465	S288	T427
	Y390	S465
	S397	
	T427	
	S434	
	S465	
	S494	
	T620	

Figure A.2: hGle1 is phosphorylated at multiple sites.

(A-C) Identification of phosphorylated residues by mass spectrometry. Sequence coverage map of (A) GFP-hGle1A protein from untreated cells (B) GFP-hGle1A and (C) GFP-hGle1B proteins from arsenite-treated cells. The yellow region indicates the peptide sequence identified by mass spectrometry and green color highlights the amino acid residues that are posttranslationally modified. (D) A list of phosphorylated amino acids that are identified by mass spectrometry.

conditions (Figure A.2A, A.2B and A.2D). We also tested if similar amino -acid residues were phosphorylated in hGle1B as observed in hGle1A. Only seven phosphorylated sites were identified in GFP-hGle1B under stress conditions (Figure A.2C and A.2D). The sequence coverage for GFP-hGle1B (51%) was lower compared to GFP-hGle1A sample (74%) (Figure A.2C and A.2D). Thus, this could be one of the potential reasons for lower number of phosphorylated sites identified in GFP-hGle1B compared to GFP-hGle1A. Importantly, all the putative phosphorylation sites identified in GFP-hGle1B were also present in GFP-hGle1A isoform (Figure A.2D). Thus, both isoforms are phosphorylated at the same sites. However, it is very likely that we might have missed phosphorylated amino acids in the unique sites of GFP-hGle1B due to lower sequence coverage. Additionally, it is possible that these isoforms might have specific post-translation modifications other than phosphorylation. Collectively, mass spectrometry data confirmed the results obtained with small-scale immunoblotting analysis, demonstrating that hGle1 is phosphorylated under basal and stress conditions.

Phosphorylated hGle1 is enriched in the cytosol during stress conditions

Endogenous hGle1 is localized at the cytoplasm, nuclear rim and in the nucleus at steady-state (Aditi et al., 2015; Kendirgi et al., 2003). Since phosphorylation can alter the localization of proteins, we investigated if phosphorylated hGle1 is enriched in one cellular compartment compared to others. To test this, nuclear and cytosolic fractions were prepared from untreated and heat-shocked cells, and hGle1 phosphorylation was analyzed by immunoblotting. Lamin B1 and GAPDH were used as markers for nuclear and cytosolic fractions, respectively. The isolations were relatively pure as determined by the lack of GAPDH in the nuclear fractions and vice-versa (Figure A.3). Surprisingly, we observed that the cytosolic fraction but not the nuclear

fraction contained phosphorylated hGle1 as determined by the higher migrating band on immunoblot (Figure A.3). The nuclear fractions, prepared under normal or stress conditions, contained hGle1 but did not show any mobility shift (Figure A.3). This data suggested that phosphorylated hGle1 is enriched at the cytoplasm. hGle1 dynamically shuttles in and out of nucleus and it is possible that phosphorylation might alter these shuttling dynamics and restrict localization to the cytoplasm.

SG formation is not required for hGle1 phosphorylation

Since the cytosolic pool of hGle1 is phosphorylated, and hGle1 is recruited to cytoplasmic SGs, we next tested if hGle1 phosphorylation is dependent upon SG formation. SG assembly is inhibited by the addition of cycloheximide, a translation inhibitor, which prevents disassembly of polysomes (Kedersha et al., 2000, 2005; Mollet et al., 2008). Therefore, we examined the status of hGle1 phosphorylation during stress in the presence of cycloheximide. In agreement with published results (Kedersha et al., 2000, 2005; Mollet et al., 2008), we noticed that cells incubated with cycloheximide failed to assemble SGs in response to heat shock, as assessed by immunofluorescence staining with SG marker G3BP (Figure A.4A). However, immunoblotting analysis of cell lysates prepared from these treatments still showed reduced mobility of hGle1 protein (Figure A.4B). Thus, hGle1 phosphorylation is not dependent upon SG assembly. Interestingly, we also noticed that hGle1 was phosphorylated when cell lysates were prepared under normal conditions in the presence of cycloheximide (Figure A.4B). Since cycloheximide is a translation inhibitor and its addition resulted in hGle1 phosphorylation,

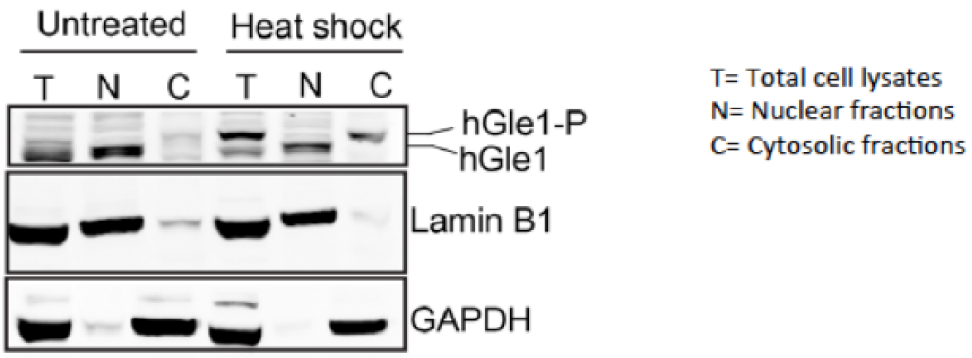

Figure A.3: Cytosolic pool of hGle1 is phosphorylated during stress.

Hela cells were either left untreated at 37°C or heat shocked at 45°C for 60 min. Cell lysates were fractionated into nuclear and cytosolic fractions. These fractions were separated by SDS-PAGE and immunoblotted with anti-hGle1, anti-Lamin b1 and anti- GAPDH antibodies.

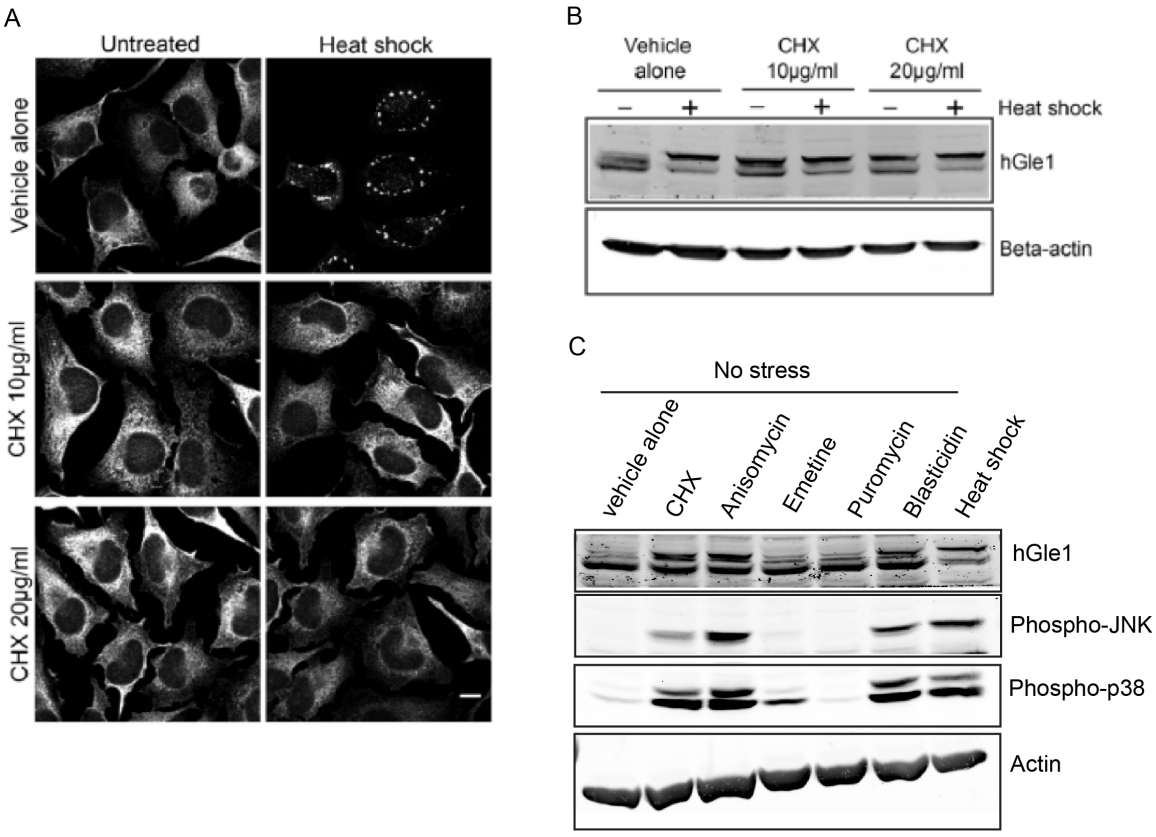

Figure A.4: hGle1 phosphorylation is not dependent on SG fromation.

Cells were either left untreated or heat shocked at 45°C for 60 min in the presence or absence of cycloheximide (CHX). SG formation was assessed by immunofluorescence using anti-G3BP antibodies. (B) Cell lysates were prepared and immunoblotted with anti hGle1 and anti-actin antibodies. (C) Hela cells were treated with various translation inhibitors as indicated and lysates were resolved by SDS-PAGE and immunoblotted with anti-hGle1, anti-phospho-p38, anti-phospho-JNK and anti-actin antibodies.

we next determined if translation inhibition is a signal for hGle1 phosphorylation.We used five different translation inhibitors – cycloheximide, puromycin, emetine, anisomycin and blasticidin that target various steps of translation. Intriguingly, hGle1 was phosphorylated when cells were treated with cycloheximide, anisomycin and blasticidin, but not with emetine and puromycin (Figure A.4C). Thus, these results argue that translation inhibition alone is not sufficient for hGle1 phosphorylation. However, it is possible that blocking translation at a specific step might be a signal for hGle1 phosphorylation. Alternatively, these three inhibitors might activate a specific cell-signaling pathway that may result in hGle1 phosphorylation.

hGle1 is phosphorylated by MAPK kinases

Cycloheximide and anisomycin have been shown to activate MAPK kinases (Zinck et al., 1995). In fact, we observed that the MAPK kinases p38 and JNK were activated when cells were treated with cycloheximide, anisomycin or blasticidin (Figure A.4C). Activation of p38 and JNK kinases is determined by immunoblotting analysis using phospho-p38 and phospho-JNK antibodies (Roux and Blenis, 2004; Xing, 2000). Since MAPK kinases are activated under stress conditions and play critical roles in a stress response, we next investigated if MAPK kinases were required for hGle1 phosphorylation. In order to test the involvement of p38, JNK and ERK kinases, a series of inhibitors were employed that target each of these MAPKs. Inhibitors targeting each individual MAPK did not affect hGle1 phosphorylation (Figure A.5A). However, inhibition of JNK and ERK together resulted in the collapse of the higher migrating hGle1 band under stress conditions (Figure A.5A). Inhibition of either JNK and p38 or p38 and ERK together did not influence hGle1 phosphorylation (Figure A.5A). These results suggested that ERK and JNK kinase might be responsible for hGle1 phosphorylation.

To further test the involvement of ERK and JNK kinases, we performed an *in vitro* phosphorylation assay with purified JNK and ERK kinases. Bacterially expressed recombinant MBP-hGle1A or MBP alone were incubated with purified active ERK and/or JNK kinases in the presence of radioactive (gamma-^{32}P)-ATP and radioactivity incorporation was measured by autoradiography. JNK kinases phosphorylated both MBP protein and MBP-hGle1to a similar degree, and thus, this analysis was inconclusive. However, incubation of MBP-hGle1A with ERK kinase resulted in a significant (gamma-^{32}P)-ATP incorporation compared to MBP alone sample (Figure A.5B). Thus, this data suggested that ERK kinase is responsible for hGle1A phosphorylation. (Figure A.5B).

MAPK kinases are categorized as proline (P)-directed kinases that usually phosphorylate proteins on a serine (S) residue followed by a proline (SP) or a threonine (T) residue followed by a proline (TP) (Roux and Blenis, 2004). hGle1 has three SP and one TP sites. Moreover, mass spectrometry results also identified two SP sites (serine 88 and serine 99) that are phosphorylated (Figure A.2) . Therefore, we mutated the codon for serine 88, serine 92, serine 96, serine 99 and threonine 102 to alanine (A) and tested whether these changes influenced hGle1 migration. Interestingly, expression of a plasmid encoding for GFP-hGle1A (S88A) resulted in loss of gel shift on a phos-tag gel. However, expression of GFP-hGle1A(S92A), GFP-hGle1A(S96A), GFP-hGle1A(S99A) and GFP-hGle1A(T102A) did not affect the gel shift (Figure A.6). These results suggested that the serine 88 residue contributes towards altered mobility of hGle1 on a phos tag gel. Currently, I am testing if recombinant, bacterially expressed MBP-hGle1A (S88A) or MBP-hGle1A (S88A/S92A/S96A/S99AT102A) are phosphorylated *in vitro* by ERK and/or JNK kinases.

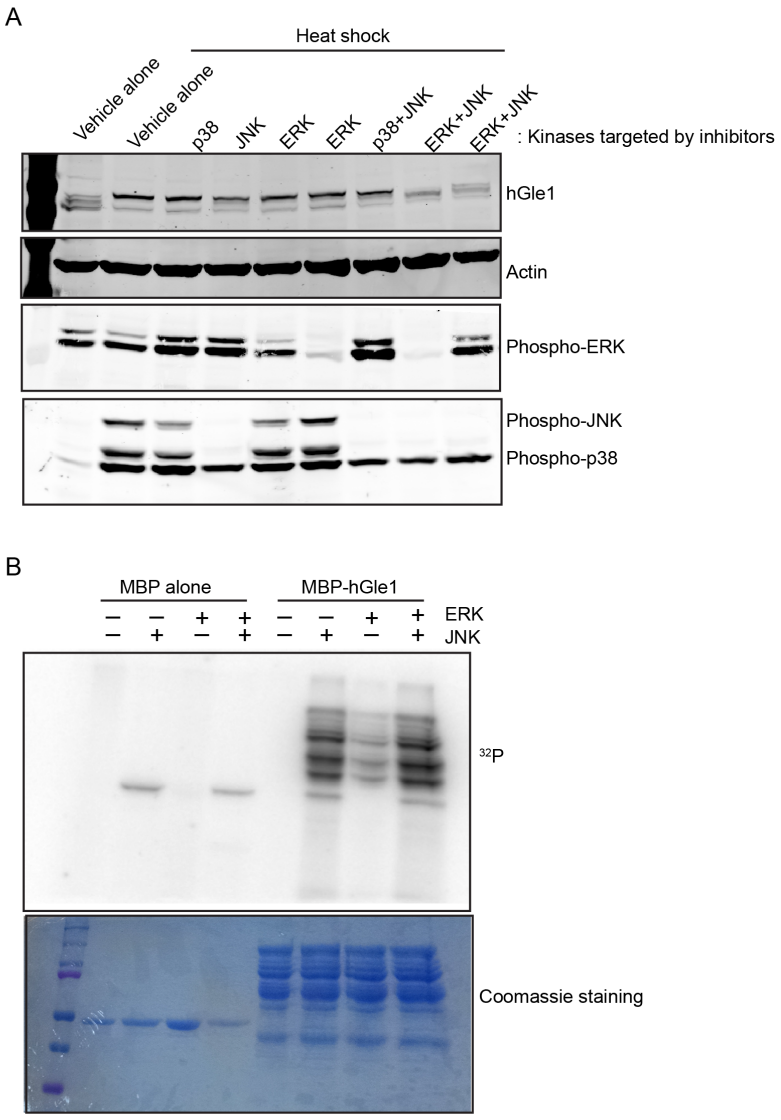

Figure A.5: MAPK kinases phosphorylate hGle1

(A) Cells were either left untreated or heat shocked at 45°C for 60 min in the presence of indicated kinase inhibitors. Cell lysates were prepared and immunoblotting was performed with anti-hGle1, anti-phospho-p38, anti-phospho-JNK, anti-phospho ERK and anti-actin antibodies. (B) MBP or MBP-hGle1A proteins expressed in bacteria were purified and incubated with recombinant active ERK and JNK kinases in the presence of radioactive (gamma-^{32}P)-ATP. Reactions were terminated by addition of 6X SDS dye and resolved on a SDS-PAGE gel. Radioactivity incorporation was measured by autoradiography. The gel was also stained with coomassie stain to confirm equal loading of proteins.

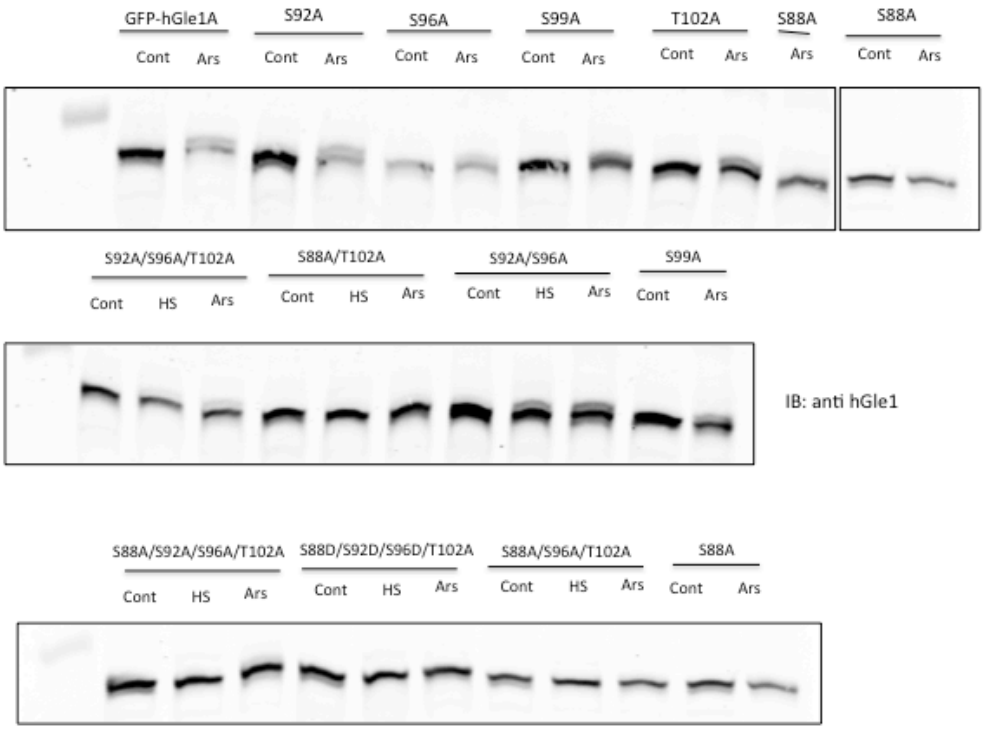

Figure A.6: Serine 88 residue is contributing towards altered mobility of hGle1 on phos-tag SDS-PAGE

Hela cells transfected with plasmids expressing the indicated EGFP-hGle1A phospho-dead proteins were either left untreated at 37°C (Cont), heat shocked at 45°C (HS) or treated with sodium arsenite (Ars) for 60 min at 37°C. Cell lysates were resolved by phos-tag SDS-PAGE and immunoblotted with anti-Gle1 antibodies.

In summary, this work has demonstrated that hGle1 is phosphorylated potentially at several sites. Moreover, we showed that hGle1 phosphorylation is not dependent upon SG formation. However, it is unclear if hGle1 phosphorylation is required for SG formation and how phosphorylation coordinates with inhibition of translation in response to stress. We also do not understand the contribution of each phosphorylation site versus phosphorylation at multiple sites and how it regulates hGle1 biological function. Future studies should be focused on investigating how phosphorylation modulates hGle1 activity to alter gene expression during stress. Investigating this aspect of hGle1 function will provide valuable insight into the mechanisms by which PTMs modulate mRNP dynamics and influence the flow of information in response to stress.

APPENDIX B

Identification of a novel isoform of *GLE1* in HeLa cells

Introduction

Eukaryotic cells rarely follow a one gene-one polypeptide concept. The majority of human genes encode more than one isoform of a protein by utilizing alternative transcription start sites, alternative splicing or alternative polyadenylation. This diversity is key in accounting for the evolution of proteome complexity in higher eukaryotes despite having relatively fewer protein-coding genes (Di Giammartino et al., 2011; Nilsen and Graveley, 2010; Shabalina et al., 2014). The *GLE1* gene is also alternatively spliced to generate at least two known isoforms-hGle1A and hGle1B (Kendirgi et al., 2003; Watkins et al., 1998). Interestingly, cDNA sequences available from public databases indicate that additional isoforms exist for hGle1. One such database (AceView) predicts nine isoforms for hGle1. The AceView database is a curated, comprehensive annotation of non-redundant cDNA-supported gene and transcripts. It aligns experimental cDNA sequences available from the public databases (GenBank, RefSeq and dbEST) onto the genome sequence and then clusters good cDNA sequences into mRNA models (Thierry-Mieg and Thierry-Mieg, 2006). According to this database, transcription of the *GLE1* gene produces eleven mRNA products (nine alternatively spliced and two unspliced forms). The nine spliced mRNAs are predicted to code for six complete and three partial proteins (Figure B.1). Interestingly, we noticed that one mRNA variant labeled as 'aAug10' is predicted to encode a 701 amino acid long protein that is very similar in sequence to the hGle1B isoform (Figure B.1 and B.2). This mRNA variant sequence is constructed from thirty-five cDNA clones isolated from various tissues and the difference in amino acid sequence is predicted to be

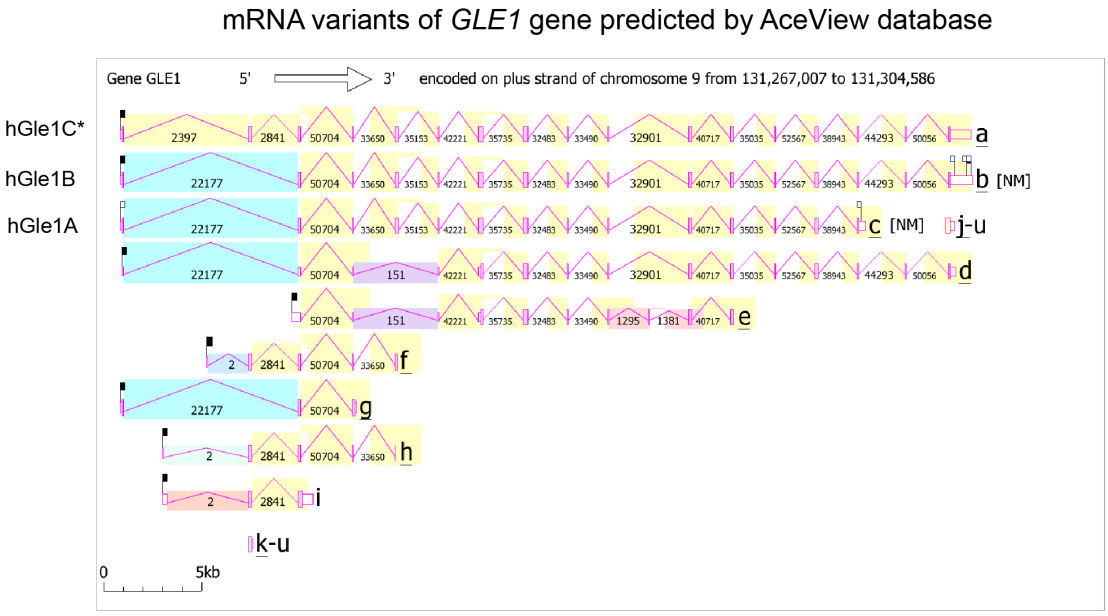

Figure B.1: Prediction of mRNA variants of *GLE1* gene by AceView database

The AceView database predicts eleven mRNA variants of *GLE1* gene. Nine alternatively spliced mRNAs are shown here and two mRNA variants encode for the known hGle1A and hGle1B isoforms. These mRNA variants shown are aligned from 5' to 3' on a virtual genome. Exon size is proportional to its length and intron size has been shrunken to a minimum length. 5' cap and 3' polyA tail flags show completeness of the transcripts.

generated by alternative splicing. The resulting protein product contains a unique 38 amino acid domain at its amino-terminus, while the rest of protein sequence is very similar to hGle1B (Figure B.1 and B.2B). Since hGle1A and hGle1B isoforms perform distinct non-overlapping functions despite sharing more than 90% sequence homology, we sought to investigate if this predicted hGle1 isoform also exists in cells and whether it performs a distinct function in the cell. These findings are discussed below.

Results and discussion

To determine if the predicted hGle1 isoform exists, we investigated if full-length mRNA sequence corresponding to the predicted protein is present in cells. We purified RNA from HeLa cells and prepared total cDNA by reverse transcription using oligo $(dT)_{16}$ primers. Next, we used gene specific primers to amplify the coding sequence of this putative mRNA from the cDNA. Using a forward primer specific to the new isoform and a reverse primer that is common to the new variant and *GLE1B* isoform, we detected a ~ 2.1 kb product on the gel (Figure B.3B). This matched well with predicted size of the putative mRNA variant. The amplified PCR product was gel purified and cloned into a MultiSite Gateway vector with a CMV/SP6 promoter and an EGFP tag at the amino- terminus (pCMV/SP6-*EGFP*-h*GLE1C*). This construct can be used to express hGle1 protein in zebrafish and mammalian cells. Importantly, plasmid DNA sequencing results confirmed that the cloned cDNA sequence matched exactly to the predicted mRNA variant coding sequence (Figure B.2A). Expression of pCMV/SP6-*EGFP-hGLE1C* in HeLa cells was further confirmed by immunoblotting using anti-hGle1 antibody. We observed a ~ 106 kD protein product on the SDS gel that matched with the predicted size of the protein (Figure B.3B).

A

mRNA sequence alignment of hGle1C with hGle1A and hGle1B isoforms

```
hGLE1C      ATGCCCATAATCCCAACACTTTGCGAGGCTGAGGCAGGACGAGTGCTTGAGGCCAGTTCA
hGLE1A      ATG--------CCGTCTGAGGGTCGCTGCTGGGAGACCTTGAAGGC-CCTACGCAGTTCC
hGLE1B      ATG--------CCGTCTGAGGGTCGCTGCTGGGAGACCTTGAAGGC-CCTACGCAGTTCC
            ***        **          **  **** *  *    **  **       ******

hGLE1C      AGCTGGGAGCCAGGCTTGGTGTCTCACACCTGCAATCCCAGCACTTTGGATGTTTTAGAA
hGLE1A      GACAAAGGTCGCCTTTGCTACTACCGCGACTGGCTGCTGCGGCGCGAGGATGTTTTAGAA
hGLE1B      GACAAAGGTCGCCTTTGCTACTACCGCGACTGGCTGCTGCGGCGCGAGGATGTTTTAGAA
              *   *  *     *     *  * *  ***    *   *      ************

hGLE1C      GAATGTATGTCTCTTCCCAAGCTATCTTCTTATTCTGGATGGGTGGTAGAGCACGTCCTA
hGLE1A      GAATGTATGTCTCTTCCCAAGCTATCTTCTTATTCTGGATGGGTGGTAGAGCACGTCCTA
hGLE1B      GAATGTATGTCTCTTCCCAAGCTATCTTCTTATTCTGGATGGGTGGTAGAGCACGTCCTA
            ************************************************************

hGLE1C      CCCCATATGCAGGAGAACCAACCTCTGTCTGAGACTTCGCCATCCTCTACGTCAGCTTCA
hGLE1A      CCCCATATGCAGGAGAACCAACCTCTGTCTGAGACTTCGCCATCCTCTACGTCAGCTTCA
hGLE1B      CCCCATATGCAGGAGAACCAACCTCTGTCTGAGACTTCGCCATCCTCTACGTCAGCTTCA
            ************************************************************
```

B

Protein sequence alignment of hGle1C with hGle1A and hGle1B isoforms

```
hGle1C      MPIIPTLCEAEAGRVLEASSSWEPGLVSHTC-----NPSTLDVLEECMSLPKLSSYSGWV
hGle1A      MP--------SEGRCWETLKALRSSDKGRLCYYRDWLLRREDVLEECMSLPKLSSYSGWV
hGle1B      MP--------SEGRCWETLKALRSSDKGRLCYYRDWLLRREDVLEECMSLPKLSSYSGWV
            **        . **  *: .: . .  .: *          ******************

hGle1C      VEHVLPHMQENQPLSETSPSSTSASALDQPSFVPKSPDASSAFSPASPATPNGTKGKDES
hGle1A      VEHVLPHMQENQPLSETSPSSTSASALDQPSFVPKSPDASSAFSPASPATPNGTKGKDES
hGle1B      VEHVLPHMQENQPLSETSPSSTSASALDQPSFVPKSPDASSAFSPASPATPNGTKGKDES
            ************************************************************

hGle1C      QHTESMVLQSSRGIKVEGCVRMYELVHRMKGTEGLRLWQEEQERKVQALSEMASEQLKRF
hGle1A      QHTESMVLQSSRGIKVEGCVRMYELVHRMKGTEGLRLWQEEQERKVQALSEMASEQLKRF
hGle1B      QHTESMVLQSSRGIKVEGCVRMYELVHRMKGTEGLRLWQEEQERKVQALSEMASEQLKRF
            ************************************************************

hGle1C      DEWKELKQHKEFQDLREVMEKSSREALGHQEKLKAEHRHRAKILNLKLREAEQQRVKQAE
hGle1A      DEWKELKQHKEFQDLREVMEKSSREALGHQEKLKAEHRHRAKILNLKLREAEQQRVKQAE
hGle1B      DEWKELKQHKEFQDLREVMEKSSREALGHQEKLKAEHRHRAKILNLKLREAEQQRVKQAE
            ************************************************************
```

Figure B.2: Sequence comparison of amino-terminal region of hGle1A, hGle1B and hGle1C isoforms.

(A) mRNA and (B) protein sequences alignments of hGle1C with hGle1A and hGle1B isoforms. * identical residues, : or., conserved residues.

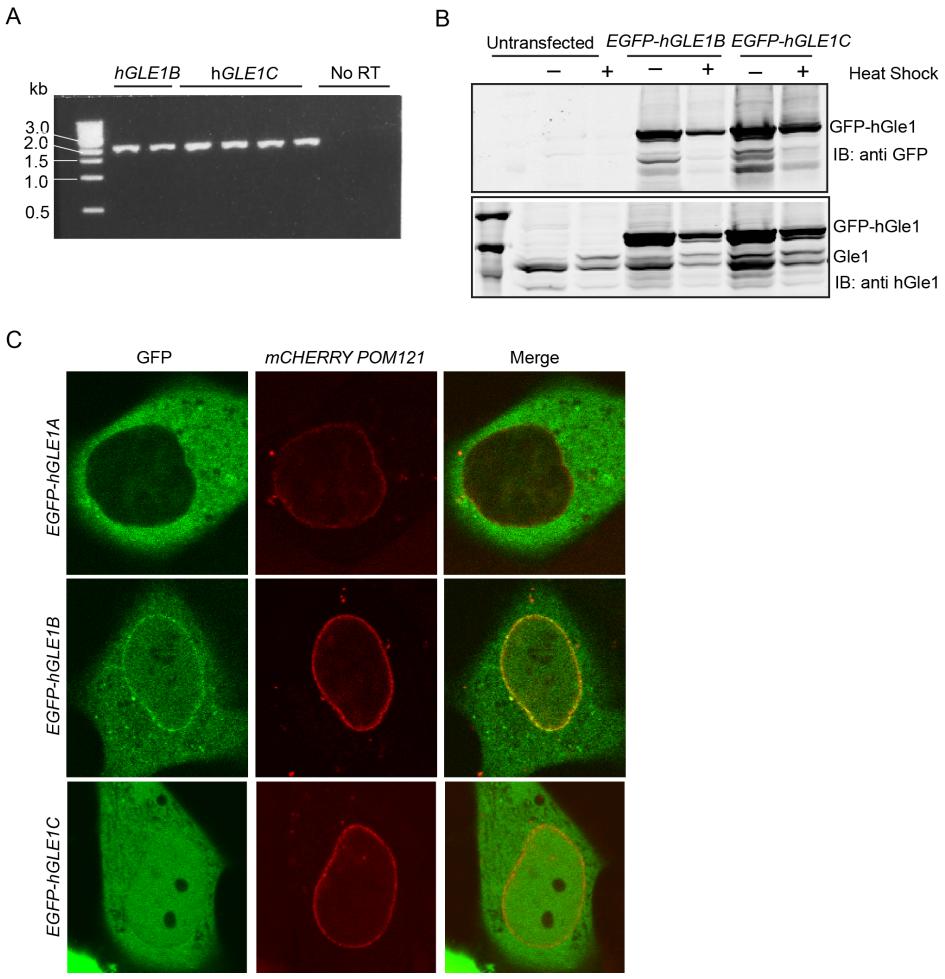

Figure B.3: *GLE1* gene encodes hGle1C isoform.

(A) RT-PCR analysis of h*GLE1C* mRNA in HeLa cells. h*GLE1C* and h*GLE1B* mRNAs were amplified using forward primers specific to h*GLE1C* and h*GLE1B a*nd a reverse primer that is common to both variants, respectively. Amplified products were separated on an agarose gel and visualized by ethidium bromide staining. (B) *EGFP-hGLE1B* and *EGFP-hGLE1C* plasmids were expressed in Hela cells and cells were either left untreated or sheat shocked at 45°C for 45 min The cell lysates were made in RIPA buffer and protein products were detected by immunoblotting using anti-hGle1 and anti-GFP antibodies. (C) Localization of hGle1 isoforms in HeLa cells. *EGFP-hGLE1A, EGFP-hGLE1B* or *EGFP-hGLE1C* and *mCHERRY POM 121* plasmids were co-expressed in cells. After 24h post-transfection, cells were imaged live using confocal microscopy. mCherry-Pom121 was used as a marker for the NPC.

These results confirmed that an additional mRNA isoform of hGle1 is expressed in HeLa cells. We designated this new isoform as hGle1C protein.

We next investigated the localization of this protein in HeLa cells. Plasmids expressing either *EGFP-hGLE1A*, *EGFP-hGLE1B,* or *EGFP-hGLE1C* and *mCherry POM121* were expressed in HeLa cells and visualized by live microscopy. Pom121 was used as a marker for the nuclear rim. Interestingly, EGFP-hGle1C did not localize to nuclear rim, but appeared to localize in the nucleus and at the cytoplasm at the steady state levels. Importantly, we observed a noticeable difference in the hGle1C localization pattern compared to hGle1A (present in the cytoplasm) and hGle1B isoforms (present at the nuclear envelope, cytoplasm and nucleus) (Figure B.3C)(Aditi et al., 2015; Kendirgi et al., 2003). Since the sequence of the first 38 amino acids of hGle1C protein is different compared to hGle1A and hGle1B isoforms, this dissimilarity could account for its distinct localization (Figure B.2B). Notably, the amino-terminal 29 amino acids of hGle1A and hGle1B proteins interact with Nup155 protein (Rayala et al., 2004), and this hGle1B domain is required for NPC localization. Therefore, we anticipate that hGle1C might not be capable of binding to Nup155. We also tested the localization of this isoform in SGs and observed that it is recruited to SGs similar to other hGle1 isoforms (Figure B.4).

Taken together, these results indicate that an additional isoform of hGle1 is expressed in human cells. This suggests that, by having multiple isoforms, hGle1 could regulate various steps of gene expression in a spatial and temporal manner. Moreover, these isoforms may have tissue - specific functions. Future experiments could be focused on understanding the biological roles of various hGle1 isoforms in a cell and tissue-specific manner and how they influence gene expression.

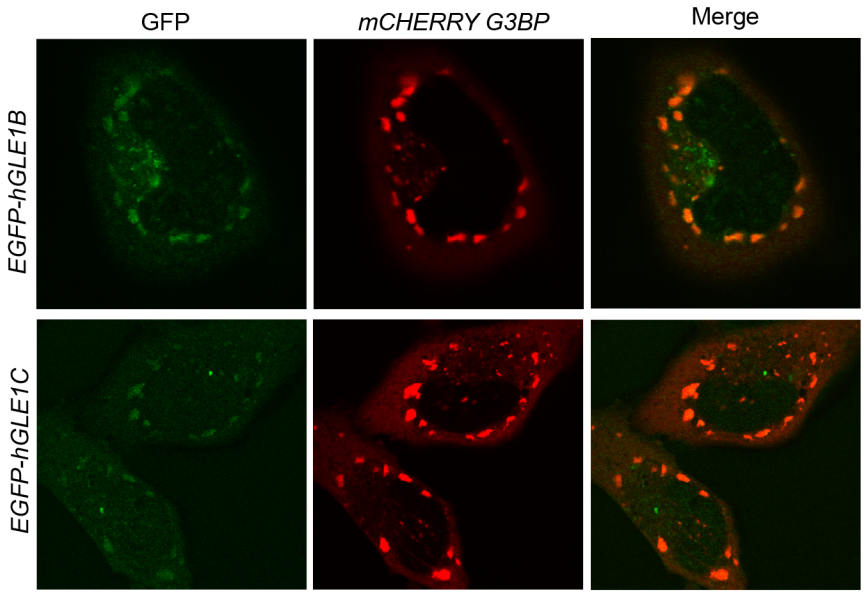

Figure B.4: hGle1C is localized to stress granules upon heat shock.

Plasmids expressing either GFP-hGle1B or GFP-hGle1C and mCherry-G3BP proteins were co-expressed in HeLa cells. Cells were heat shocked at 45°C for 45 min and imaged live by confocal microscopy.

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