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Stress granules at the intersection of autophagy and ALS

Zachary Monahan[1], **Frank Shewmaker**[1], and **Udai Bhan Pandey**[2,3,4,*]

[1]Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD

[2]Department of Pediatrics, Division of Child Neurology, Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA

[3]Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA

[4]Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal disease caused by loss of upper and lower motor neurons. The majority of ALS cases are classified as sporadic (80-90%), with the remaining considered familial based on patient history. The last decade has seen a surge in the identification of ALS-causing genes – including *TARDBP* (TDP-43), *FUS*, *MATR3* (Matrin-3), *C9ORF72* and several others – providing important insights into the molecular pathways involved in pathogenesis. Most of the protein products of ALS-linked genes fall into two functional categories: RNA-binding/homeostasis and protein-quality control (*i.e.* autophagy and proteasome). The RNA-binding proteins tend to be aggregation-prone with low-complexity domains similar to the prion-forming domains of yeast. Many also incorporate into stress granules (SGs), which are cytoplasmic ribonucleoprotein complexes that form in response to cellular stress. Mutant forms of TDP-43 and FUS perturb SG dynamics, lengthening their cytoplasmic persistence. Recent evidence suggests that SGs are regulated by the autophagy pathway, suggesting a unifying connection between many of the ALS-linked genes. Persistent SGs may give rise to intractable aggregates that disrupt neuronal homeostasis, thus failure to clear SGs by autophagic processes may promote ALS pathogenesis.

Keywords

Amyotrophic lateral sclerosis; autophagy; motor neuron diseases; neurodegeneration; neuromuscular diseases; P-bodies; protein degradation pathways; rapamycin; stress granules

[*]**Corresponding author:** Udai Bhan Pandey, Department of Pediatrics, Human Genetics, Neurology, Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh PA, Ph (412)-692-3192, udai@pitt.edu.

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1. Introduction

ALS is the most common adult-onset motor neuron disorder, typically striking in the fifth to seventh decades of life, though juvenile disease also exists. It is characterized by rapid degeneration of motor neurons, and subsequent atrophy of innervated muscle groups. Death is generally secondary to failure of respiratory muscles (Ravits and La Spada, 2009; Turner et al., 2013). ALS occurs globally in all races, ethnic and socioeconomic groups. There are no pharmacological interventions for the underlying molecular pathogenesis (Miller et al., 2012).

Neurodegenerative diseases often share two clinicopathological properties. First, by definition, they affect highly translationally-active neurons preferentially to other cell types; second, they are often associated with mutations in components of protein-quality control (PQC) (Hetz et al., 2009; Kabashi and Durham, 2006). It is perhaps not surprising that perturbations to PQC pathways would have significant impact on cells that are both especially translationally active and long-lived. In the case of ALS, a number of different proteins and metabolic pathways have been linked to pathogenesis, but issues of proteostasis (*e.g.* protein folding, aggregation and quality-control) appear to be the most common pathogenic theme (Andersen and Al-Chalabi, 2011; Renton et al., 2014). Many ALS-associated proteins have intriguing properties with regard to self-association, aggregation-propensity, and interaction with cytoplasmic stress granules (SGs) (Bosco et al., 2010; Colombrita et al., 2009; Daigle et al., 2013; Dewey et al., 2012; Guo et al., 2011; Liu-Yesucevitz et al., 2010; McDonald et al., 2011; Sun et al., 2011; Vance et al., 2013). Other ALS-associated proteins have explicit functions in PQC pathways, including autophagy. Below we discuss the intersections between protein aggregation, SGs and autophagy in ALS pathogenesis.

2. Stress Granules – Discrete Stress-Induced Cytoplasmic Sites of Ribonucleoprotein Accumulation

Ribonucleoprotein (RNP) granules are cellular sites dedicated to RNA processing. Well-characterized types of RNP granules include transport RNPs, processing bodies (P-bodies) and stress granules (SGs); all of which have distinct roles in mRNA regulation (Anderson and Kedersha, 2008; Kedersha et al., 2005). Transport RNPs ensure localized neuronal translation of RNAs by facilitating their transport along cytoskeletal elements while maintaining temporary translational repression (Kiebler and Bassell, 2006). SGs and P-bodies are phenotypically similar, non-membrane-bound, discrete cytoplasmic structures visible by light microscopy (Buchan and Parker, 2009; Guil et al., 2006). They contain many of the same proteins, but each has exclusive constituents; P-bodies are enriched for proteins involved in RNA degradation, while SGs are preferentially composed of translation initiation factors (Reineke and Lloyd, 2013). Thus, P-bodies are classified as foci of RNA breakdown and turnover, and SGs are thought to be sites of paused translation initiation and global translation repression (Anderson and Kedersha, 2008; Li et al., 2013; Parker and Sheth, 2007; Thomas et al., 2011). Both SGs and P-bodies have the ability to exchange mRNAs with bulk cytoplasm depending on cellular conditions (Decker and Parker, 2012).

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The formation of SGs is believed to be a conserved, protective response to various cell stresses. Some example stresses include: oxidative (Anderson and Kedersha, 2002; Anderson and Kedersha, 2008; Bosco et al., 2010; Daigle et al., 2013), mitochondrial (Buchan et al., 2011; Chalupnikova et al., 2008; Stoecklin et al., 2004), proteasomal (Fournier et al., 2010; Mazroui et al., 2007) and viral (Emara and Brinton, 2007; Raaben et al., 2007). Interestingly, many external stimuli/stressors do not induce SG formation in mammalian cells, suggesting SGs are a specific response not common to all stress (Kedersha et al., 1999). There are several proposed means by which they exert their protection. SGs may offer direct protection for certain mRNAs from damaging stressors (Kedersha and Anderson, 2002). Alternatively, SGs may sequester unwanted mRNAs, preventing their translation, such as viral RNAs during infection (Beckham and Parker, 2008), or less critical mRNAs during stress conditions (Li et al., 2013; Unsworth et al., 2010; Wolozin, 2012). Thus, SGs may offer prioritization of specific protein products (Scheu et al., 2006). More generally, SGs may decrease protein stress through the global repression of translation by binding mRNAs that would otherwise be translated. The apparently causal role of phosphorylated eIF2α in facilitating SG formation supports this hypothesis, as eIF2α has an established role in translation repression (Kedersha et al., 1999).

SGs contain polyadenylated mRNAs, translation initiation factors, small ribosome subunits and several RNA-binding proteins (Anderson and Kedersha, 2008; Daigle et al., 2016). Putative SG functions all demand the intimate association of these components within a discrete cytosolic space, removed from the majority of cellular machinery. Importantly, RNPs containing specific mRNAs are critical for transport and localized translation in neuronal dendrites. Different types of RNPs (SG, P-body, transport) share similar components (Decker and Parker, 2012), thus neurons may be particularly sensitive to disruption of RNP homeostasis.

SGs are assembled and disassembled through the formation and dissolution of a “liquid-liquid phase-separated state”, in which the components that form SGs “demix” from the bulk solution to create a unique micro-environment. This transient phase-separated state presumably allows for a rapid, reversible response to stress (Elbaum-Garfinkle et al., 2015; Lin et al., 2015; Molliex et al., 2015). Several proteins, as well as mRNA, are implicated in driving the physical phase separation (Zhang et al., 2015). The protein TIA1, for example, is critical to the early stages of SG assembly. TIA1 has three amino-terminal RNA recognition motifs and a carboxy-terminal domain, which has low-complexity composition similar to the intrinsically-disordered domains that drive yeast prion proteins to form self-propagating amyloid fibrils. In fact, a peculiarity about many of the proteins that are both linked to ALS and SG formation is they possess yeast prion-like domains (discussed below). Substitution of this domain of TIA1 with the actual prion-forming domain of yeast prion protein Sup35, results in a restoration of SG formation, which is lost following native TIA1 prion-like domain deletion (Gilks et al., 2004).

The evolutionary conservation of SGs in eukaryotic cells indicates that they serve critical cellular functions. However, the promiscuous, *en masse* sequestration of mRNA transcripts in cytosolic granules would clearly have dramatic implications for cell survival. As with any metabolic pathway, SG formation must be balanced with mechanisms to ensure disassembly.

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Intracellular component turnover relies on multiple pathways, including autophagy and ubiquitin-mediated proteolysis (Ciechanover, 1994; Cuervo et al., 2005; Glickman and Ciechanover, 2002; Levine et al., 2008; Reed, 2003).

3. Autophagy – a Mechanism for Clearing Protein Aggregates

Autophagy is a well-studied system for disposal of a variety of intracellular species. First identified in the context of hormone studies in rats, it has since been appreciated as a mechanism for nearly all eukaryotic cells to dispose of a wide variety of intracellular components deemed unnecessary or maladaptive (Deter et al., 1967; Gomes and Scorrano, 2013). Autophagy involves an autophagosome, a double-membrane bound structure that forms from extant membrane-bound organelles (Chan and Tang, 2013). The autophagosome engulfs regions of the cytosol and fuses with the lysosome to become the autophagolysosome where its contents are catabolized (Deter et al., 1967; Gomes and Scorrano, 2013). This membrane-enclosed mechanism is sometimes more specifically called macroautophagy to distinguish it from other types of autophagic processes. Not all autophagy processes employ the formation of a new autophagosome. Chaperone-mediated autophagy involves the direct targeting of substrates to the lysosome via chaperone intermediates and then active translocation of the substrates across the lysosomal membrane. Microautophagy involves the direct engulfment of cytoplasmic content by invagination of the lysosomal membrane (Mizushima et al., 2008).

Autophagy has historically been considered a non-selective process of cellular digestion, in contrast to the ubiquitin-proteasome system (Gomes and Scorrano, 2013; Meijer et al., 2007). In general, less selectivity may be a consequence of the extremely diverse homeostatic functions of autophagy (Cuervo et al., 2005). Gross protein turnover via autophagic pathways, for instance, is likely a critical means to balance anabolic pathways with a free source of available amino acids (Harris et al., 2004; Meijer, 2009). This last point is strongly supported by the observation that amino acid deprivation is a very potent inducer of autophagocytic pathways in mammalian cells (Ghislat et al., 2015; Mortimore and Schworer, 1977). However recent findings suggest a greater degree of specificity and overlap with the proteasome system (discussed below).

a. Selective Macroautophagy

Despite the historical perspective of autophagy being a generic pathway, recent work suggests importance of a selective function with implications for human disease (De Duve and Wattiaux, 1966; Gomes and Scorrano, 2013). Specifically, ubiquitin tagging may have a role in targeting selective autophagy (Heo et al., 2015; Kirkin et al., 2009). Moreover, selective autophagy is particularly well-suited to clearance of large (> 2 um) protein aggregates and RNP granules that need to be disassembled, in part because large protein aggregates and persistent RNP granules are known to be resistant to ubiquitin-proteasome degradation (Venkatraman et al., 2004).

The general model of selective autophagic engulfment of substrate is shown in Figure 1. Initially, the phospholipid-conjugated LC3 protein (Atg8 in yeast) facilitates the formation of membranous phagophore structures that recognize the targeted material through various

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acceptor and receptor proteins. The phagophore envelops the substrate to form an autophagosome that fuses with the lysosome, forming the autophagolysosome (Klionsky et al., 2014). This structure hosts proteolysis, facilitating amino acid export to the cytosol for re-use (Harris et al., 2004; Klionsky et al., 2014; Meijer, 2009).

Many ubiquitinating factors and adapter proteins enable specificity in selective macroautophagy. For example, ubiquillin-2 is implicated in facilitating interaction between an autophagosome and its target (N'Diaye et al., 2009; Rothenberg et al., 2010). Proteins p62 and NBR1 function as selective cargo receptors, linking ubiquitin tags with autophagosome receptors (Lamark et al., 2009; Pankiv et al., 2007). NBR1 in particular has been shown to mediate selective autophagy in plant cells dealing with otherwise intractable protein aggregates (Zhou et al., 2013a). Many of these adaptor proteins recognize the phagophore through “LC3-interacting region” motifs in their peptide sequence (Birgisdottir et al., 2013). Moreover, mutations in several adapters are linked to sub-types of ALS (discussed below).

b. Autophagic Clearance of Protein Aggregates and Stress Granules

Autophagy has been linked to the clearing of the large protein aggregates that feature prominently in several neurodegenerative diseases. For example, the protein alpha-synuclein forms intracellular fibrils in neurons of patients with Parkinson’s disease (Baba et al., 1998). Formation of these fibrous aggregates correlates with a massive increase in the presence of autophagosomes in cultured cells (Shintani and Klionsky, 2004; Stefanis et al., 2001). This could be a consequence of increased formation or impaired clearance of autophagosomes (Shintani and Klionsky, 2004). In a similar fashion, aggregates of mutant huntingtin protein, which are linked to Huntington’s disease, are also associated with an abundance of autophagosomes (Kegel et al., 2000). In the case of both huntingtin and alpha-synuclein, promotion of autophagy via pharmacological stimulation by rapamycin results in an increase in autophagic degradation of the two proteins, as well as a corresponding reduction in toxicity (Ravikumar et al., 2002; Shintani and Klionsky, 2004; Webb et al., 2003).

Similarly, autophagy is involved in the clearance of RNP granules. Buchan and colleagues demonstrated that SG breakdown in particular was dependent on selective autophagy. Strikingly, and very similarly to previous experiments with huntingtin and alpha-synuclein, rapamycin administration promoted SG clearance, while selective autophagy inhibitors had the opposite effect (Buchan et al., 2013). Likewise, when autophagy-initiating proteins (Atg proteins) were partially deleted in yeast, cytosolic SG persistence was dramatically increased. Beyond the canonical Atg family, an additional protein of interest is valosin-containing protein (VCP/p97), which appears to specifically target SGs to autophagic pathways for degradation (Buchan et al., 2013; Ju et al., 2009). When VCP is silenced with siRNA or with specific chemical inhibitors in stressed HeLa cells, SGs accumulate (Buchan et al., 2013).

Other mechanisms are linked to SG clearance, such as decapping SG mRNAs, returning sequestered mRNAs to active translation, and direct 5’ to 3’ digestion of constituent mRNA molecules. Such fates likely involve dynamic handling of mRNAs among P-bodies, SGs, and polysomes (Bhattacharyya et al., 2006; Brengues et al., 2005; Buchan et al., 2013; Sheth and Parker, 2003). Intriguingly, Buchan and colleagues showed that upon inactivation of

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these pathways via genetic knockout, targeting of SGs to autophagy appeared to increase (Buchan et al., 2013). The metabolic and homeostatic mechanisms of mRNA translation and degradation likely reflect a very dynamic, complex cycle, of which autophagy is a critical player.

c. Autophagy in Animal Models of ALS

Connections between autophagy and ALS have been observed in animal models. The first gene linked to familial ALS was superoxide dismutase 1 (SOD1). Induction of the autophagy pathway has been observed in a transgenic mouse model of SOD1-ALS, as well as spinal cord tissues from SOD1-ALS patients, supporting the idea that pathogenic mutations in SOD1 impair, or require, autophagy pathways (Morimoto et al., 2007; Sasaki, 2011). Since autophagy is critical to managing the burden of misfolded and toxic proteins, it is not unexpected that perturbed autophagy has also been observed in the postmortem tissues of sporadic ALS patients (Morimoto et al., 2007). Retinoic acid has been demonstrated to act on PQC pathways including autophagy, and interestingly, control rats maintained on a retinoid-free diet show ALS-like symptoms (Anguiano et al., 2013; Castillo et al., 2013; Corcoran et al., 2002; Kolarcik and Bowser, 2012; Rajawat et al., 2010; Rajawat et al., 2011; Riancho et al., 2015; Riancho et al., 2016). Furthermore, treatment with Bexarotene, a retinoid-X receptor agonist, or induction of autophagy in a SOD1 mutant mouse model drastically delayed motor symptoms and ALS pathology suggesting that targeting PQC machinery could be a good therapeutic target for SOD1-ALS (Castillo et al., 2013; Crippa et al., 2010; Riancho et al., 2015; Riancho et al., 2016).

Autophagic dysfunctions have also been implicated in subtypes of ALS caused by mutations in TDP-43 and FUS (discussed more below). It has been suggested that TDP-43 is involved in regulating autophagy in general and particularly autophagosomal and lysosomal biogenesis (Bose et al., 2011; Filimonenko et al., 2007; Xia et al., 2016; Ying et al., 2016). Interestingly, TDP-43 is managed by both proteasome and autophagy pathways where the soluble form of TDP-43 is degraded by the proteasome; and oligomeric and aggregated forms of TDP-43 are cleared by autophagy (Xia et al., 2016). Given that TDP-43 is a highly aggregation-prone protein, it is not surprising to observe that impaired turn-over of TDP-43 directly correlates with motor dysfunctions and reduced autophagy-related proteins in a mouse model of TDP-43 proteinopathy (Caccamo et al., 2015). Similarly, pathogenic mutations in FUS have been shown to impair the autophagy pathway in cellular models and ALS patient cells (Soo et al., 2015). Importantly, pharmacological induction of autophagy ameliorates neurodegenerative symptoms as well as TDP-43 protein mislocalization in Drosophila and rodent models of TDP-43 proteinopathy, further supporting a notion that autophagy could be a potential therapeutic target for ALS (Barmada et al., 2014; Caccamo et al., 2009; Cheng et al., 2015; Wang et al., 2012).

4. Intersections Between ALS, Stress Granules and Autophagy

A curious feature of many SG proteins is low-complexity domains resembling the prion-forming domains of certain yeast proteins. Moreover, many SG proteins are also found in the pathological inclusions in ALS patients' motor neurons. In fact, many ALS-associated

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proteins have significant structural or functional overlap with SG proteins. This predicted overlap is due to the presence of RNA-binding domains and prion-like domains (Arai et al., 2006; Ju et al., 2011; Kwiatkowski et al., 2009; Maekawa et al., 2009; Neumann et al., 2006; Udan and Baloh, 2011; Vance et al., 2009), as well as observed functional interactions in experimental models (Acosta et al., 2014; Baron et al., 2013; Daigle et al., 2016; Dewey et al., 2012; Di Salvio et al., 2015; Lenzi et al., 2015; McDonald et al., 2011; Parker et al., 2012; Sama et al., 2013). These observations encourage the idea that RNA homeostasis and prion-like mechanisms of protein self-association are critical to ALS pathogenesis.

Prion-like mechanisms have been implicated in a variety of neurodegenerative diseases, most notoriously the transmissible spongiform encephalopathies (TSEs) (Gielbert et al., 2015). In TSEs, auto-catalytic processes drive the propagation of misfolded protein isoforms within the nervous tissue of infected animals. This propagation results in the accumulation of insoluble protein aggregates that spatially correlates with cell death (Jeffrey et al., 1995). Soluble misfolded oligomeric species, resistant to proteolytic degradation, presumably precede the formation of macroscopic aggregates (Bessen et al., 1997; Jeffrey et al., 1995). Together, these oligomeric and larger aggregates are thought to exert cellular toxicity. Thus, the fact that ALS-linked SG proteins have prion-resembling domains that are aggregation-prone suggests that ALS could have a prion-like mechanism in which toxic misfolded proteins self-propagate.

Given the potential that prion-like mechanisms underlie disease pathologies, as well as SG dynamics, interest in the intersection between disease and SG homeostasis is unsurprisingly robust (Acosta et al., 2014; Baron et al., 2013; Bentmann et al., 2013; Daigle et al., 2016; Dewey et al., 2010; Dewey et al., 2012; Di Salvio et al., 2015; Lenzi et al., 2015; Li et al., 2013; McDonald et al., 2011; Parker et al., 2012; Sama et al., 2013; Walker et al., 2013) . The high local concentration of proteins with prion-like domains in SGs is thought to facilitate the transient phase-separated granule structure. However, self-association between these domains is not static (Burke et al., 2015). Instead, under certain conditions, the normal internal interactions are hypothesized to evolve into terminally-aggregated species that are not easily cleared by the cell. Specifically, an inappropriate persistence, or excessive mRNA binding, of SGs may be a critical element in neurodegeneration. If SG pathology overwhelms autophagy pathways, cellular pathology may follow. Potential consequences of ALS-associated genetic mutations are listed in Table 1 and discussed below.

a. FUS, TDP-43, Matrin-3 and hnRNPA2/A1 as Stress Granule-Associated Proteins

The proteins fused-in-sarcoma (FUS) and TAR DNA binding protein-43 kDa (TDP-43) are well-established DNA/RNA-binding proteins that are implicated in similar functions, including transcription regulation and RNA splicing (Lagier-Tourenne et al., 2010; Lagier-Tourenne et al., 2012). They have similar structures, including nucleic acid binding domains and prion-like domains of low intrinsic complexity. Mutations of both proteins, characteristically in domains regulating subcellular localization, are correlated with development of ALS. In diseased cells, a characteristic pattern of cytoplasmic foci and structural association with SGs has been widely reported in diverse organisms (Acosta et al., 2014; Arai et al., 2006; Barmada et al., 2010; Baron et al., 2013; Bentmann et al., 2013;

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Dewey et al., 2012; Di Salvio et al., 2015, Daigle, 2016 #305; Johnson et al., 2008; Ju et al., 2011; Kwiatkowski et al., 2009; Lenzi et al., 2015; Li et al., 2010; Maekawa et al., 2009; McDonald et al., 2011; Neumann et al., 2006; Parker et al., 2012; Sama et al., 2013; Sun et al., 2011; Udan and Baloh, 2011; Vance et al., 2009; Walker et al., 2013).

Both FUS and TDP-43 have default nuclear localization consistent with their RNA-related functions. They also demonstrate an ability to regulate their own expression (Avendano-Vazquez et al., 2012; Budini and Buratti, 2011; D'Alton et al., 2015; Dormann and Haass, 2011; Zhou et al., 2013b). An abundance of intranuclear FUS causes exon 7 skipping in FUS transcription, driving FUS transcripts to nonsense mediated decay and preventing pathological protein buildup (Zhou et al., 2013b); an accumulation of FUS outside of the nucleus could thus result in self-perpetuating high expression, exacerbating the potential for misfolding in the cytoplasm. FUS is also implicated in DNA repair. It accumulates at sites of DNA damage and is implicated in regulating DNA repair pathways (Hicks et al., 2000; Lagier-Tourenne et al., 2010; Mastrocola et al., 2013; Patel et al., 2015a; Rulten et al., 2014; Sama et al., 2013; Wang et al., 2013). This self-association is likely mediated by its prion-like domain, leading to intranuclear phase-separated droplets that facilitate DNA repair by recruiting specific proteins (Patel et al., 2015a; Rulten et al., 2014).

Despite the intranuclear roles described above, there is very clearly a cytosolic role for both proteins, as evidenced by the presence of a nuclear export sequence (Lorenzo-Betancor et al., 2014). This cytoplasmic shuttling follows various types of cell stress (Sama et al., 2013), perhaps facilitating physiological SG metabolism. FUS, for example, localizes to the cytoplasm following post-translational modifications, including methylation and phosphorylation (Deng et al., 2014; Scaramuzzino et al., 2013; Tradewell et al., 2011). Normally, an intact nuclear localization signal (NLS) likely ensures a prompt return to the nucleus, especially in the context of SG disassembly (Sama et al., 2013). In the case that the proteins contain an NLS mutation, persistent cytosolic localization of FUS or TDP-43 results (Acosta et al., 2014; Barmada et al., 2010; Dormann and Haass, 2011). The subsequent cytosolic co-localization with SGs is perhaps a consequence of FUS/TDP-43 having prion-like domains similar to the domains found in many SG constituent proteins (Gilks et al., 2004; Udan and Baloh, 2011). Co-localization may modify the properties of SGs. Both FUS and TDP-43 have domains that would presumably bind resident mRNAs (Schwartz et al., 2013; Ugras and Shorter, 2012). Thus, cytoplasmic localization of both proteins may drive inappropriate interactions within SGs. Subsequent modification of SG homeostasis, such as excessively promiscuous SG-mRNA binding or inappropriate persistence of SGs, may overwhelm regulatory processes and lead to intractable aggregation. Such a model is especially attractive in light of the biophysical properties of liquid-liquid phase-separated FUS droplets (Burke et al., 2015; Patel et al., 2015a; Zhang et al., 2015), which have been shown *in vitro* to evolve into irreversible aggregates in a time-dependent manner. If FUS and SGs behave so similarly in isolation, such mechanisms may drive pathology when driven into cytosolic juxtaposition by ALS-associated mutations.

The interaction of FUS and TDP-43 with cytosolic SGs represents a putative cytosolic gain-of-function toxicity pathway. This hypothesis is supported by a variety of studies (Ju et al., 2011; Kryndushkin et al., 2011; Lanson et al., 2011; Scaramuzzino et al., 2013; Sharma et

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al., 2016; Sun et al., 2011). In model systems, ectopic expression of human FUS or TDP-43 results in cytoplasmic aggregation into discrete FUS or TDP-43-positive foci, as well as cellular toxicities. Toxicity can be partially ameliorated by disruption of either the prion-like domain or the RNA-binding domains (Daigle et al., 2013; Sun et al., 2011), suggesting that both of these domains are critical to exerting toxicity. Interruption of the prion-like domain may interfere with protein association, while the RNA-binding interruption may prevent RNA from being irreversibly sequestered in the aggregates. Moreover, disruption of RNA-binding capacity may hamper further protein association by preventing RNA-nucleation of protein interactions (Schwartz et al., 2013). Importantly, in a transgenic FUS mouse line, postnatal knockdown of FUS did not promote motor neuron degeneration, whereas expression of FUS mutants was dominant negative for motor neuron survival ((Sharma et al., 2016).

While gain-of-function toxicity appears to be a major part of the FUS/TDP-43 story, deletion of these proteins has also been shown to have deleterious consequences for mammalian cells (Ayala et al., 2008; Chiang et al., 2010; Fiesel and Kahle, 2011; Hicks et al., 2000; Kuroda et al., 2000; Orozco et al., 2012; Sama et al., 2014; Shan et al., 2010). Thus, a loss of intranuclear function must be considered as a contributor to pathology. First, it may lead to a loss of nucleic-acid regulation as well as other intranuclear functions ascribed to both FUS and TDP-43 (Avendano-Vazquez et al., 2012; Budini and Buratti, 2011; D'Alton et al., 2015; Dormann and Haass, 2011; Hicks et al., 2000; Lagier-Tourenne et al., 2010; Mastrocola et al., 2013; Patel et al., 2015a; Rulten et al., 2014; Sama et al., 2014; Wang et al., 2013; Zhou et al., 2013b). Second, the loss of autoregulation of expression could lead to unchecked transcription, cytosolic translation, and continued cytoplasmic aggregation with SGs as described above. Specifically, TDP-43-linked ALS cases were recently shown to exhibit an abundance of so-called cryptic exons in nervous system tissue (Pochet et al., 2015). TDP-43 has a role, similar to FUS autoregulation, to repress expression of the cryptic exons. Loss of intranuclear TDP-43 to cytoplasmic SGs/aggregates may lead to excessive cryptic exon expression, driving nervous system pathology in ALS patients (Ling et al., 2015).

Additional ALS-linked proteins Matrin-3 and hnRNPA2/A1 have DNA/RNA-binding properties. Matrin-3 is, like FUS and TDP-43, a resident of the nucleus and is implicated in various RNA-related functions. Matrin-3 was shown to interact with TDP-43 in cytosolic aggregates in spinal neurons. Also, ALS-linked mutations appear to modulate the degree of this interaction (Gallego-Iradi et al., 2015; Johnson et al., 2014; Millecamps et al., 2014). While less well characterized than FUS and TDP-43, the potential for Matrin-3 to interact with SGs, perhaps even via a direct association with TDP-43, would have obvious functional implications.

In the case of hnRNPA2/A1, we again see nucleic acid binding capacity joined by the presence of a prion-like domain. Proteins harboring mutations linked to ALS phenotypes display an increased *in vitro* aggregation propensity relative to wild-type, capacity to propagate as a yeast prion when swapped with the native Sup35 prion-domain in yeast, as well as an enhanced recruitment to stress granules (Kim et al., 2013). All of these

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observations strongly support the importance of SG association and protein self-association in facilitating pathways of toxicity.

While the roles of FUS, TDP-43, Matrin-3, and hnRNPA2/A1 in ALS will likely continue to be clarified, there are intriguing hints of a link between these proteins and SGs and autophagy. Indeed, the clearance of FUS and TDP-43 aggregates by autophagy has been reported (Caccamo et al., 2009; Ryu et al., 2014; Wang et al., 2010). Recent reports suggest that autophagy of FUS aggregates can be enhanced by overexpression of the critical autophagy protein Rab1 (Soo et al., 2015). This suggests aggregates are overwhelming autophagic machinery in degenerating motor neurons.

b. C9ORF72 and VCP as Regulators of Autophagy

Beyond the prion-like nucleic acid binding proteins discussed thus far, a number of other proteins have been associated with ALS pathogenesis. These proteins also offer pathogenetic connections with SGs and autophagy. In fact, the most common genetic abnormality linked with familial ALS is a disordered intron in the nucleic acid sequence located on chromosome 9, open reading frame 72 (C9ORF72). Specifically, ALS has been associated with a hexanucleotide repeat expansion (HRE) GGGGCC inherited in an autosomal dominant fashion (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The potential avenues by which the HRE may drive neurotoxicity are numerous, and include both nucleic acid-driven and protein-driven explanations.

It is yet not entirely clear how HRE causes motor neuron degeneration associated with ALS. It has been demonstrated that the C9 expanded hexanucleotides abnormally bind and sequester RNA binding proteins in nuclear foci in ALS patient neurons (Donnelly et al., 2013). Expression of the HRE is enough to cause the formation of SGs under stress conditions. These SGs sequester other disease-associated proteins such as hnRNPA1 and hnRNPA2/B1 (Farg et al., 2014). Moreover, repeat expression leads to elevated levels of a protein called p62 in motor neurons differentiated from C9 induced pluripotent stem cells (Almeida et al., 2013). Importantly, p62 has been implicated as an adaptor protein for selective autophagy of protein aggregates (Pankiv et al., 2007). In addition to this, C9 hexanucleotide repeat causes increased sensitivity to cellular stress induced by autophagy inhibitors, further supporting the role of autophagy in C9ORF72 mediated neurodegeneration (Almeida et al., 2013).

In addition to RNA foci, C9ORF72 repeat transcripts can produce unconventional, repeat-associated non-ATG mediated (RAN) translation products. These RAN peptides, translation of which occurs without a start codon, have been observed in ALS patient tissues as well as in different experimental models (Gendron et al., 2013; Mori et al., 2013; Wen et al., 2014). RAN translation can occur in all possible reading frames, producing dipeptide repeat proteins in the sense and antisense direction (antisense: poly-PR and poly-PA; sense: poly-GA and poly-GR; both sense: poly-GP). These C9 RAN products have been examined in cellular and animal models where it has been shown that GR and PR dipeptides are extremely toxic as well as being especially aggregation-prone (Mizielinska et al., 2014; Wen et al., 2014). Additionally, GA proteins have been shown to form neuronal inclusions that stain positive for the autophagy-mediator p62 (Schludi et al., 2015). Proteins known to

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sequester in SGs such as PABP-1 localize to TDP-43 positive inclusions more frequently in patients carrying expansions in C9ORF72 (McGurk et al., 2014).

Interestingly, it has been demonstrated that the C9ORF72 translation product colocalizes with proteins implicated in regulating autophagy and endocytic transport such as Rab1, Rab5, Rab7 and Rab11 in mammalian neuronal cells and human spinal cord motor neurons. Perhaps most intriguing, depletion of C9ORF72 led to an increase in autophagosome dysregulation, specifically by disrupting function of receptors Trk-beta and LC3. Moreover, pharmacological inhibition of the 26S proteasome in addition to C9ORF72 overexpression led to an accumulation of SGs (Farg et al., 2014). These findings suggest that C9ORF72 is a regulator of endosomal trafficking, functions with critical importance to autophagy. This role in regulating endosomal and vesicular trafficking is further evident from the fact that C9ORF72 colocalizes with other ALS-linked proteins in ubiquilin-2 and LC3-positive vesicles. Protein interaction by C9ORF72 also includes heterogeneous nuclear ribonucleoproteins, Matrin 3, hnRNPA2/B1 and hnRNPA1 (Donnelly et al., 2013; Farg et al., 2014; Haeusler et al., 2014).

The link between dysregulated autophagy, SGs, and development of an ALS phenotype is further enhanced by studies of Valosin-containing protein, VCP. Ju and colleagues showed the importance of intact VCP in allowing for normal autophagic pathways (Ju et al., 2009). Specifically, this study demonstrated the accumulation of both autophagosomes arrested before association with lysosomes, and TDP-43 positive protein inclusions in cells depleted of wild type VCP (Ju et al., 2009). Moreover, Buchan *et al.* showed that VCP helps target SGs to the autophagosome for disassembly, while Cherkasov and workers showed that this disassembly was chaperone mediated (Buchan et al., 2013; Cherkasov et al., 2013). Surprisingly, another report showed that impaired VCP led to a reduction in the size and formation of SGs, suggesting that VCP may play an important role in both SG assembly and targeting to the autophagosome (Seguin et al., 2014). This same study showed a concomitant accumulation of ubiquitinated proteins with a reduction in SG formation.

c. Optineurin, TBK1 and Ubiquilin-2 as Modulators of Stress Granule Clearance by Autophagy

Optineurin is an additional protein implicated in both ALS (Maruyama et al., 2010) and autophagic pathways, specifically by binding to the autophagosome receptor LC3 (N'Diaye et al., 2009; Rothenberg et al., 2010; Wong and Holzbaur, 2014). Optineurin has been shown to act as an adaptor for selective autophagy, similarly to p62 previously described (Pankiv et al., 2007; Wild et al., 2011). It is well documented that optineurin binds LC3, and that this association facilitates mitophagy (autophagy of mitochondria). A protein called Parkin, which has been associated with Parkinson’s disease, enhanced the stability of the association between optineurin and mitochondria (Wong and Holzbaur, 2014). Moreover, Shen and colleagues demonstrated that overexpression of wild-type optineurin facilitated the autophagy-mediated clearance of cytosolic aggregates containing the aggregation-prone protein mutant huntingtin (Shen et al., 2015).

Optineurin has a robust aggregation propensity in addition to its ability to regulate autophagy (Kryndushkin et al., 2012). The case of optineurin actually offers a very

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compelling example of how complicated is the interaction between aggregation propensity and autophagy. Mutations in the ubiquitin-binding domain of optineurin have been shown to reduce its ability to perform its autophagic function (Shen et al., 2015). Mutations in this domain include the E478G mutation linked to ALS (Maruyama et al., 2010). Optineurin E478G, despite its reduced ability to bind ubiquitin, still has the potential to associate with and trap wild-type optineurin in the cytosol. This prevents the association of wild-type optineurin to autophagic machinery, and therefore the appropriate maturation of autophagosomes. The authors used LC3 turnover as a measure of autophagic flux to show that this wild-type optineurin trapping effect of E478G led to measurable decreases in autophagy for the cell, as well as a subsequent increase the abundance of cytosolic aggregates (Shen et al., 2015).

As described above, optineurin may offer an example of how aggregation propensity and dysregulation of autophagy are directly related in the cell. If a key function of autophagy is to clear intracellular protein aggregates, it makes sense that major regulators of autophagy would have the capacity to interact with protein aggregates. It is perhaps via this property of self-association that proteins are able to physically interact with aggregates, and then modulate autophagy via other protein domains.

Optineurin is also implicated in ALS pathogenesis as a result of interaction with kinase pathways. Phosphorylation is perhaps the most widely used cellular signal for countless metabolic processes. Unsurprisingly, kinases like TBK1 have been implicated in diverse human pathologies from hepatic dysfunction to neurodegeneration (Bonnard et al., 2000; Freischmidt et al., 2015). Indeed, TBK1 was first linked to ALS in 2015 (Cirulli et al., 2015). Subsequent studies showed that TBK1-linked ALS was likely due to haploinsufficiency of this kinase, an observation that builds on many of the ideas so far discussed in this review (Freischmidt et al., 2015). Perhaps the simplest explanation for TBK1-mediated ALS pathogenesis arises from the observation that TBK1 phosphorylates optineurin in cell models (Heo et al., 2015; Morton et al., 2008; Wild et al., 2011). Indeed, this phosphorylation has been shown to enhance autophagy in the context of autophagic anti-microbial innate immunity (Pilli et al., 2012; Radtke et al., 2007; Wild et al., 2011). Despite the differing context, there is clearly the potential for insufficient phosphorylation of optineurin to impair autophagosome formation and maturation.

Optineurin and TBK1 have also been discovered co-localizing in protein aggregates, and the phosphorylation of optineurin at serine 177 has been shown to be critical to its function in mediating clearance of aggregated proteins via autophagy (Korac et al., 2013). Again, the aggregation propensity of optineurin may facilitate its role as a modulator of autophagy-mediated aggregate clearance, and TBK1 association with optineurin in aggregates may be an additional factor necessary for this process. Specifically, the phosphorylation event may drive the conversion of optineurin from simply a ubiquitin/aggregate-associated protein to an LC3-mediated autophagy receptor (N'Diaye et al., 2009; Rothenberg et al., 2010; Wong and Holzbaur, 2014). This would specifically facilitate the autophagy of ubiquitinated and aggregated proteins, precisely the role of selective autophagy (Lee et al., 2010).

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The above model makes sense in the context of disease-associated optineurin mutations. For instance, open-angle glaucoma has been associated with an E50K mutation in optineurin. This mutation has been shown to enhance the TBK1-optineurin interaction, as well as reducing appropriate intracellular solubility of optineurin (Kryndushkin et al., 2012; Minegishi et al., 2013). A reduced solubility of the TBK1-optineurin complex could be expected to deplete the pool of available phosphorylated optineurin to act as an autophagy adaptor. Moreover, insufficient availability of TBK1 would clearly impair autophagy, and may simply lead to the uncontrolled association of optineurin with aggregates. This itself may actually promote more and more promiscuous cytosolic binding of cytosolic SG-associated proteins.

Like optineurin, mutations in the X-linked protein ubiquilin-2 are associated with familial ALS (Deng et al., 2011). Ubiquilin-2 has been well characterized for its function in delivering ubiquitin-tagged proteins to the proteasome for degradation (Deng et al., 2011; Walters et al., 2004) (Williams et al., 2012; Zhang et al., 2014). It has also been implicated in delivering polyubiquitinated substrates to the autophagy receptor LC3 (Rothenberg et al., 2010). Reduction in ubiquilin-2 is associated with concomitant reduction in autophagosome formation (Rothenberg et al., 2010). Given its role in protein turnover, the simplest explanation for the association of ubiquilin-2 and ALS would be a loss of function and the resulting accumulation of intracellular aggregates. SGs are enriched for ubiquitinated species (Kwon et al., 2007), thus failure to identify these species by ubiqulin-2 could reduce SG turnover by autophagy, leading to SG persistence.

d. Other Proteins Linked to ALS and Autophagy

The final step in autophagy is the fusion of the autophagosome with the lysosome. This organelle maturation appears to be mediated by the cytosolic protein HDAC6, which contains ubiquitin-binding and dynein-interacting domains (Kawaguchi et al., 2003; Lee et al., 2010). It has been implicated as a regulator of diverse cellular processes through deacetylating and destabilizing microtubules, mediating the transport of ubiquitinated proteins into aggresomes and facilitating autophagosome-lysosome fusion (Kawaguchi et al., 2003; Lee et al., 2010; Pandey et al., 2007a; Pandey et al., 2007b). Interestingly, HDAC6 physically interacts with G3BP, a known component of SGs, and is sequestered into SGs under different stress conditions. Moreover, genetic or pharmacological inhibition of HDAC6 impairs SG formation, suggesting that HDAC6 is essential for the assembly of SGs (Kwon et al., 2007).

The impairment of HDAC6 has been further implicated in pathogenesis of certain types of ALS (Gal et al., 2013), though its precise effect remain unclear (Gal et al., 2013; Taes et al., 2013). In a mouse model of SOD1 disease, HDAC6 promoted increased life span and autophagolysosome formation (Chen et al., 2015). Recent studies have shown that the autophagy-adapter p62 localizes with HDAC6 and controls its deacetylase activity (Yan et al., 2013). Mutations in p62 itself have also been linked to cases of ALS in multiple studies (Fecto et al., 2011; Teyssou et al., 2013). While their relationship to autophagy and protein aggregation in driving ALS pathogenesis is likely complicated, their implication serves to further bolster the importance of autophagy-related proteins in motor neuron disease.

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5. Pharmacological Modulators of Autophagy in ALS Models

Numerous studies have found autophagy to be involved in the degradation of misfolded proteins associated with many neurodegenerative diseases. Several autophagy-modulating molecules have been evaluated for their specific effects on ALS models (Cipolat Mis et al., 2016). In a cell model of TDP-43-linked ALS, the autophagy-inhibitor 3-methyladenine was found to significantly inhibit the degradation of TDP-43 species (Scotter et al., 2014). Likewise, activation of autophagy by rapamycin, decreased the severity of motor dysfunction in a mouse model of TDP-43/ALS (Wang et al., 2012) and locomotive defects in a TDP-43/ALS Drosophila model (Cheng et al., 2015). Similar observations were made in cultured neuronal cells expressing mutant ALS-causing FUS; rapamycin reduced FUS-positive SGs, as well as neurite fragmentation and cell death in neurons expressing mutant FUS under oxidative stress (Ryu et al., 2014). Also, inducing autophagy with rapamycin was shown to improve *in vivo* pathology of transgenic mice harboring a disease-causing VCP mutation (Nalbandian et al., 2015). Other autophagy modifiers have also been shown to have varying success in ALS models, including trehalose, lithium and Withaferin A (Castillo et al., 2013; Fornai et al., 2008; Patel et al., 2015b). Small molecule library screens have also yielded authopagy-modulating compounds that show promise in reducing ALS-linked cytotoxicity in primary neuronal models (Barmada et al., 2014). Thus, in conclusion, autophagy enhancement remains a promising strategy for future ALS therapeutics.

6. Discussion and Future Directions

The proposed pathways described here relating to RNP formation, autophagy, and protein aggregation in the context of ALS are admittedly hypothetical and complex. It appears likely that autophagy is critical to modulating ALS, at least in part, because it facilitates RNP granule turnover. With every newly-identified ALS-related protein, it becomes clear that many different quality-control pathways play critical disease-related roles. Understanding why certain quality-control pathways are central to any given neurodegeneration model suggests the opportunity to predict novel proteins that may be associated with various neurodegenerative diseases beyond ALS, as well as identifying new therapeutic targets. Exploring a metabolically basic function like autophagy can also have a multiplier effect because so many related neurodegenerative diseases could benefit from properly regulated autophagic processes. Thus, understanding important players in autophagic pathways will help identify therapeutic targets that could have wide applications.

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Highlights

- ALS is a fatal adult-onset motor neuron disease for which no cure is available
- Pathogenic mutations in ALS-associated genes have been shown to perturb autophagy pathways
- Many ALS-associated proteins accumulate in cytoplasmic stress granules
- Mutant ALS-causing proteins such as FUS, TDP-43, and SOD1 have been shown to be cleared by autophagy in cellular and human stem-cell derived neurons

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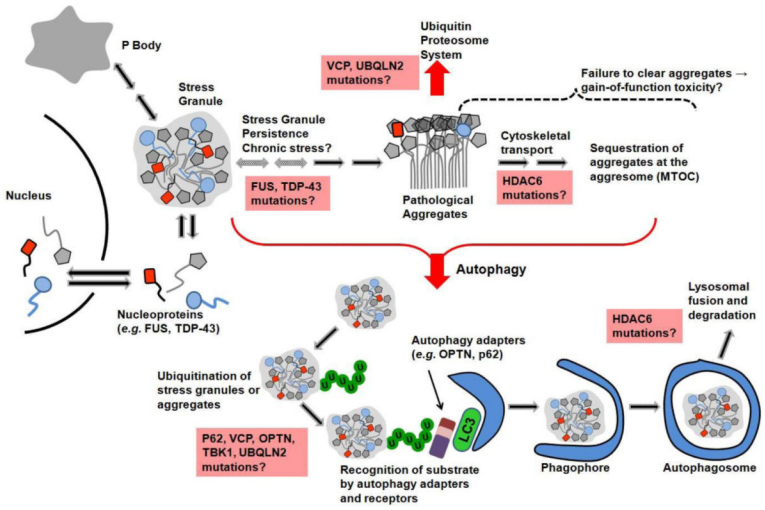

Figure 1.

Schematic showing the connection between stress granules, protein aggregation and autophagy. Together, these structures and processes are commonly dysfunctional in ALS. Boxes in pink indicate mutant genes that could be affecting the indicated process.

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Table 1

Summery of ALS linked proteins, their broad functions and potential link to the disease

Gene	Protein Function	Proposed Link to Disease	Stress Granule Component	Present in Pathological Inclusions
ANG	Neuroprotection and angiogenesis	Enhanced susceptibility to cellular stress		
ATXN2	Unknown	Persistent cytosolic aggregates Cytotoxic dipeptides,	Yes	Yes
C9ORF72	Facilitates intracellular endosomal trafficking	impaired autophagy, RNA-mediated aberrant protein sequestration		Ambiguous[2]
CHMP2B	Facilitation of endosomal trafficking	Impaired autophagy		
FUS	DNA/RNA homeostasis	Persistent cytotoxic RNP aggregates, loss of genome integrity	Yes	Yes
HDAC6	Facilitates autophagolysosome maturation	Impaired autophagy		Yes
MATR3	RNA/DNA homeostasis	Persistent cytotoxic RNP aggregates, loss of genome integrity		Yes
NEFH	Structural neurofilament	Loss of neuronal function		
OPTN	Facilitates autophagy	Impaired autophagy		Yes
P62/SQSTM1	Autophagy adapter	Impaired autophagy		Yes
PFN-1	Actin binding protein	Persistent cytotoxic RNP aggregates, impaired cytoskeletal transport	Yes	Yes
PRPH	Structural neurofilament	Loss of neuronal function		
SOD1	Reducing enzyme for superoxide radicals	Persistent cytotoxic aggregates		Yes
TBK-1	Facilitates autophagy	Impaired autophagy		Ambiguous[1]
TDP-43	DNA/RNA homeostasis	Persistent cytotoxic RNP aggregates, loss of genome integrity	Yes	Yes
UBQLN-2	Facilitates autophagy/proteasome degradation	Impaired autophagy/proteasome function		Yes
VAPB	Facilitates unfolded protein response in protein quality control	Persistent cytotoxic aggregates		Yes
VCP	Facilitates autophagy	Impaired autophagy		Yes

[1] Implicated in intracellular inclusions in viral infections mediated by viral proteins

[2] Hexanucleotide repeat encodes aggregation-prone dipeptide repeats