Published in final edited form as:

Adv Biol Regul. 2016 September ; 62: 25–36. doi:10.1016/j.jbior.2015.11.001.

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

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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 (IP₆) binding for function. Interestingly, one *hGLE1* mutation (c.1965-2A>C) results in a novel 88 amino acid C-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. 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.

Keywords

stress granules; nuclear pore complex; RNA-binding protein; amyotrophic lateral sclerosis

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,

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RNA binding proteins (RBPs) 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 (RBPs) is a primary mediator of an mRNA's fate (Mitchell and Parker, 2014). For example, in *Saccharomyces cerevisiae*, association of the RBP 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 (Lund and Guthrie, 2005; Tran et al., 2007; Brockmann et al., 2012). 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 dysregulation 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; Ramaswami et al., 2013; Philips et al., 2015; Fox and Tibbets, 2015; Nussbacher et al., 2015).

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; Kwiatowski et al., 2009; Vance et al., 2009), hnRNP1 and hnRNP2 (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 (Alcazar-Roman et al., 2008; Murphy and Wente, 1996; Folkmann et al., 2013), translation (Bolger et al., 2008; Alcazar-Roman et al., 2010; Bolger and Wente, 2011) and stress granule dynamics (Aditi et al., 2015). By inducing the release of select RBPs, 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 (Kendirgi et al., 2003), Kendirgi et al., 2005; Rayala et al., 2004; Folkmann et al., 2013). 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 (IP₆), 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 C-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 dysregulated fashion. Considering that a defining hallmark of ALS motor neuron pathology is cytoplasmic inclusions of aggregated RBPs (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., 2011) 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 IP₆, also relocalizes to SGs upon stress (Brehm et al., 2007). We speculated that the C-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°C for 45 minutes or left untreated at 37°C, and imaged by live cell microscopy. At 37°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 1). The cytoplasmic distribution of GFP-hGle1-IVS14-2A>C with exclusion from the nucleus was indistinguishable from previously reported hGle1A localization (Kendirgi et al., 2003; Kaneb et al., 2015; Aditi et al., 2015), and had no similarity to hGle1B localization (Kendirgi et al., 2003; Folkmann et al., 2013). Interestingly, as previously observed for both hGle1A and hGle1B, GFP-hGle1-IVS14-2A>C was recruited to SGs upon heat shock at 45°C (Figure 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.

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°C for 60 minutes, fixed and SG formation was assessed by indirect immunofluorescence of the SG protein G3BP (Tourriere 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 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 2A and 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 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 equilibrium (Kedersha et al., 2000; Aditi et al., 2015). 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; 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). In fact, no significant difference was detected for SG morphology in cells expressing hGle1A versus hGle1-IVS14-2A>C (Figure 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 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 4, right column). 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 5A and 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 6A and 6B). However, TDP-43 did colocalize with the “fibrous” GFP-Gle1A and GFP-hGle1-IVS14-2A>C aggregates (Figure 6A and 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, which has been suggested to modulate the stability and degradation of mutant protein aggregates in neuronal inclusions (Lou 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 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 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 IP₆, hGle1B plays an integral role in this process by regulating mRNP remodeling at the NPC cytoplasmic face (Folkman 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 not support mRNA export. Using our previously validated knockdown:add-back approach of hGle1 depletion and exogenous expression (Folkman 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. 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 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 (Murphy and Wente, 1996; Bolger et al., 2008; Bolger and Wente, 2011). 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 nonoverlapping 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. 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 (Nousianen et al., 2008; Folkmann et al., 2013, 2014).

In this study, we characterized the functional capacity of a rare ALS-linked *hGLE1-c1965-2A>C* mutation that alters the C-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 C-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 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-c.1965-2A>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 dysregulation 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 dysregulation of the hGle1-cofactor IP₆ in neurodegenerative disease. First, activation of the caspase-independent apoptosis pathway is associated with familial ALS, and is provoked through phosphorylation of IP₆ following cytoplasmic translocation of IP₆ kinase (reviewed in Ghavami et al., 2014). Second, IPK1 is recruited to stress granules and increased levels of IP₆ are neuroprotective, suggesting that IP₆ production in SG might facilitate disaggregation of proteins sequestered there (Brehm et al., 2007; Xu et al., 2008; Anekonda et al., 2011; Lv et al., 2015). 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; Jović et al., 2015; Zhang et al., 2015). 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; Johnson et al., 2008; Dormann et al., 2011; 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 RBPs such as TDP-43 and thereby promote ALS by the gain- and/or loss of native RBP 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 RBPs 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 (Johnson et al., 2010; Buchan et al., 2013). 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 C-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.

Acknowledgements

The authors thank members of the Wentz laboratory for discussions and critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (R37GM051219 to S.R.W.) and A. was supported in part by the Vanderbilt International Scholar Program.

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Fig. 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 μ m.

Fig. 2.

ALS-linked hGle1-IVS14-2A>C rescues SG assembly defects in hGle1-depleted cells similar to that of hGle1A. CTRL or *hGle1* siRNA-treated cells were transfected with either *GFP* or *GFP-hGle1-c.1965-2A>C^R* 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).

Fig. 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-hGLE1A^R*, or *GFP-hGLE1-c.1965-2A>C^R* 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 $\pm$ 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 .

Fig. 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 μ m.

Fig. 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-hGle1^R*, or *GFP-hGle1-c.1965-2A>C^R* plasmids. 24 hours after transfection, cells were fixed and processed for indirect immunofluorescence using anti-DDX3 and anti-G3BP antibodies. Scale bar represents 10 μ m.

Fig. 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-hGLE1^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 μ m.

Fig. 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.

Fig. 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 μ m. (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.

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Table 1

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

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	+	+	+	+	+	–

[†]
(Kaneb et al., 2015)

^{*}
Rescues defect in hGle1-depleted