Common Themes in RNA Subcellular Transport, Stress Granule Formation and Abnormal Protein Aggregation

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Abstract: Control of protein synthesis and quality are critical steps to support eukaryotic cells' maintenance and survival. Two very distinctive mechanisms emerge as key checkpoints of protein synthesis regulation. The first one is the delivery of mRNA molecules, packed into ribonucleoprotein (mRNP) granules, to specific subcellular regions in order to restrict protein synthesis to distinct cytoplasmic domains. In the presence of cellular stress or injury, translation is aborted by sequestering mRNA molecules into a sub-type of RNP particles called stress granules (SGs). The second mechanism deals with the folding state and further processing of synthesized proteins. Misbehavior of a particular protein, affecting its processing, functioning, and/or conformation can cause the formation of protein inclusions called aggresomes. Interestingly, self-aggregation of abnormal proteins is one of the leading causes of neurodegenerative disorders. Recently, intracellular transport directed by microtubule-motors, has emerged as an important step in the assembly and dynamic of SGs and aggresomes. This mechanism allows for a precise temporal and spatial trafficking of RNA and protein complexes. Furthermore, it facilitates the regulation of the RNA silencing domains and targets abnormal protein aggregates for degradation. In this review we will explore the specific and common features of mRNA transport and of SG and aggresome formation, and will provide details on the role of the microtubule network and motors in their movement and dynamics.

Keywords: Abnormal protein aggregates, aggresome, cell stress, cytoskeleton, dynein, kinesin, RNP, stress granules.

1. INTRODUCTION

A fundamental aspect of gene expression control is the modulation of mRNA function in the cytoplasm. Here, the translation, stability, and subcellular localization of messenger RNAs are strictly controlled in a coordinate manner, and are often interconnected. For instance, the subcellular distribution of a given transcript is vital for proper translation initiation, and translation repression helps mRNA transport [1]. Furthermore, mRNA decay and translation repression use similar proteins [2, 3]. The existence of cytoplasmic granules containing translationally silent mRNAs has been identified in germ cells, embryos and neurons. These macromolecular aggregates are collectively called RNA granules, and the term defines a broad spectrum of entities, ranging from neuronal RNA transport granules to specific structures for the storage of maternal mRNAs. Two additional ubiquitous granules have been recently discovered, termed "Processing Bodies" (PBs) and "Stress Granules" (SGs). While SGs and PBs both share similar substrate mRNA and proteins components, and exhibit similar dynamic properties, they also contain unique components and perform independent functions. Each can exist as separate entities, but are frequently found tethered together. SGs assemble transiently under environmental stress conditions or cellular injuries that jeopardize the normal translation process (Fig. **1**). PBs are constitutive, but are enhanced during the stress response. SGs have many translation initiation components, which are excluded from PBs, which generally contain the mRNA decay machinery mRNA decay machinery [4-7]. Both SGs and PBs are related to neuronal RNA granules and germ granules, which play important roles in the localization and control of mRNAs in neurons and embryos, respectively [7-9]. More recently, other cellular structures such as abnormal protein aggregates and aggresomes have been shown to present similarities with SGs. Additionally, unrelated neurodegenerative diseases are characterized by protein misfolding and accretions, which results in the formation of insoluble aggregates, inclusion bodies and/or aggresomes [10].

Aggregated particles move at speeds exceeding those of simple diffusion and active transport by molecular motors underlies their movement and aggregation [11]. First, we will introduce how the cellular transport machinery contributes to RNA localization, through the active movement of RNA granules, and present a recently described model based on the use of cultured *Drosophila* S2 cells to study microtubule-based transport. Then, we will describe how antagonistic molecular motors govern the transient formation of SGs. Finally, we will discuss common aspects underlying the SG nucleation and abnormal proteins aggregation, and will speculate on how aggresomes and related protein aggregates potentially affect SG motility and function, and that of RNA granules in general.

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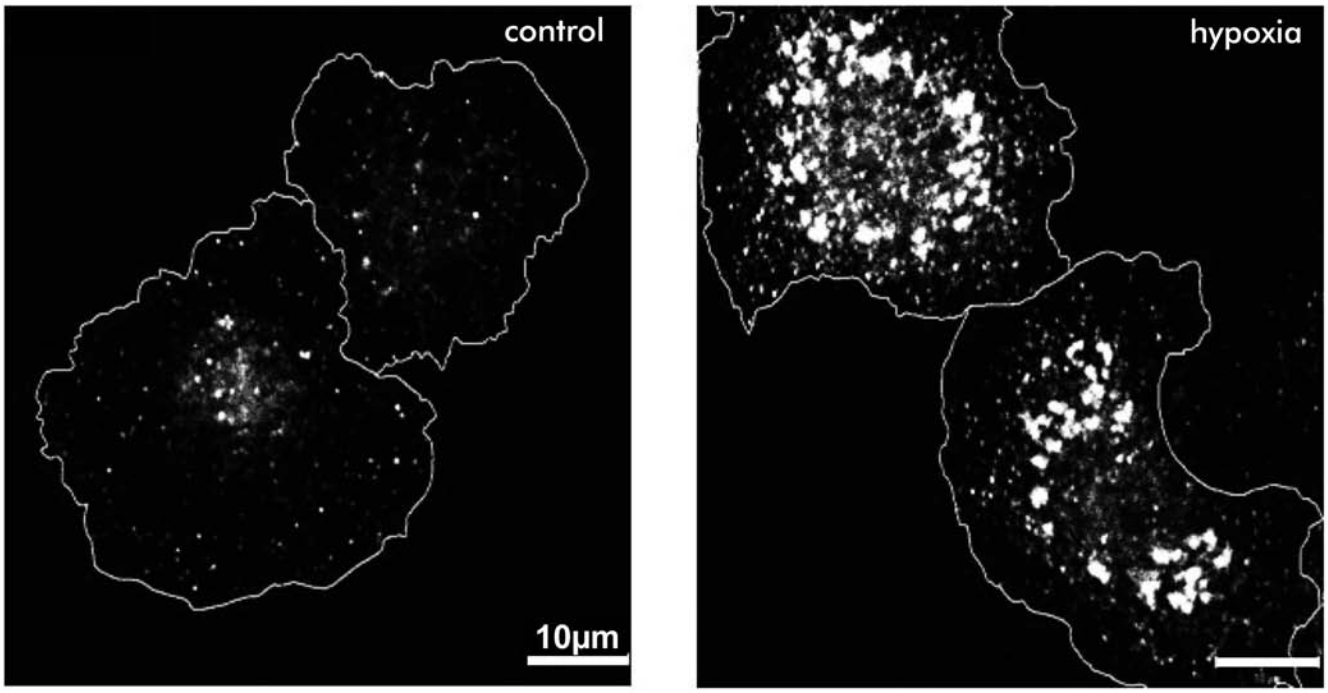

Fig. (1). SG formation in *Drosophila* S2 cells.

S2R+ cells were exposed to hypoxia as indicated [155], and SGs were identified by staining for PABP, a surrogate marker of polyadenylated RNA. The cell edge was determined by actin staining (white line).
Most PABP signal is diffuse in control cells, and a low number of small PABP-particles are also present. Upon hypoxic stress, most polyadenylated RNA concentrate in SGs (see text for details), which surround the nucleus and occupy most of the cell body (right panel).

2. MESSENGER RNA CYTOPLASMIC TRANSPORT

2.1. The Microtubule Network and Motor Proteins

Subcellular transport of a wide variety of particles and organelles is accomplished by specific molecules, termed molecular motors, which move along cytoskeletal tracks. Actin filaments and their cognate motors, the myosins, are usually implicated in short-distance movements, while long-distance transport is commonly guided by kinesin and dynein motors along microtubule tracks. The relevance of myosins and microfilaments in RNA transport is emerging in yeast cells, but the evidence of their participation in mammalian or insect cells is scarce [12]. Similarly, myosin-driven movement has not yet been implicated in SG dynamics or abnormal protein aggregation, and therefore, we will focus in this section on the microtubule-dependent subcellular transport.

Many cell types depend on a polarized microtubule cytoskeleton and the activity of specific molecular motors for proper mRNA localization. Microtubules are polymers of α and β-tubulin dimers, and can be highly dynamic. Microtubules polymerize outward from the microtubule organizing center (MTOC), and undergo then a stochastic transition, resulting in a very rapid depolymerization. In the *Drosophila* oocyte at mid-oogenesis, microtubules grow from the anterior and lateral cortex of the cell and show some degree of overall polarization.

Active transport along microtubule tracks is directed by molecular motors that use the energy of ATP hydrolysis to transport cargo along the extensive cytoskeleton network. Microtubule motors include members of the kinesin superfamily and cytoplasmic dynein. During translocation along the cytoskeleton, the head domains of these motors alternate in a hand-over-hand mechanism, whereby the ATP/ADP status of each head determines the binding affinity to the cytoskeletal track [13, 14]. Each motor is characterized by an orientation of movement. The majority of kinesins move in an anterograde fashion toward the plus end of microtubules, usually oriented toward the cell periphery, while dynein moves in a retrograde manner towards the minus end of the cytoskeletal track. The combined effect of the motors yields a bidirectional movement of the cargo molecule [15, 16]

2.2. RNA Transport

In all organisms and cell types, messenger RNAs are usually transported packed in large granular ribonucleoparticles, which are microscopically visible. The movement of these RNA granules depends on the action of specific molecular motors. The transport of *gurken* mRNA, which encodes a transforming growth factor alpha member, to the anterior-dorsal corner of the *Drosophila* oocyte depends on specific subsets of microtubules and the motor protein dynein, whose function switches to that of a static anchor when the RNA reaches its target site [17-19]. Experiments in which dynein is inactivated indicate that at the same time, *bicoid* mRNA is transported in a dynein-dependent manner to the microtubule minus ends at the anterior of the oocyte [20, 21]. Interestingly, live imaging of fluorescently tagged *bicoid* mRNA has revealed that the bulk of the RNA is local-

ized and maintained at the anterior pole by a dynamic process involving continuous active transport of *bicoid* by dynein on anterior microtubules. Similarly, mRNA tagging and live imaging have shown that kinesin-1 transports *oskar* mRNA to the posterior pole of the oocyte in a random walk on a weakly polarized cytoskeleton [22]. During early embryogenesis pair-rule transcripts, such as *hairy*, are localized apically by bidirectional transport on microtubules. *Cis*-acting localization elements on the mRNA dictate the number of motors associated with the mRNAs and thereby determine the speed, frequency, and duration of movement, and ultimately the localization of the mRNPs. Two proteins, Egalitarian and Bicaudal D (BicD), appear to function as adapters that mediate the association of dynein-heavy-chain (DHC) with the localization elements. Besides being instrumental for RNP movement, these molecules contribute to retain the mRNAs in their final destination [23]. In neurons, where the lengths along which mRNAs are transported are especially long, microtubules have also been demonstrated to play a critical role. Hirokawa and colleagues [24] demonstrated a role for the microtubule anterograde motor KIF5 (mammalian kinesin-1) in transporting many dendritically localized transcripts and further showed that alterations in the concentrations of KIF5 modulated the dendritic localization of RNA granules in neurons. Studies of Staufen-dependent dendritic mRNA transport have highlighted a fundamental role for microtubules in neuronal transport [25, 26]. Genetic, pharmacological, and siRNA-mediated inhibition of kinesin-heavy-chain (KHC) have been shown to inhibit FMRP transport to dendrites and have further indicated that FMRP interacts with at least two distinct kinesin proteins, KLC -the light chain component of KIF5- [27] and KIF3C [28]. The finding that FMRP can use two kinesin motors indicates that molecular motors may have overlapping roles in mRNA transport. Other studies have indicated that neuronal activity modulates the transport of mRNAs into dendrites [29], thus it will be informative to determine whether this modulation occurs as a result of post-translational changes in the RNA-binding proteins, in the composition of RNA granules, or modifications of microtubules or motor proteins.

2.3. *Drosophila* S2 Cells as a Model System to Study Subcellular Transport

There are still many key questions remaining on the subcellular transport of RNA particles. Which motors are involved in these processes and which cargo components do they interact with? When multiple types of motors bind a single cargo, do they collaborate or compete? How is this regulated and how does it generate an optimal speed and location? It is important to find a suitable model to study these questions. Several models have been used to study RNP granule formation and intracellular transport [30-35]. Recently, cultured *Drosophila* cell lines have become an increasingly popular model system for cell biological and functional genomic studies. Some of the most commonly used lines are *Drosophila* S2 and S2R+ cells, a sub-line of the parental S2, with higher adhesive properties [36, 37]. Both cell lines are particularly useful as they are easy to grow and maintain in the lab, they are highly susceptible to gene inhibition using RNAi, and are well suited for high-resolution light microscopic assays [38]. Consequently, they have been used extensively to study the mechanisms of intracellular trafficking.

Pioneer work from the group of Gelfand *et al* has successfully established S2 cell cultures as a model system to study microtubule dynamics, and intracellular transport of organelles and/or protein complexes [32, 39-42]. Target molecules utilized in these reports have high degree of similarity between mammalian and flies, and show similar and reproducible behaviors in different cell types. In addition, a particular set of the proteins used in transport studies, such as dFMRP and NF2/Merlin are associated with neurodegenerative and cancer related syndromes [32, 40, 43] (Fig. **2A, B**).

Drosophila S2 cells can be induced to form long processes filled with uniformly oriented and unfragmented microtubules [32] (Fig. **2A**, **B and D**). In these cells, at least 95% of microtubules are oriented with plus ends pointing toward the tips of processes as indicated by the localization of EB1, a microtubule binding protein that localizes at the plus-end of microtubules [44]. The homogenous microtubule orientation in these processes combined with the easy manipulation described above makes S2 cells a suitable model to study the bidirectional transport of any target molecule or organelle (see model in Fig. **2C, D**).

RNA granules are closely linked to SGs, and they share several protein components. In particular many of the RNA binding proteins that form RNA transport granules in neurons are recruited to SGs. Two of them, Staufen and FMRP, are particularly interesting, in light of their relevance on RNA transport, which was studied in both mammalian and *Drosophila* systems. Staufen-containing RNPs are thought to mediate mRNA transport and localization both in mammalian and invertebrate systems [9, 45-47]. FMRP or dFMRP, the *Drosophila* homolog, a translational repressor that is also involved in RNA targeting also forms granules [48]. Movement of FMRP particles has been studied using the S2 cell culture mentioned above [32]. *Drosophila* FMRP granules are transported bidirectionally along microtubules by the molecular motors kinesin-1 and cytoplasmic dynein (Fig. **2C**, **D**). Biochemical data showed that Staufen and dFMRP co-immunoprecipitate with KHC and DHC and the knock down of any of the microtubule motors causes FMRP particles to stall and accumulate in the body of S2 cells [32].

Noteworthy, Staufen and FMRP are present in SGs induced upon several stressors. Recent reports indicate that mutations in these proteins affect SG dynamics, which depends on microtubule motors, as described below [30, 49-52]. Whether the interaction of Staufen or FMRP with the transport apparatus directly affects the trafficking of mRNPs between the cytosol and SGs remains an open question.

3. STRESS GRANULES

The stress response in eukaryotic cells inhibits translation initiation and leads to the formation of stress granules as mentioned above. These contain non-translated mRNAs, translation initiation components, and many additional proteins that affect mRNA function [53]. Stress granules have been described in different cell types –neurons, glia and *Drosophila* cells, among others– and can be induced by a

wide variety of stressors such as oxidative stress, UV radiation or hypoxia (Fig. **1**). SGs affect mRNA translation and stability and have been linked to apoptosis and nuclear processes [4, 53]. Recently, SGs have been linked to protein aggregates present in neurodegenerative diseases [7]. In addition to the accumulation of abortive mRNAs translation complexes triggered upon cellular stress, SG assembly is influenced by two other important factors: a) protein and RNA modifications; and b) protein-protein interactions.

3.1. Protein and RNA Modifications Underlying SG Formation

Reversible post-translational modification of mRNP components is an effective mechanism to modulate mRNA function during a stress response, which requires a rapid and reversible adaptation. The stress response involves important post-translational modifications of several SGs components. The most common modifications are phosphorylation, acety lation, ubiquitination and O-glycosylation [54-56]. These modifications work coordinately to modulate SG dynamics. Specific SG components are modified, thus assisting in the formation and overall maintenance of these silencing *foci*. For instance, SG assembly requires the O-glycosylation of ribosomal proteins, a reversible modification that modulates protein self-aggregation [57]. Several signaling molecules have also been implicated in SG dynamics. Stress-activated JNK kinase and MKK7, as well as the small GTPase RhoA and the ROCK1 kinase are recruited to SGs upon oxidative stress [58, 59]. In addition, transient SG assembly is controlled by other stress-induced protein kinases or SPAKs, which are required for microtubule-motor activity [33, 58, 60]. On the other hand, SG disassembly is linked to ubiquitination. Depending on the stress response, SG components are tagged for degradation, providing an additional level of SG modulation. This notion is validated with the observed increase in SG formation upon knock-down of the E3-ubiquitin ligase EDD [61] and the effect of proteasome inhibitors. The deubiquitylating enzyme USP10 and its partner G3BP are polysome-associated proteins that move with untranslated RNPs to SGs [62]. Depletion of USP10 or G3BP in mammalian cells impairs SG assembly, suggesting that deubiquitination of unknown SG components facilitates SG assembly. This process is regulated by the phosphorylation of G3BP, which inhibits its interaction with USP10 and impairs SG assembly [56, 63].

In addition to protein modifications, RNA modification is also important in SG formation. A recent report links inosine-containing double-stranded RNA (I-dsRNA) to SGs [64]. These RNAs are derived from non-coding RNAs that contain inverted repeat sequences or from viral RNA in virus-infected cells. Adenosine deaminases that act on RNA (ADARs) catalyze the hydrolytic deamination of adenosine to inosine [65]. Ribosomes will decode inosine as guanosine resulting in selective editing that has the potential to alter the coding capacity of mRNAs. The most frequent targets of editing are high copy number repetitive elements [66-68]. Inosine-modified double stranded RNA bind strongly to specific SG components, and inhibit translation of specific transcripts, downregulating both endogenous and reporter gene expression in *trans* [64].

Another example of modified RNA molecules are retrotransposons. Retrotransposition has been a driving force of mammalian evolution, but they also pose an ongoing threat to the integrity of the genome [69]. LINE-1 (L1) retrotransposons encode a 40-kDa RNA-binding protein (ORF1p) and a 150-kDa protein (ORF2p) with endonuclease and reverse transcriptase activities. Recent reports revealed the localization of ORF1p in SGs together with YB-1, Argonaute (Ago2) and dFMRP among others [70-73]. Some of these proteins are also components of the RNA-induced silencing complex (RISC) that regulates the translation and decay of many mRNAs [74-76]. The targeting of ORF1p, and L1 RNP to stress granules is likely to have evolved as a mechanism for retrotransposition and the associated genetic and cellular defects. It has been shown that the cell recognizes high levels of ORF1p as a stress signal, and targets the protein and its bound RNA to SGs, reducing the number of L1 RNA molecules available for translation and nuclear import, thereby reducing retrotransposition [73].

The combined data described above indicates that although protein post-translational modifications are an invaluable mechanism to control the assembly of SG during the stress response, RNA modifications are just as important. For instance, RNA inosination presents an even earlier detection method to facilitate SG formation under unfavourable cellular conditions.

3.2. Protein Aggregation Nucleates SGs

A second aspect of stress granule assembly is the presence of protein-protein interaction domains found on numerous RNA binding proteins known to participate in self-aggregation and dimerization. Most proteins that nucleate SGs contain distinct domains that mediate homotypic aggregation, in addition to domains that bind to RNA directly (see ref [7] for a list of SG-nucleting proteins). TIA-1 and TIAR possess glutamine-rich prion-related domains (PRDs) at their carboxyl termini [77], which are essential for SG assembly. When expressed in COS-7 cells, full-length recombinant TIA-1 nucleates the assembly of *bona fide* SGs, whereas recombinant TIA-1 lacking the PRD does not. PRD from the well-characterized yeast translation termination factor Sup35p can substitute for the PRD of TIA-1 to promote SG assembly, indicating that protein self-aggregation is required for TIA-1-mediated SG assembly [77]. Several nucleating proteins possess glutamine-rich motifs (e.g. RCK, CPEB, G3BP) that might promote SG assembly by a similar mechanism. Like their prion relatives, the aggregation of TIA-1 or TIAR is regulated by molecular chaperones [77] and is blocked by HSP70 overexpression. This finding suggests that HSP70 is involved in a feedback loop that promotes SG disassembly when HSP70 levels, which are titrated by the unfolded proteins that accumulate during stress, return to normal. In this model, minimal constitutive levels of HSP70 are continuously required to prevent spontaneous TIA-1 aggregation. Stress-induced denaturation of other cytoplasmic proteins recruits both HSP70 and ATP for protein renaturation, thus diverting HSP70 away from TIA-1 and promoting TIA-1 aggregation, consequently nucleating SGs. The subsequent TIA-1 disaggregation promotes SG disassembly. It has also been reported that metabolic inhibitors such as mitochondrial poisons [78] provide an alternative mechanism

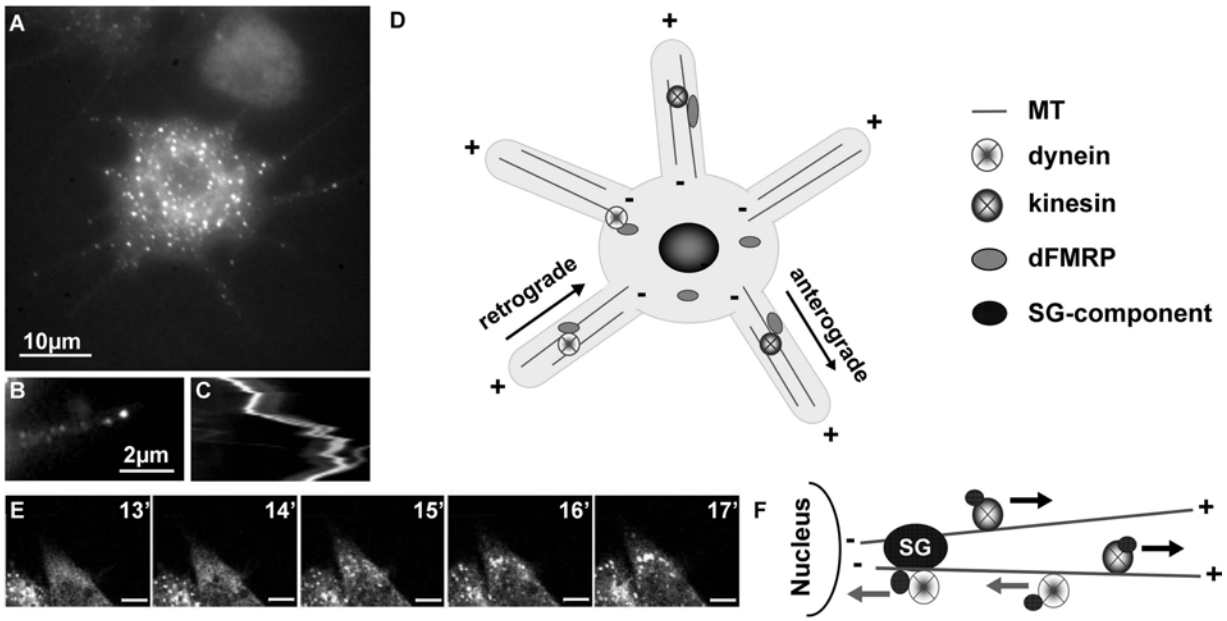

Fig. (2). Movement of RNPs in *Drosophila* and mammalian cells

A-D. FMRP particles move bidirectionally in S2 cells. *Drosophila* S2 cells expressing Fragile X protein (dFMRP) fused to GFP were treated with microfilament depolymerizing drugs to allow the formation of cell processes that contain oriented microtubules (A-white box and D). Under non-stress conditions, GFP-dFMRP forms granules that are distributed both in the cell body and processes (A and B). (C) dFMRP granules move bidirectionally along these processes as shown by kymograph analysis. (D) Microtubule (black lines) plus ends (+) are oriented outwards, and minus ends (-) are oriented towards the cell body. dFMRP particles move bidirectionally by means of anterogarde and retrograde motors under normal conditions, and collapse into SGs upon stress induction (not shown, see text for details). The *Drosophila* S2 cell culture is an ideal model to study microtubule-dependent RNP transport (see reference [32] and text for details). E-F. SGs containing PABP, FMRP and TIA1, among other RNA binding proteins, forms in *Drosophila* and mammalian cells exposed to stress insults. (E) Real time analysis of SG formation in NIH3T3 cells transiently transfected with EGFP-TIA1. Time (minutes) after oxidative stress induction is indicated. Note that TIA1 begins to form small accretions that are very dynamic and that show bidirectional movement. These cytoplasmic aggregates continue to fuse to form larger granules. *Scale bar, 10μm*. (F) Schematic representation of the intracellular transport of SG components guided by molecular motors.

to promote SG assembly. HSP70-induced conformational changes are ATP dependent [79], thus, ATP depletion prevents the HSP70-induced solubilization of TIA-1 PRD resulting in the aggregation of the PRD, thus promoting SG assembly. In addition, HSP70 was recently reported to disassemble SGs induced in response to proteasome inhibition [61]. Similarly, other mechanisms of regulated aggregation contribute to SG assembly. For instance, self-aggregation of G3BP, an important nucleator of SGs, is regulated by phosphorylation at Ser149 [80].

As we will describe in the next section, self-aggregation is key to the formation of aggresomes, thus linking the physiology of SGs and abnormal protein aggregates in a yet unknown manner.

4. AGGRESOMES

Aggresome formation is part of a highly organized and regulated process aimed to deliver abnormal polypeptides and the degradation machinery to a single locale. Under normal cellular conditions, the cell produces a relative small amount of abnormal polypeptides. Up to 30% of the ribosome-synthesized proteins are defective and are degraded shortly after their synthesis [81]. Emerging evidence suggests that aggresome formation may actually serve a protective role as a way to confine potentially toxic polypeptides when degradation is impaired. However, abnormal molecules do not always aggregate under normal, physiological conditions, despite their continued production, due in part to the existence of a cellular "quality control" mechanism, which involves a joint effect of chaperones and proteins, and the degradation of defective molecules. The system ensures proper folding of cytosolic and ER-synthesized proteins and maturation in the final compartments of the secretory pathway [82]. If refolding or maturation of the polypeptide chain and post-translational modifications is not possible, as in the case of mutated or truncated proteins, degradation follows. Most defective proteins in mammalian cells are degraded by the ubiquitin-proteasome system, usually after proteins are tagged with a polyubiquitin chain [83, 84]. This occurs in specific cytosolic compartments, which are characterized by high proteasome-dependent proteolytic activity, and are therefore termed "proteolysis centers" [85, 86]. The existence of these proteolysis centers is supported by the finding that treatment of cell lines with a selective proteasome inhibitor provoked the formation of a single aggregate. This aggregate localizes frequently in the perinucleus and contains proteasomes and ubiquitinated proteins, rather than a generalized accumulation of ubiquitinated proteins throughout the cell [86].

Several labs have attempted to elucidate the link between protein aggregation and cell dysfunction by inducing protein aggregation by means of over-expression of wild type or mutant polypeptides in cultured cells. These studies have helped to define key features of aggresome formation, including the coalescence of protein deposits at the centrosome, and the collapse of the vimentin intermediate filaments, which reorganize and form a cage surrounding the deposits. It was originally proposed that aggresome formation is a general cellular response to the accumulation of misfolded protein [10]. There is recent evidence that protein aggregates in animal models of human neurodegenerative diseases resemble aggresomes [87-90]. However, aggresome formation is not an obligate reaction against protein misfolding, and several human neurodegenerative diseases involving abnormal proteins occur without visible aggresomes, which suggests that cellular management of protein misfolding *in vivo* is a complex process with multiple pathways [91].

The deposition of protein aggregates is an important pathological feature of a large number of diseases that affect the nervous system and/or peripheral organs [92-94]. Many neurodegenerative diseases, such as Alzheimer`s, Parkinson's, Amyotrophic Lateral Sclerosis (ALS), Huntington's and several hereditary conditions have strikingly similar cellular and molecular mechanism. The clinical symptoms and neuronal death usually correlate with the accumulation of abnormal polypeptides, which form distinct accretions. Properties of these accumulations vary with the disease, and may form in different cellular compartments or extracellularly. Intracellular aggregates of abnormal protein in these pathologies are frequently identified as aggresomes or related to them. For example, Parkinson's disease patients display intracytoplasmic Lewy bodies with α-synuclein as a protein component. Patients with Alzheimer's and prion disease develop extracellular amyloid plaques enriched in β-amyloid or prion protein, respectively. The pathological hallmark of frontotemporal lobar degeneration (FTLD) and of Amyotrophic Lateral Sclerosis (ALS) is the presence of TDP-43 inclusions. Neurons suffer different environmental stresses, such as osmotic or oxidative stress, which can interfere with the folding of nascent polypeptides. The formation of SG resembling aggresomes was recently described in neurons exposed to a variety of stimuli (reviewed in [7]).

5. MOLECULAR MOTORS GOVERN THE TRANSIENT FORMATION OF SGs

In general terms, intracellular transport is a common mechanism required for the movement and assembly of all types of RNP granules and aggresomes. In this section, we will review how the subcellular transport machinery helps the transient formation of SGs. Later we will explore common features to both cytoplasmic structures during transport and assembly.

RNPs are normally dispersed in the cell helping to localize certain mRNAs to specific cellular domains, such as the distal processes of myelinating cells or neuronal synapses. Therefore, the formation of SGs in these cells involves an important component of retrograde transport. As mentioned above, mRNA transport by microtubules motors has been well established under normal cellular conditions. Recently, the interest has focused on the role of intracellular transport during the stress response and cellular injury that cause the formation of SGs. These silencing *foci* are virtually absent from the distal region of stressed cells and mostly localized at the cell body and branching points of neurons and oligodendrocytes [95]. The process of SG assembly is gradual and begins with the formation of numerous small aggregates that then coalesce into larger but fewer granules [7] (Fig. **2E**). This change in distribution is fast as mRNPs undergo a net movement of about 50-100 μm with time frames as short as 30 min. In addition, it has been reported that during recovery, and depending on the strength of the stress stimuli, oligodendrocyte processes and neuronal dendrites are refilled with mRNPs within a few minutes. Other cell types such as fibroblasts show even shorter recovery times. The rapid and coordinated assembly of SG and their subsequent dissolution suggest that SG components are actively transported by molecular motors. This idea is strengthened by the presence of dynein and kinesin motors in stress granules as observed by immunoflorescence [33] and the fact that microtubule-depolymerizing drugs such as nocodazole affect SG formation [55, 96-98]. Relevantly, SGs are not static and mRNAs are not irreversibly trapped inside these *foci*, and both mRNAs and protein components shuttle in and out of the SGs (Fig. **2F**). For example, the polyA binding protein spends around 5 seconds as part of the SG complex before it dissociates [99]. All these observations suggest that a constant delivery of mRNPs by molecular motors is required to avoid premature release of mRNPs and SG dissolution.

5.1. Dynein-Mediated Assembly of SGs

Dynein inhibition or knockdown has been shown to decrease the number of stress granules formed after induction of oxidative stress or ER stress [33]. Also, increased protease sensitivity of TIA-1 aggregates provides additional evidence for a role of dynein in stress granule formation [55, 100]. Therefore, microtubules appear to assist in SG assembly by multiple mechanisms. Since these granules are relatively non-mobile compared to PBs [78], the assembly defects caused by microtubule disruption may partially reflect impaired mRNP transport in and out of stress granules. A possible model is that microtubules provide a scaffold for translationally inactive mRNPs and translation initiation factors, thus facilitating SG formation. In this light, it is striking that the initiation factor eIF3, which is required for SG assembly in mammals [56], contains a microtubule binding domain [101]; and co-localizes and co-immunoprecipitates with microtubule proteins [55, 101]. This strongly suggests that microtubules play an important role in forming stress granules by independently concentrating untranslated mRNAs and SG-forming factors.

Formation of stress granules in fly cells is mediated by cytoplasmic dynein (DHC), while dynein heavy chain isoform 1 (DHC1) is the motor responsible for SG assembly in mammals. *Drosophila* bicaudal D as well as its mammalian homolog BicD1 helps aggregation by actin as adapters. Thus, SG aggregation shares mechanisms with RNA transport (see model in Fig. **2F**). How stress-silenced mRNPs contact specific molecular motors subunits is still unknown, but the role of Egalitarian-related molecules is anticipated.

Another open question is whether dynein subunits and/or adaptors act as anchoring factors.

5.2. Kinesin-Mediated Disassembly of SGs

SG assembly is followed by a rapid disassembly. The most likely explanation is that an anterograde motor participates in this process to balance the effect of dynein-dependent assembly. Supporting this notion, kinesin-1 is present in stress granules in COS-7, HeLa and NIH/3T3 cells, among others [33]. Moreover, knockdown of the ubiquitous kinesin heavy chain KIF5B in mammalian cells, or of the *Drosophila* homolog KHC in S2 cells significantly delays the rate of SG dissolution [33]. This indicates that the microtubule motor kinesin-1 participates in the process of SG disassembly. Metazoan kinesin-1 is a tetrameric molecule composed of two heavy chains (KHC) and two cargo-linking light chains (KLC) [102, 103]. Both KHC and KLC subunits were found as components of mRNPs suggesting that kinesin-1 dependent disassembly may utilize a cargo adaptor protein. Interestingly, Loschi *et al* [33] recently showed that depletion of the KLC1 isoform provoked a prolonged persistence of SGs similar to KHC knockdown, but no effect was observed upon RNAi against KLC2. Thus, the KLC1 isoform is likely the adaptor protein required for kinesin1-dependent SG disassembly. Thus, SG dispersion reminds kinesin-dependent RNA transport, which may or not require KLC subunits.

Double knockdown (KD) experiments in mammalian cells showed that the effect triggered by DHC1 KD is partially reverted by kinesin silencing, suggesting that kinesin is antagonizing the dynein-mediated aggregation [33]. Conversely, the effect of kinesin KD on SG dissolution is partially compensated by dynein KD, again suggesting a competition between motors [33]. How anterograde and retrograde movements of RNP components are balanced to efficiently regulate SG dynamics remains an open question. Some studies indicate that differential activity of various kinases, such as PKC isoforms, can modulate the balance between anterograde and retrograde transport [58, 104] considering the possibility that stress-activated signaling sequentially regulates dynein and kinesin at the level of motor recruitment and/or activity.

In addition to mRNA transport mechanisms (see models in Fig. (**2D**, **F**), the assembly of SGs shares many common features with the formation of aggresomes. In the next section, we will briefly review the role of subcellular transport on aggresome formation with particular emphasis on the similarities with SG assembly.

6. COMMON THEMES IN SG AND AGGRESOME FORMATION

Similarly to RNA granules and SGs, specific cellular machineries are actively involved in aggresome formation. Assembly of these cytosolic inclusions is a multi-step process in which small protein aggregates originated in the cell volume and periphery travel to the MTOC located in the vicinity of the nucleus [55, 105, 106]. Garcia-Mata and collaborators [11] have studied aggresome formation using an aggregation-prone 250-amino acid fragment of a protein called p115 fused to GFP (GFP-250). Time-lapse analysis in living cells showed that small aggregates of GFP-250 initially form at the cell periphery and then travel to the MTOC where they merge to form a single large inclusion [11]. This is comparable to SG formation visualized by real-time confocal microscopy in NIH-3T3 cells transfected with EGFP-TIA1 (Fig. **2E**, **F**). Similar to SG nucleation, protein aggregates lose their motility upon treatment with nocodazole, which disrupts microtubules [10, 11].

Dynamitin/p50 is a major subunit of the microtubule-associated dynactin complex that is required for stabilization and attachment of cargo to dynein motors [107, 108]. Overexpression of dynamitin/p50 causes the dissociation of the dynactin complex and inhibits dynein-mediated transport, thus disrupting aggresome formation and leading to the accumulation of peripherally distributed small protein aggregates [11, 109]. This response also reminds of the effect of dynamitin/p50 on SG assembly, which is dramatically impaired by overexpression of this dynactin subunit in mammalian cells [33]. Highlighting a protective role for dynein in neurodegeneration, heterozygous mutations in the gene encoding $p150^{glued}$, a subunit of dynactin that binds to dynein and microtubule, have been found in a number of patients with sporadic and familial ALS [110], and also in a family with both ALS and frontotemporal dementia (FTD) [111], although it is unclear whether these mutations represent the primary causative factor. Furthermore, work with experimental animals indicates that disruption of dynein function by mutations in the dynein heavy chain [112], or by overexpression of the dynactin subunit dynamitin/p50 [113] are sufficient to cause progressive motor neurodegeneration in mice.

Several proteins related to motor complexes have been implicated in aggresome formation. Histone deacetylase 6 (HDAC6) localizes to aggresomes formed in cell culture [114, 115] and Lewy bodies in Parkinson's disease [116]. HDAC6 has been reported to play an essential role in aggresomal protein degradation because it can bind to both polyubiquitinated proteins and dynein proteins, thereby recruiting protein cargo to dynein motors to transport misfolded proteins to aggresomes [116, 117]. siRNA-mediated depletion of HDAC6 profoundly attenuated the formation of aggresomes [116]. It is currently unclear how HDAC6 deacetylase activity relates to aggresome formation. HDAC6 may regulate aggresome formation *via* the deacetylation of one of its identified substrates (α-tubulin, Hsp90, cortactin) or of an unidentified substrate. Given the role of dynein-mediated transport in aggresome formation, the recent finding that inhibition of HDAC6 results in high levels of α-tubulin acetylation at lysine 40 and a consequent increase in motor protein binding and microtubule-dependent transport is particularly noteworthy [118, 119].

In direct connection with this, HDAC6 appears to be important for SG formation, as genetic ablation of HDAC6 significantly impairs SG formation [55]. HDAC6 is present in SGs induced by a variety of stressors, namely arsenite, UV irradiation, mitochondrial stress, or heat shock, and interacts with an important SG component, G3BP (Ras-GTPase-activating protein SH3 domain-binding protein 1), which also helps SG assembly [55]. HDAC6 enzymatic activity is important, as pharmachological inhibition abrogates

SG assembly, suggesting that deacetylation of specific proteins facilitates SG formation. In addition, these authors reported that the HDAC6 ubiquitin-binding domain is required. The link between ubiquitination of SG components, -such as G3BP-, protein deacetylation and SG formation is still unknown, but likely involves regulation of retrograde transport [55]. However, Dompierre *et al.* [118] suggest that the mechanism involving HDAC6 in aggresome formation does not involves HDAC6-mediated regulation of microtubule-dependent transport, and this is compatible with additional HDAC6 functions governing both aggresome and SG formation. Further studies aimed to determine the relevance of local tubulin acetylation and other targets by HDAC6 in aggresome and SG formation will shed light to this point.

SG aggregation depends on the presence of self-aggregating proteins and is regulated by the chaperon activity of HSP70. As mentioned above, self-aggregation is common in neurodegenerative pathologies, like Huntington's disease, where polyglutamine proteins appear to undergo a conformational change and self-aggregate forming characteristic inclusion bodies/aggregates [120]. HSP70 and related chaperones are instrumental in controlling aggresome and other abnormal protein aggregates. HSP70 and HSP90 are components of the chaperone cycle that seems to be altered in disease. It was demonstrated that there is an association between the complex HSP70/CHIP, a co-chaperone, and pathological α-synuclein detected in Lewy bodies in Parkinson`s disease [121]. Furthermore, CHIP can facilitate either lysosomal-or proteasomal-mediated routes of degradation for α-synuclein [122], further suggesting the need for a functional HSP70/CHIP system in degrading disease-related proteins. Therefore, is not surprising that inhibition of the ubiquitin-proteasome system via specific drugs induces stress granule formation [61], and affects intracellular protein aggregates in several cases [123]. This is a particular point where the molecular mechanisms underlying abnormal protein aggregation and SG formation overlap considerably. Additionally, the presence of ubiquitinated or O-glycosylated proteins is a hallmark of both stress granules and aggresomes [55, 61]. In ALS and FTLD, it was recently reported that intraneuronal aggregates contain ubiquitinated TDP-43 [124]. Also ubiquitin immunoreactivity is strongly associated with neurofibrillary tangles and Aβ plaques in the brain of Alzheimer`s patients [125]. Ohn *et al*. showed that several components, which reversibly modify proteins with O-linked N-acetylglucosamine (O-Glc-NAc) in response to stress, are required for SG and PB assembly [56].

SGs and aggresomes arise as a consequence of different cellular responses and, as expected, there are also remarkable differences between these intriguing structures. SGs are specifically induced upon cellular stress and a few hours after the stress induction these *foci* begin to dissolve synchronously. The time-course of SG assembly and dissolution observed during different stresses, namely oxidative stress, heat shock, UV radiation or endoplasmic reticulum stress is very similar [4, 7]. SGs are highly dynamic and constantly exchange mRNAs and proteins with the cytosol [7]. Conversely, aggresomes are long-lived structures difficult to dissolve or degrade. As shown by various groups, protein aggregates tend to be more static, and have extremely slow dissociation kinetics. In a few cases, there is an equilibrium between higher order protein aggregates and their oligo- and monomeric counterparts. Most protein aggregates do not dissolve, and can be eventually destroyed by an autophagic process. Large aggresomes do not dissolve nor degrade, and are inherited by daughter cells, in most cases in an asymmetric way, so that one descendent cell lineage is protected. This has been elegantly shown in both mammalian and *Drosophila* models [126]. Rujano *et al*. [126] followed the fate of aggresomes during the asymmetric cell division of fly neuroblasts in intact flies, and demonstrated that aggresomes are inherited asymmetrically, allowing the generation of neural cells free of abnormal proteins. In contrast, SG formation is impaired in mitotic cells [7].

SGs are still intriguing structures that combine properties of both, normal RNA granules, which are structures specific for mRNA regulation, and abnormal protein aggregates formed in pathological conditions. A clear example of these characteristic features is given by the biology of SG and TDP-43 aggregates. TDP-43 was recently identified as the major disease protein in the ubiquitinated inclusions characteristic of sporadic and familial forms of ALS and the most frequent pathological form of FTLD [124, 127]. TDP-43 is a 414 aminoacid protein that contains two RNA recognition motifs (RRMs) and a Gly-rich c-terminal region that allows binding to single-stranded DNA, RNA, and other proteins [128]. TDP-43 is highly conserved, widely expressed, and predominantly localized to the nucleus. However, in pathological conditions TDP-43 undergoes mislocalization and forms cytoplasmic insoluble inclusions. Also TDP-43 suffers several modifications like ubiquitination and hyperphosphorylation [129]. The mechanisms governing TDP-43 inclusion formation are poorly understood. Increasing evidence indicates that TDP-43 regulates mRNA metabolism by interacting with mRNA-binding proteins that are known to associate with RNA granules (Fig. **3A**). Zhang *et al*. [130] demonstrated that caspase-3 can mediate cleavage of TDP-43 to generate 25- and 35-kDa fragments when progranulin is down-regulated (as in familial FTLD). Moreover, the 25-kDa c-terminal fragment of caspase-cleaved TDP-43 (TDP-25) leads to the formation of toxic cytoplasmic inclusions [130, 131]. Several publications have recently showed that TDP-43 is capable to respond to an environmental insult by assembling into SGs, both in culture cells and in pathological brain tissue [132-134]. Co-localization of TDP-43 with SG proteins could result from direct binding to SG proteins such as TIA-1, TIAR or G3BP, or via indirect binding mediated by mRNA. However overexpression of TDP-25 provokes protein accumulation in the cytoplasm and more than 80% of these aggregates does not colocalize with SG markers (Fig. **3B**). Liu-Yesucevitz *et al*. [135] propose to take advantage of the reversibility of SGs to suppress or disperse inclusions containing full length TDP-43, and disperse inclusions composed of TDP-43 cleavage fragments. These results suggest that the TDP-43 might contribute to RNA-linked functions, and reversible SG formation represents a compelling example of the versatility of prion-based protein aggregation as a cellular strategy for the regulated assembly of these structures. The precise link between these structures and how their interplay contributes to neurodegeneration remains to be investigated. A tempting hypothesis to explore is whether

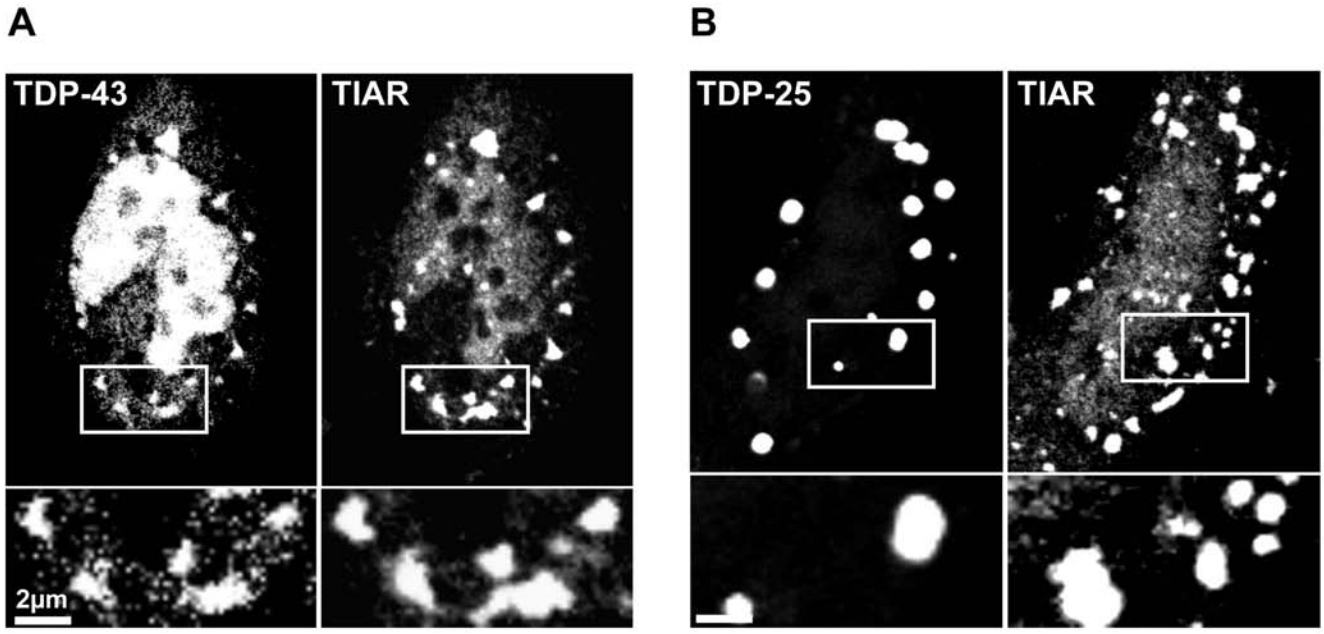

Fig. (3). Abnormal protein aggregates are related to SGs.

TDP-43, a protein that forms cytosolic aggregates in neurodegenerative conditions, or a fragment of it, termed TDP-25, also frequent in abnormal protein aggregates in neurons, are expressed in a human cell line, U2OS, fused to EGFP. Both proteins aggregate when cells are exposed to proteasome inhibitors, which halt degradation of abnormal proteins and induce SGs. (A) TDP-43 (left panel) aggregates colocalize with SGs, identified by the SG marker TIAR (right panel). (B) In contrast, most TDP-25 aggregates (left panel) do not associate with SGs (right-TIAR marker). *Scale bar, 2μm*. These observations highlight the relationship between SGs and abnormal protein aggregates associated to neurodegenerative disorders (See text for details).

abnormal protein inclusions affect SG dynamics, thus interfering with the survival response to stress.

7. CONCLUSIONS

In this review we aim to provide a comprehensive description on the main molecular features and dynamics of distinctive cytoplasmic structures, RNA transport granules, stress granules and aggresomes [7, 10]. The aggregation of proteins into aggresomes, and the assembly of RNA molecules into stress granules are protective mechanisms employed by all eukaryotic cell types. They seem to have evolved to preserve the cells from unnecessary protein synthesis or to relieve the cells from misfolded polypeptides causing protein overload [133]. The field of cell maintenance is advancing quite quickly and the subject is constantly growing. Accumulation of toxic protein aggregates as well as interference with the formation of RNA silencing *foci* are now gaining more relevance in the field of cell injury, such as in cancer therapies, infectious diseases like those involving prion-like proteins and in neurodegenerative disorders. One of the major points highlighted in this review is the importance of microtubule-dependent intracellular transport for the assembly and subcellular distribution of RNA granules, SGs and aggresomes, and the relevance of self-aggregation to the last two structures.

The work discussed here strongly supports the idea that the microtubule network and molecular motors play a substantial role in the overall dynamics of these cytoplasmic regulatory components [33]. Subcellular distribution of either normal proteins or RNA to specified regions of the cells is as important as the final destination of polyubiquitinated protein accretions or translationally abortive transcripts. The participation of motor proteins in the formation of the aforementioned structures is key to preserving cellular homeostasis. Molecular motors are multi-subunit complexes that interact with different granules or aggresome components. It is now well known that cytoplasmic dynein, together with bicaudalD1 (BicD1) are fundamental mediators of SG aggregation. Conversely, kinesin-1 heavy chain (KIF5B) and the light chain KLC1 are the mediators of SG dissolution [33], thus resembling RNA cytoplasmic transport. This is also a conserved and shared characteristic with aggresomes, known to utilize cytoplasmic dynein for assembly, though the role of cargo adaptor relies on the dynactin subunit instead of BicD. It is unclear however, which ones are the major components that facilitate the recruitment of molecular motors and subsequent transport. It is likely that several adaptor proteins participate in this role to provide specificity to an otherwise general transport mechanism. Since functional specificity is also linked to signaling path-

ways it is possible that a discrete cell signaling or a reversible post-translational modification determines the association of molecular motors with different adaptors.

In line with this, it has been recently shown that similar to aggresomes, SGs contain ubiquitinated proteins, and that SG assembly requires histone deacetylase 6 (HDAC6), a protein that binds ubiquitin and interacts with the dynein adaptor dynactin [61, 124]. It is noteworthy that HDAC6 deacetylates the α-tubulin subunit in microtubules increasing bundling and stabilizing the microtubule network [136-140]. Bundling of microtubules also correlates with an increase in cargo transport observed by a greater recruitment of kinesin-1 and dynein [118, 119, 140, 141]. This observation presents a conundrum as to whether acetylation really favors assembly of SGs or aggresomes since these processes are dependent primarily on dynein transport. Though this remains as an open question it is possible that dynein adaptor proteins are more sensitive to acetylated microtubules than kinesin adaptor proteins. This could potentially result in a shift of balance towards association of cargoes with dynein motors thus favoring assembly versus dissolution of the particles. Alternatively, the interaction with the cargo may remain intact but is the direction of movement what is modified to result in assembly. Although this hypothesis has yet to be confirmed with endogenous proteins, viral infection provides a clear example of motor preference. Viruses have developed strategies to profit from the host cellular transport machinery for efficient infection. For instance, the herpes virus HHV-8 is able to enhance microtubule acetylation by activation of RhoA and its effector protein Dia2, which accelerates the dynein dependent delivery of viral DNA to the nucleus [142]. Adenovirus act in a similar way [143].

Whether the formation of aggresomes and SGs upon stress exposure is somehow coordinated is unknown. However, there are protein components found in SGs that are known to form inclusions when aberrantly processed. The most recent example is TDP-43. Under cellular stress TDP-43 colocalizes with HuR and TIAR, two important markers of SG assembly. In addition, missense mutations in TDP-43 form aggresomes of misfolded protein. These protein inclusions have been found in autosomal dominant ALS families, suggesting that mutant TDP-43 may be a primary cause of motor neuron degeneration [144-149]. Other studies have also shown that truncation of specific polypeptides leads to the formation of intracellular aggregates and neurodegeneration [150-153, 154]. This clearly identifies a hidden link between the process of SG assembly and aggresome formation. It is therefore possible that other proteins and RNA accretions may also utilize common components, thus revealing a common denominator among all these cell survival mechanisms.

Finally, we reviewed the advantages of using a *Drosophila* cell culture system, which is a reliable and easy-to-manipulate model to study SG and aggresome formation. We envision that the use of S2 and S2R+ cells will remain as a landmark in the study of SGs and aggresomes, and would potentially reveal interesting aspects of the control of gene expression as well as proteinopathies associated with neurodegenerative diseases.

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