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STRESS GRANULES AND PROTEIN QUALITY CONTROL: TWO CONVERGING PATHWAYS IN CELL HEALTH AND DISEASE

Coordinatore:
Chiar.mo Prof. Vittorio Gallese

Tutor:
Chiar.mo Prof. Serena Carra

Co-Tutor:
Dott.ssa Laura Mediani

Dottoranda: Arianna Dorotea Carrà

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Introduction:

1. Protein homeostasis

Proteins are one of the most important structural and functional macromolecules of living organisms. They are involved in all biological processes such as catalysis of chemical reactions, transport of nutrients and oxygen, cellular signal transduction, cellular growth, as well as cell death by apoptosis or necrosis (Molecular Biology of The Cell, 2015).
In order to carry out their proper biological functions, proteins need to fold into a unique three-dimensional structure, denominated “native state”. The information for the folding of each protein into its native state is encoded in its primary sequence, which is the sequence of amino-acids held together by peptide bonds. Each amino-acid in the chain interacts with the surrounding amino-acid’s groups. These interactions may be based on polarity, hydrophobicity or charge and they induce an associated fold. The resulting fold represents the secondary structure, which can be α-helix, β-sheet and β-turn. Associations between these folds result in tertiary structures and for proteins composed by several subunits, in quaternary structures, which allow the protein to reach its native and functional state (Molecular Biology of The Cell, 2015).

Although the folding of a protein into its native state is dictated by its primary sequence and should occur spontaneously, the protein folding process is particularly challenging within the cellular environment and could fail for several reasons. Firstly, the newly synthesized proteins are in an unfolded state and this causes the exposure of hydrophobic regions into an intracellular hydrophilic context. This allows promiscuous interactions with other proteins that expose hydrophobic patches. Secondly, a substantial portion of newly made proteins need to associate with specific binding partners to achieve their native conformation. Thirdly, cells are frequently exposed to proteotoxic stress that leads to unfolding or fragmentation (wrong post translation cleavage) of mature proteins. Therefore, the preservation of the native state within the intracellular crowded environment can be extremely challenging. In addition, in mammalian cells, the concentration of several proteins is closed to supersaturation, which may also contribute to affect their solubility and favour the loss of their native state and their aggregation. These supersaturated proteins represent a metastable fraction of the proteome that has implications for human disease and ageing (Cyram et al, 2015). Besides supersaturation,

also genetic mutations can increase the propensity of a protein to misfold and aggregate, causing loss or toxic gain of function and leading to the formation of aggregates that can exert cytotoxic effects (Labbadia and Morimoto, 2015).

When a protein unfolds or misfolds it can either lose its physiological function or gain some toxic properties. Both processes can have a negative impact on cell function and viability. Thus, maintaining the proper folding of all proteins, which is referred to as protein homeostasis, is crucial for cellular and organism survival. In agreement, deficiencies in proteostasis (e.g. in case of misfolded protein aggregation) can facilitate the manifestation or progression of many neurodegenerative and muscular diseases such as Amyotrophic Lateral Sclerosis (ALS), Huntington's disease (HD), Alzheimer's disease (AD). Some of these human disorders increase in prevalence with ageing (Hartl and Hayer-Hartl 2002). This observation suggests that the pathways necessary to preserve proteostasis and suppress misfolding and aggregation events are gradually compromised with age, eventually leading to disease onset (Tuite and Melki, 2007). Indeed, these diseases are usually characterised by the deposition of specific misfolded proteins or by the development of detergent-insoluble inclusions and aggregates in cells, tissues and organs. These inclusions compromise the functionality of tissues and organs, with deleterious consequences, such as tissues' atrophy that subsequently causes organs degeneration and eventually organism death.

2. Protein Quality Control system (PQC)

To ensure adequate protein homeostasis, prokaryotic and eukaryotic cells have developed a proteostasis network that monitors and assists protein folding and maintains their correct functions (Roth and Balch 2011). The core of the proteostasis network is the Protein Quality Control system (PQC). As mentioned above, any failure of the PQC leads to the accumulation of misfolded and aggregate-prone proteins and is often associated with neurodegenerative diseases such as ALS, HD, and AD (Ciechanover and Kwon 2017).

PQC includes two degradative pathways: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system, which degrade proteins at the end of their life or damaged and aggregated-proteins. The PQC also comprises molecular chaperones and co-

chaperones that help the folding of the proteins and assist the targeting to the degradation systems of proteins that failed to reach and maintain their native state (Hartl et al, 2011).

2.1 Molecular chaperone and co-chaperones

Molecular chaperones are proteins that assist the correct folding and assembly of other proteins, without being part of the final folded structure. Some chaperones help the folding of a very restricted subset of proteins, while others interact with a large variety of polypeptides. Molecular chaperones constitute up to ca. 10% of the proteome. Molecular chaperones are able to recognize the hydrophobic regions of misfolded or unfolded proteins, including newly synthesized polypeptides. These hydrophobic regions are highly unstable in a hydrophilic cellular environment. Consequently, these regions are usually not accessible in properly folded proteins, since they are buried in the "core" of the protein (Hartl and Hayer-Hartl 2002).

The main functions of molecular chaperones are:

- assisting the folding of newly synthesized proteins;
- assisting mature proteins during the assembly/disassembly into protein complexes;
- preventing promiscuous interactions and aggregation between the interactive surface of proteins;
- assisting the targeting of misfolded proteins to the degradation machineries.

Molecular chaperones exist in two different functional states: low or high affinity state for misfolded substrates. A shift between the two states allows cycles of binding and release of the substrates, giving them repeated opportunities for renaturation or degradation. According to the mechanism of action, it is possible to divide the chaperones in two classes: ATP–independent chaperones (like small Heat Shock Proteins, sHSPs) and ATP-dependent chaperones (like Hsp100, Hsp70, Hsp90, Hsp60, and Hsp40). The chaperones have the ability to refold misfolded proteins in three ways. Firstly, ATP-independent chaperones can hold unfolded-misfolded proteins (also called clients) in an unfolded state until they spontaneously achieve their fold. Secondly, ATP-dependent chaperones can use the energy produced by the hydrolysis of ATP to convert the unfolded state into natively refoldable species (Ranford et al, 2000). Thirdly, some chaperones act as disaggregases: they use the

energy of ATP hydrolysis to solubilize aggregates into natively refolded proteins (Mosser et al, 2004; Kim et al, 2013).

Chaperones generally work in multi-chaperone complexes that also include other co-factors such as the co-chaperones. The co-chaperones are non-client-binding partners of chaperones. They help and regulate the chaperone activity, in order to ensure the maintenance of proteostasis (Mogk et al, 2003; Ehrnsperger et al, 1997). Co-chaperones can dictate the fate of a substrate bound by a chaperone; for example, the co-chaperones of the BAG family of proteins BAG1 and BAG3 promote the degradation of Hsp70-bound clients by the proteasome or autophagy systems, respectively (Hohfeld and Jentsch 1997; Gamerding et al, 2011).

When the action of chaperones and co-chaperones is not enough to revert misfolding or aggregation of proteins, the molecular chaperones can also promote the targeting of these deleterious proteins to degradation. In this way the cell protects itself from toxic and dangerous protein species and restores or maintains proteostasis, which is essential for cell functionality and viability.

2.2 Heat Shock Proteins

Molecular chaperones can be grouped in several classes with specific functions and cellular compartment localization. A large number of chaperones were discovered in 1962 by Ferruccio Ritossa, who found out that changes in the temperature leads to the synthesis of new factors, which were called Heat Shock Proteins (HSPs) (De Maio, 2011).

Although they were initially discovered to be expressed following temperature upshift, subsequent studies demonstrated that some genes encoding for HSPs are constitutively expressed in cells growing at physiological temperature. Therefore, based on their expression profile, HSPs can be classified as constitutive or inducible HSPs. The former is present in resting conditions, such as for example the heat shock cognate Hsc70/HSPA8, and the latter are only induced upon stress conditions, such as for example HSPA6. These stress conditions include, besides heat shock, oxidative stress, changes in pH and changes in nutrients (De Maio, 2011).

HSPs are named according to two different nomenclatures. The first one, based on the molecular weight of the HSPs, was published in 1996 (Tavaria et al, 1996). Afterwards, a

new nomenclature based on the systematic gene symbols that have been proposed by the HUGO Gene Nomenclature Committee (HGNC) has been adopted. This is used as the primary identifier in databases such as Entrez Gene and Ensemble (Kampinga et al, 2009). The need for a new nomenclature derived from the fact that HSPs of the same molecular weight, and therefore with the same name, in specific cases may not be equivalent in different species, bringing to confusion. For example, Drosophila melanogaster HSP22 is a mitochondrial small HSP that is different from human HSP22, whose new nomenclature is HSPB8. Nevertheless, the scientific literature still makes use of both nomenclatures, although the scientists in the field often specify both names for a given HSP, in order to avoid misunderstanding.

Thus, from an historical point of view, HSPs have been classified into six major families based on their molecular weight. Here, we will report the old and new nomenclature of these families. Hsp100/HSPH (100-110 kDa), Hsp90/HSPC (83-90 kDa), Hsp70/HSPA (66-78 kDa), Hsp60/HSPD (60 kDa), Hsp40/DNAJ (40 kDa) and small heat shock proteins (sHSPs)/HSPB (15-30 kDa) (see Table 1).

HSPs	LOCALIZATION	FUNCTION
Hsp100	*Cytosol*	*Helps stress tolerance*
Hsp90	*Cytosol and Endoplasmic reticulum (ER)*	*Role in signal transduction, refolds and maintains role in cell cycle and proliferation*
Hsp70	*Cytosol, ER and mitochondria*	*Autoregulation of the heat shock response, inter-organellar transport and antiapoptotic activity*
Hsp60	*Cytosol, ER and mitochondria*	*Role in refolding and preventing aggregation*
Hsp40	*Cytosol*	*Essential co-chaperone activity with 70*
Small Hsps	*Cytosol*	*Role in countering aggregation, heat inactivation*

Table 1. The families of heat shock proteins (HSPs) (Published in: Byung CY., Seung YP., Young-Don L., Jung NL., Yu JH., Heung KP. The Expression of Heat Shock Protein 60 kDa in Tissues and Cell Lines of Breast Cancer. J Breast Cancer, 2008).

The HSPs are constitutively transcribed in physiological conditions, but in stress conditions their expression is regulated via the so-called heat shock response (HSR) pathway. The activation of the HSR promotes the expression of the inducible forms of HSPs, which is mediated by the heat shock transcription factors (HSFs). The human genome encodes for three human HSF genes, namely HSF1, HSF2, HSF4. The HSF1 is the best characterised and it is essential for the heat shock response, it works as a sensor of cellular stress conditions (Westerheide and Morimoto, 2005). In homeostatic conditions HSF1 exists as an inactive monomer either in the cytoplasm and in the nucleus; after exposure of the cells to stress, HSF1 trimerizes and accumulates in the nucleus. Here, HSF1 undergoes a series of post-translational modifications that activates the trimer (Morimoto, 1993). Consequently, HSF1 binds with high affinity to the heat shock elements (HSEs) that are DNA sequences located in the promoter regions of the stress response genes. As a consequence, HSF1 strongly activates the transcription and the synthesis of heat shock proteins such as Hsp70 and Hsp90.

HSP expression is not only regulated by HSFs; the heat shock genes can contain other regulatory sequences that are recognized and bound by other transcription factors such as STAT1, STAT3, and NF-IL6, thereby regulating their expression (Stephanou and Latchman, 2011). Accordingly, the cooperation of these pathways leads upregulation of HSPs that is fundamental to enable cell response and adaptation to stress conditions, including heat shock and proteotoxic stress conditions, which lead to protein misfolding. In this way the cells enhance the refolding capacity upregulating HSPs is therefore crucial to restore the proteostasis (Morimoto and Santoro, 1998).
In this thesis, we mainly focus on the Hsp70 and Hsp90 chaperones.

2.3 The HSP70 system

The 70 KDa Heat Shock Protein (HSP70/HSPA) family is one of the most conserved family of proteins in evolution. In humans, this family consists of 13 members, including both stress-inducible and constitutive proteins: HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA5,

HSPA6, HSPA7, HSPA8, HSPA9, HSPA12A, HSPA12B, HSPA14, HSPA15. HSPA8 is constitutively expressed, while other members are only induced upon stress. Examples include the HSPA1A and the HSPA1B proteins, which differ only for two amino acids, and are also referred to as Hsp70, and HSPA6 (Daugaard et al, 2007).

Hsp70s can be located inside and outside the cells and based on their location they can exert different functions. Intracellular Hsp70s govern protein folding and transport across membranes (Horwich et al, 2006). It participates to a wide variety of biological processes, such as stress response, regulation of cell cycle, cell death and senescence. As consequence, Hsp70s are implicated in cancer cell growth and division (Rohde et al, 2005). Instead, extracellular Hsp70s display immunomodulatory functions and act as stimulator of the innate immune response (Mansilla et al, 2014).

Hsp70s are ATP-dependent chaperones and they can promote the folding and assembling of *de-novo* proteins that emerge from the ribosome as well as of non-native proteins. The Hsp70 system is highly versatile due to its ability to recognise a specific motif consisting in a core of five residues enriched in hydrophobic amino acids, flanked by regions enriched in positively charged amino acids. A specific portion of Hsp70, the C-terminal domain recognizes this hydrophobic motif (Rudiger et al, 1997). This motif takes place every 30–40 residues in almost all proteins. However, in the native state such regions are arranged in the internal hydrophobic core of the protein, in order to protect these regions from the hydrophilic cellular environment. In case of *de novo* protein folding or denaturation, these hydrophobic motifs are exposed to the surroundings (Mayer, 2013). When this occurs, specific intermolecular interaction among the hydrophobic surfaces of structural subunits of partially folded or unfolded intermediates takes place, promoting the aggregation of these species. Hsp70s bind the hydrophobic amino acids exposed by unfold/misfolded peptides and prevent the formation of protein aggregates and assist their acquisition of a correct folding. If this process fails, Hsp70s acting in concert with other co-factors, assist the degradation of the unfolded/misfolded proteins, thereby maintaining protein homeostasis (Mayer and Bukau, 2005).

From the structural point of view, Hsp70 is a monomeric chaperone. It consists of the N-terminal part, also called N-terminal ATP-binding domain (ABP or NBD) and the C-terminal region, named Substrate-binding domain (SBD). These two domains are connected one

each other by a highly conserved interdomain linker (figure 1). The NBD is composed of four subdomains organised into two lobes that form the ATPase binding pocket. The SBD is subdivided in two layers: a twisted β-sandwich subdomain that contains the peptide binding pocket and an α-helical subdomain, which serves as a lid to cover the substrate binding site. Another important part in the SBD is a variable region (EEVD sequence) that acts as a docking site for co-chaperones such as HOP (Hsp70-90 organizing protein), TRP-proteins (Tetratricopeptide repeat) and HIP (Hsc70 interacting protein) (Kundrat et al, 2010; Mayer and Bukau, 2005). Hsp70 activity depends on co-chaperones, such as the family of Hsp40 (called also DNAJ proteins (JDPs) in the new nomenclature) and several families of nucleotide exchange factors (NEFs).

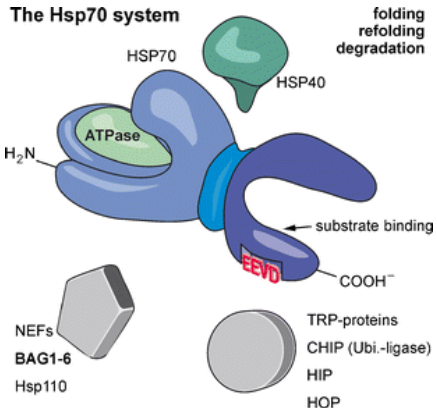

Figure 1: The Hsp70 chaperone.
The Hsp70 chaperone consists of the N-terminal domain (light blue) where there is the ATP binding site, the interdomain linker and the C-terminal domain (blue). The C-terminal presents the substrate binding pocket and a specific sequence EEVD that forms the docking site of co-chaperones (HOP, HIP, TRP-proteins). The figure shows a J-domain protein, e.g. Hsp40 and a Nucleotide Exchange Factors (NEF, BAG, Hsp100) that are important in the Hsp70 chaperone cycle (Published in: Gamerding M., Carra S., Behl C. Emerging roles of molecular chaperones and co-chaperones in selective autophagy: Focus on BAG proteins. Journal of Molecular medicine, 2011).

The initial binding of the unfolded client protein is done by the Hsp40/DNAJ-proteins, which recognize and transfer clients to Hsp70. The DNAJ protein family is defined by the presence of the J-domain that is crucial for the binding of the Hsp70-ATPase domain, in particular to the EEVD region of the protein Hsp70. Once bound, the DNAJ proteins regulate the chaperone activity of Hsp70s by stimulating the ATP hydrolysis. Hsp40/DNAJ can stabilise the interaction between Hsp70 and the substrate. Besides, DNAJ proteins carry other domains and motifs that are involved in the binding of specific clients. Based on these domains three classes of DNAJ proteins have been identified: DNAJAs, DNAJBs and DNAJCs (Kampinga and Craig, 2010).

After the recognition, DNAJ proteins target misfolded or unfolded proteins, wherein called client proteins, onto Hsp70-ATP. In particular, when Hsp70 is associated to ATP (low-affinity state for clients), the N-terminal nucleotide binding domain together with ATP induces some conformational changes in the adjacent SBD, which opens the substrate binding pocket and its helical lid (Tamadaddi and Sahi, 2016). The binding of DNAJ protein-client to Hsp70 first triggers the ATP hydrolysis (Hsp70-ADP; high affinity state for clients), then the transfer of the client and the concomitant closure of the SDB, where the clients remain bound and protected and finally DNAJ protein is released. Once the client has been refolded or marked for further processing, Hsp70 acquires a low affinity state (ATP bound), which is catalysed by NEFs. Three general classes of NEFs have been identified, based on the molecular mechanism governing the nucleotide release: pure NEF, HSPA-like NEF and BAG-family of NEF (Kampinga and Craig, 2010). All NEFs replace ADP with ATP; this turns Hsp70 to a low affinity state and leads to the clients' release. Following the release, the client may be folded, re-enter the Hsp70 cycle or be marked for degradation (figure 2).

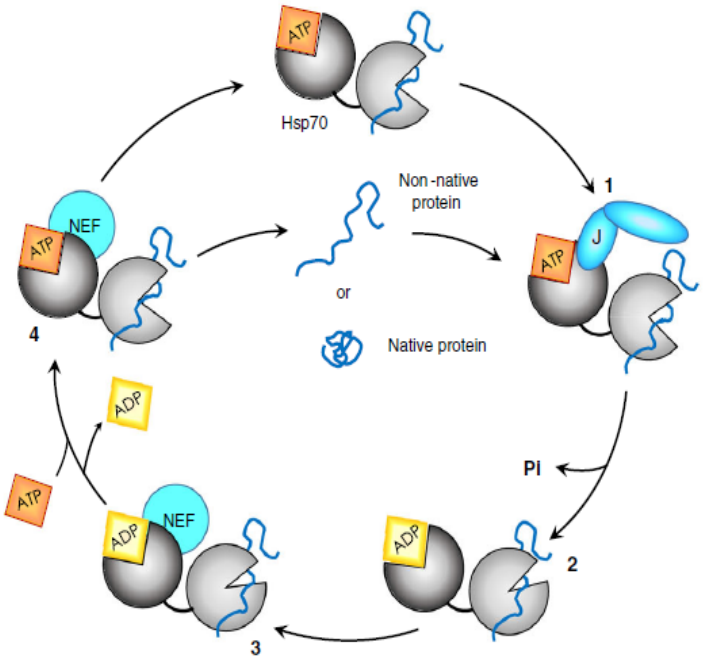

Figure 2: Hsp70 cycle.
Non-native client protein (blue line) binds Hsp70 (in grey) and DNAJ protein (light blue) stimulates ATP hydrolysis. Release of DNAJ protein and P group leads to stabilized client interaction with Hsp70 in ADP bound state. Nucleotide exchange factor (light blue) binds Hsp70 and replaces ADP with ATP, this leads to low affinity state of Hsp70 for client. Client is released either in native functional form or non-native form which can go for further rounds of binding/release (Published in Tamadaddi and Sahi. J domain independent function of J proteins. Cell Stress and Chaperones, 2016).

Recent proteomic studies demonstrated that Hsp70 is phosphorylated in mammalian cells. Using variants that mimic the phosphorylated or non-phosphorylated state of Hsp70, it has been shown that Hsp70 phosphorylation can regulate acquired thermotolerance, cell viability, as well as the ability of prion propagation (in yeast models). Hsp70 phosphorylation sites are conserved in higher eukaryotes. Beltrao and colleagues in 2012 have identified several phosphorylated sites and in particular two hot spots: the first is adjacent to the nucleotide-binding domain (at C-terminal domain) and the second is near the substrate-binding domain (at N-terminal domain) (figure 1). Considering the relevance of these sites, it is plausible that the phosphorylation state has an important impact on the Hsp70chaperone function and may regulate the binding to co-chaperones (Nitika et al, 2017). Interestingly, the C-terminal domain of Hsp70 contains an essential phosphorylation site. When the site is phosphorylated, the chaperone may bind to Hsp90 and form a ternary complex with HOP; this complex allows the client refolding. Differently, if the sites are not phosphorylated, Hsp70 binds to CHIP, which mediates the degradation of the client protein (see later) (Muller et al, 2012). Thus, based on these studies, it was concluded that the post-translational modifications are important to regulate the chaperone activity.

2.4 The HSP90 system

Another important ATP-dependent chaperone system for mammalian cells is represented by the 90-KDa Heat Shock Protein (HSP90) family. This includes four members: HSP90AA1 and HSP90AB1, which are cytoplasmic members, GRP94, which is located in the endoplasmic reticulum, TRAP1, which is located in the mitochondria and HSP90N that is a membrane-associated member. HSP90AA1 is also called Hsp90α and is heat shock inducible, while HSP90AB1, also named Hsp90β, is constitutively expressed (Hsp90s) (Ullrich et al, 1986).

From the structural point of view, Hsp90 is a homodimer. Each protomer contains three regions: the amino-terminal domain (N-domain), the middle domain (M-domain) and the carboxylic-terminal domain (C-domain) (figure 3). The N-domain has a pocket for ATP binding and also a specific co-chaperone interacting motif. It is joined to the C-domain through the M-domain. The M-domain presents a hydrophobic region and it is responsible for the client-protein recognition. The C-terminal domain (C-domain) is involved in the

dimerization process and its last five C-terminal residues (MEEVD) form a docking site for a variety of co-chaperones (Pearl and Prodromou, 2000; Rohl et al, 2013). During the Hsp90 chaperone cycle the homodimer undergoes conformational changes driven by ATP hydrolysis (figure 3). Indeed, in absence of ATP, the dimer exists in an open conformation that is also called V-conformation in which the two N-terminal domains are separated but the C-terminal is helded together. After a fast ATP binding in the N-terminal domain, inactive clients bind the M-domain causing the closure of the nucleotide-binding pocket. The ATPase activity is modulated by interactions with client proteins, chaperones and co-chaperones. Indeed, the binding of inactive client cause the ATP hydrolysis, which in turn activates the client. At the same time, Hsp90 undergoes a remodelling process to regenerate the open state, promoting the release of inorganic phosphate, active clients, chaperone and co-chaperones (Leach et al, 2012; Li and Buchner, 2013).

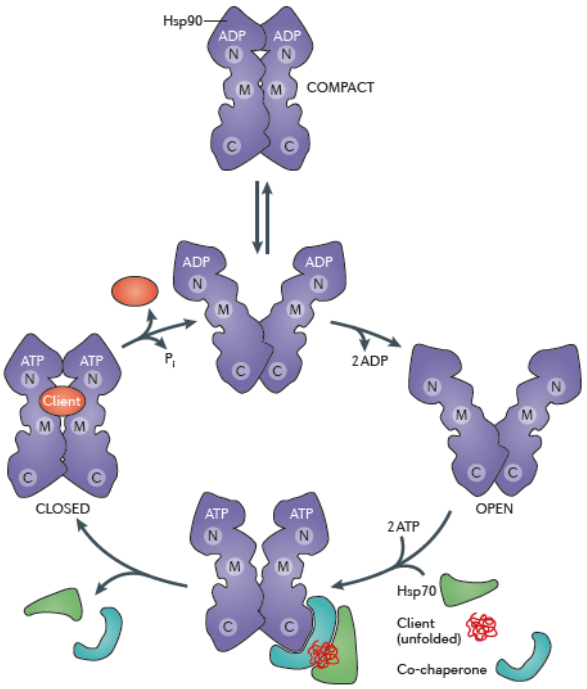

Figure 3: The Hsp90 chaperone cycle.
Hsp90 dimer are structured as follow: the amino-terminal (N), middle (M) and carboxy-terminal (C) domains. Hsp90 can take up dynamic conformations in its ADP or ATP-bound state, in which the N-terminal domains can be open or close. The Hsp90 dimer can be in open state then the release of ADP. The rapid association with ATP is linked to the interactions with co-chaperones such as, Hsp70. The chaperone and co-chaperone are associated with partial folded/unfolded client proteins, which are subsequently transfer to Hsp90. This is followed by the slow formation of a closed complex and the release of Hsp70 and co-chaperones. The folded client protein is then released, and the ATP is hydrolysed to ADP (Published in Leach et al, Fungal Hsp90: a biological transistor that tunes cellular outputs to thermal inputs. Nature reviews microbiology, 2012).

Hsp90 function is regulated by post-translation modifications as phosphorylation, nitrosylation and acetylation. Hsp90 plays an essential role in many cellular processes and it is required for the correct maturation and activation of more than 200 different substrates, which are called Hsp90 clients. Hsp90 clients include key players in the control of cell cycle, cell survival, cell growth (Falsone et al, 2005; Wu et al, 2012). Many Hsp90 clients are kinases that regulate signalling pathways, transcription factors and steroid receptors (Jackson, 2013). Importantly, in contrast to Hsp70, Hsp90 is not required for *de novo* protein folding, but it promotes and helps the final maturation of its clients (Kundrat and Regan, 2010). When the refolding of a specific Hsp90 client fails, the chaperone guides it towards degradation. The final fate of the Hsp90-bound client, folding versus degradation, is determined by the co-factors that interact with Hsp90. When Hsp90 forms a ternary complex with Hsp70 and the co-factor HOP, the client will be folded. Instead, the ternary complex that contains Hsp70, Hsp90 and the E3 ubiquitin ligase CHIP will promote the ubiquitination and subsequent proteasome-mediated degradation of the Hsp90-bound client. Of note, the folding (Hsp70-Hsp90-HOP) and degradation (Hsp70-Hsp90-CHIP) machineries do not co-exist in one single complex (Kundurat and Regan, 2010).

It is becoming increasingly clear that Hsp90 deregulation can be associated with many pathologies ranging from cancer to protein misfolding disorders and neurological diseases (Luo et al, 2010). Akt, one of the many oncogenic proteins client of Hsp90, is a player of the phosphatidylinositol 3-Kinase (PI3K)/Akt/mTOR (mammalian Target of Rapamycin) signalling pathway that controls apoptosis, cell proliferation, translation, ribosomal protein synthesis and cellular growth. Deregulation of this pathway through Akt mutation has been shown to increase cellular growth and survival, leading to cancer (Mundi et al, 2016). Considering that Akt is a Hsp90 client, it is plausible that the deregulation of Akt signalling pathway might be counteracted by modulating Hsp90. Indeed, by reducing the chaperone activity, Akt maturation is compromised and it is consequently degraded, limiting the effect of the pathway on tumor cells growth. For this reason, drugs that produce an inhibitory effect on Hsp90, such as Geldanamycin and 17-allylamino-17-demethoxygeldanamycin, are extensively used in cancer treatment (Xiao et al, 2007).

2.5 The small HSPs (sHSPs/HSPBs)

Another family of chaperones is represented by the small HSPs (sHSPs), which are characterized by a low molecular weight, ranging from 15-40 kDa and are ATP-independent, in contrast to the Hsp70 and Hsp90 systems. sHSPs are poorly conserved and phylogenetically widespread and they are present in all kingdoms of life; they have been identified and studied in species ranging from bacteria, to archea, to plants, to yeast and mammals.

The human genome encodes for 10 small heat shock proteins, which according to the new nomenclature have been named HSPBs (HSPB1-HSPB10). Mutations in human HspBs lead to particular pathologies. For instance, variants of HSPB5/αB-crystallin cause congenital cataract (Litt et al, 1998) and desmin-related myopathy (Vicart et al, 1998). Mutations in HspB1, HspB8 and HspB3 are linked to peripheral motor neuropathies (Irobi et al, 2004; Egrafov et al, 2004).

All sHSPs contain a conserved sequence of 80-100 amino acids in their C-terminal domain called the α-crystallin domain (ACD), which constitutes the signature of this family (Bakthisaran et al, 2015). The α-crystallin domain is responsible for intra- and intermolecular interactions that lead to the formation of homo- or hetero-dimers. sHSPs dimers are considered as the basic unit with chaperone activity; dimers can dynamically associate to form larger oligomers, with up to 24 subunits. Both homo and heterotypic oligomers and dimers can exist, generating a large variety of sHSP complexes that can display different affinities for a given substrate, and therefore, can potentially exert diverse functions. The sHSPs have two main types of activities (Carra and Landry, 2006).

i) sHSPs can bind denatured proteins and prevent their irreversible aggregation. sHSPs exert this function differently from ATP-dependent chaperones like Hsp70-Hsp90. For sHSPs, the cycle of chaperone-substrate association/dissociation is not ATP dependent. Instead, the association/dissociation cycle correlates to the dynamic assembly of sHSPs into oligomers of variable size. In fact, one typical feature of sHSP is their ability to form dimers or oligomers that show different binding affinities for a given denaturated substrate. Since sHSPs are APT-independent, the dissociation of the bound substrate is thought to be regulated by interaction with Hsp70s. Experimental studies demonstrated that the release of the substrates can be promoted by ATP-dependent chaperones, like Hsc70/Hsp70, and

co-factors that allow the transfer of the client hold by the sHSPs to the Hsp70 machinery which are essential for the renaturation or degradation of unfolded substrate captured by sHSP (Bryantsev et al, 2007). Therefore, sHSPs are considered as chaperone "holder", which maintain the client in a folding-competent state until the folding condition is restored. In addition, the release of the substrate can be due to a destabilization of the sHSP chaperone structure. For example, in response to signal-induced phosphorylation, oligomeric sHSPs can dissociate into dimers; this, in turn, can change the chaperone activity and affinity for the substrate that is, then, released (Shashidharamurthy et al, 2005).

ii) The second general function of the mammalian sHSPs is their ability to interact and modulate the structure and dynamics of the cytoskeleton. When exposed to stress, cells respond by drastically modifying their cytoskeletal network; for instance, the microtubule are disassembled, microfilament collapse and actin microfilament lose their organization. Concomitantly, cells rapidly induce the expression of selective sHSPs, such as HspB1, HspB5 and HspB6, which interact with intermediate filaments and actin microfilaments sustaining their stability and assembly/disassembly, especially upon proteotoxic stress (Lavoie et al, 1993; Perng et al, 1999). During the formation of microtubule network, HspB1 binds to microtubule (MT) and enhances their stability and maturation; upon MTs stabilization, HspB1 is released. Mutations in HspB1 linked to neuropathic conditions such as Charcot-Marie-Tooth seem to affect this particular function of HspB1. Mutated HspB1 binds more strongly microtubules, promoting their hyper-stabilization; this, in turn, alters microtubule dynamism. In addition, HspB1 mutants remain bound to mature MTs rather than being released after their maturation and this is also thought to contribute to increase neuronal vulnerability and disease (Almedia-Souza et al, 2011). Also, HspB5 interact with intermediate filaments, in particular with desmin that regulates sarcomere architecture in muscle cells (Hein et al, 2000). While wild-type HspB5 stabilizes desmin, mutated R120G HspB5 can increase affinity for desmin causing aberrant desmin aggregation; this, in turn, alters sarcomere structure and leads to desminopathy (Clemen et al, 2013).

Since these discoveries, many researchers have provided evidence supporting the importance of HspBs in the fitness of several types of cells and tissues under both physiological and pathological conditions. Elevated levels of sHSPs inside cells provide protection against a multitude of environmental/physical insults and stress situations.

Recently, HspBs were even suggested to play a general role in organismal lifespan (Morrow et al, 2004; Vos et al, 2016).

2.6 Co-translational folding and de novo/existing protein folding

The proteins folding is finely regulated by chaperone machineries such as Hsp70s and Hsp90s. This process can be divided in co-translational folding and post-translational folding.

Co-translational folding:

In prokaryotic and eukaryotic cells, the protein synthesis takes place in the ribosomes. Here, the folding of the nascent protein occurs simultaneously to the translational process. During translation, the N-terminal part of the nascent protein (30–40 amino acids) emerges through the polypeptide exit tunnel of the ribosome. Each time an amino acid is added to the C-terminal part of the growing peptide chain, the chain slides within the ribosomal tunnel and new amino acids are exposed. The N-terminal part is folded co-translationally, while the C-terminal part of the nascent protein is being translated. The ribosome guides protein co-translational folding through a characteristic ring in the exit tunnel. This ring is formed by important ribosomal proteins that work as a central docking site for chain-interaction factors. For instance, in this site are recruited chaperonins, chaperones and connector proteins. All together, these macromolecules participate in the folding process during translation (Frydman, 2001).

In eukaryotes, co-translational folding is essential to fold large multidomain proteins (Hartl and Hayer-Hartl, 2009) preventing non-native interdomain contacts during the protein elongation. Furthermore, the translation process is not uniform and sometimes it is slower than the folding event. The slower translation speed (≈4 amino acids per second in eukaryotes versus ≈20 amino acids per second in bacteria) facilitates the regulation of folding, targeting and other enzymatic processes (Gloge et al, 2014).

Co-translational folding is assisted by specific chaperones and co-factors. One of the first reported chaperones involved in the co-translational folding is Hsp70 (Beckmann et al, 1990). However, recent findings show that Hsp70 is involved also in *de novo* folding of proteins (Mayer and Bukau, 2005).

Post-translational folding:

Some of the newly synthesized proteins are released from the ribosome in an unfolded state. Unfolded proteins are prone to form aggregates. To avoid this phenomenon, *de novo* proteins (also call newly synthesized proteins) are subjected to post-translational folding. This process is strictly controlled by chaperones, associated with co-chaperones and adaptor proteins, which guide the acquisition of a correct fold. The Hsp70s family is a central component involved in folding of *de novo* proteins and refolding of stressed-denaturated proteins (figure 2) (Weibezahn et al, 2005). However, proteins that cannot be completely folded by Hsp70s can be transferred to a subset of other proteins or chaperones, such as Hsp90s. Indeed, specific subset of unfolded/misfolded proteins need an additional folding performed by the Hsp90 machinery and several co-factors that help the interaction between the Hsp70 and Hsp90 systems.

One of these co-factors is HOP (Hsp70-Hsp90 Organizing Protein), a modular protein that has tetratricopeptide repeat (TPR) domains. The TPR1 domain recognizes the carboxy-terminal region of Hsp70, while, at the same time, the TPR2 domain binds the carboxy-terminal pocket of Hsp90 (figure 4) (Shiying and Smith, 1998). This simultaneous binding of HOP to both Hsp70 and Hsp90 allows the transfer of the substrates from Hsp70 to Hsp90. This brings to the complete folding and maturation of client proteins via the Hsp90 cycle (figure 3) (Kundrat and Regan, 2010; Kravats et al,2018).

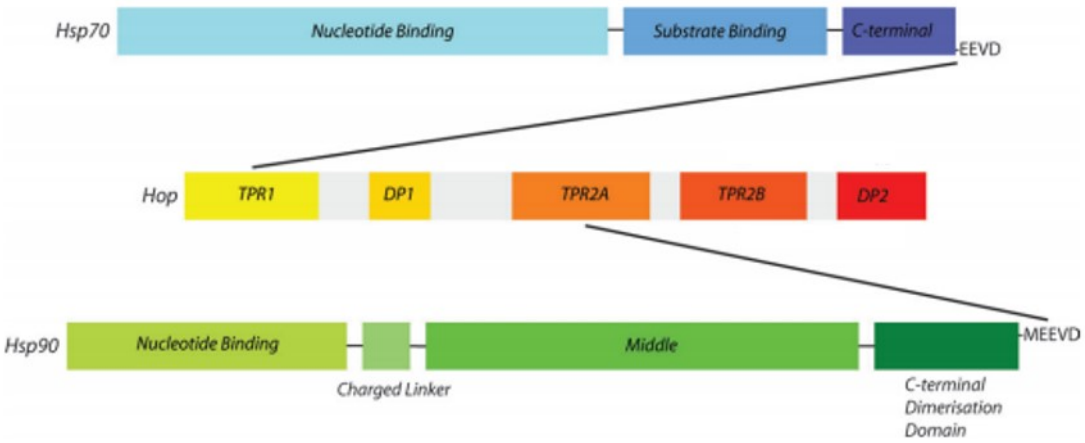

Figure 4 Overview of the domains of Hsp70, Hsp90 and Hop.
Domains of Hsp70, Hop and Hsp90 a solid line shows the interactions between Hop and Hsp70/Hsp90 (Published in Travers S. and Fares M. Functional coevolutionary networks of the Hsp70-Hop-Hsp90 system revealed through computational analyses. Mol.Biol.Evol, 2007).

2.7 Degradation systems: Ubiquitin proteasome system and autophagy

In mammalian cells, the protein quality control system is responsible for the recognition and clearance of misfolded proteins. This comprises molecular chaperones and degradation systems that recognize misfolded proteins and promote their refolding into their native state, whenever possible. If the repair fails, the misfolded protein can be escorted by chaperones towards the two main degradative pathways that exist in mammalian cells: the ubiquitin proteasome system and the autophagy machinery, which represent the final defence against proteotoxicity (Ciechanover, 2005).

The Ubiquitin proteasome system (UPS):

The UPS is responsible for degradation of the majority of abnormal proteins in the cell. First of all, the UPS adds ubiquitin (Ub), or ubiquitin-chains, to abnormal proteins in order to mark the molecules to degraded. The linkage of ubiquitin chains to the target protein is strictly regulated and it is mediated by an enzymatic cascade. The process is catalysed by Ubiquitin activating enzyme (E1), Ubiquitin conjugating enzyme (E2), Ubiquitin ligase (E3), and sometimes Ubiquitin chain elongation factor (E4) (figure 5). Subsequently, polyubiquitinated proteins (polyUb-proteins) are degraded through the proteasome complex.

The proteasome complex (26S proteasome) is an ATP driven multi subunit complex that consists of a proteolytic core (the 20S proteasome) and two regulatory subunits (the 19S proteasomes). The 19S complex recognizes the substrate and unfolds it using ATP. It, then, removes the ubiquitin tags, which are released as a pool of free ubiquitin molecules that can be recycled for further uses. At this stage, the deubiquitinated substrate enters in the 20S proteasome, where it undergoes the proteolytic degradation (figure 5) (Furlow, 2011). The digested peptides are finally released into the cytoplasm, where they are rapidly digested into constituent amino acid by cytosolic endo-aminopeptidases. In this way, the amino acid can be reused to synthesize new proteins (Rogel et al, 2010).

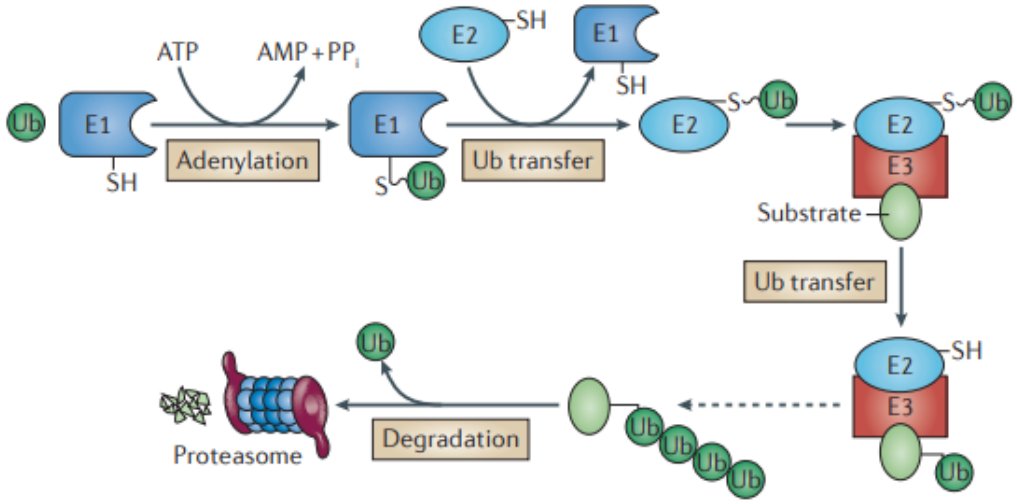

Figure 5 Schematic representation of Ubiquitin Proteasomal System:
To initiate ubiquitylation, E1 ubiquitin-activating enzyme adenylates the C-terminal of ubiquitin using ATP. This promotes the activation of ubiquitin that forms an intermediate with E1. The E1–ubiquitin intermediate transfers ubiquitin to an E2 ubiquitin-conjugating enzyme. Then, ubiquitin is transferred from E2 to the protein target (or substrate) with the assistance of E3. When the client protein is modified with ubiquitin, additional ubiquitin can bind to generate polyubiquitin chains. The ubiquitin chains, in particular Ub on Lys48, are signals for degradation by proteasomes. The 26S proteasome is formed by two 19S subunits (dark red) that flanks the 20S subunit (blue) on both sides. The 19S regulatory subunit binds the poly ubiquitinated protein and it uses the ATP molecules to unfold the protein and insert it into 20S core. Inside the 20S core, the breakdown of the protein into smaller peptides (about seven to nine amino acids in length) takes place and the ubiquitin molecules are removed. At the end of the process ubiquitin molecules and small peptides are released and can be recycled by the cell (Published in: Furlow J. Proteasomes and protein conjugation across domains of life. Nature review microbiology, 2011).

The UPS requires the concomitant engagement of chaperones and co-chaperones to improve the protein disposal, for example the Hsp70-BAG1-CHIP complex.

The BAG (Bcl-2 associated athanogene) family is a multifunctional group of proteins. The human genome encodes for 6 BAG genes: BAG1, BAG2, BAG3, BAG4, BAG5 and BAG6. All these proteins contain a conserved domain, called the BAG domain, which allow them to bind to Bcl2 and Hsc70/Hsp70 (figure 1). All BAG proteins function as NEFs that regulate the fate of Hsc70/Hsp70-bound substrates and induce the hydrolysis of ATP to ADP in order to release the client protein from the chaperone (figure 2). BAG1 and BAG3 are the most studied members of the BAG family. In particular, BAG1 is involved in the UPS-mediated degradation pathway, while BAG3 is involved in the autophagy-mediated degradation pathway (see later).

BAG1 also contains a ubiquitin-like domain that promotes the binding to the proteasome, thereby favouring the degradation of the Hsc70/Hsp70 bound substrates by the proteasome (figure 6A). To promote the UPS-mediated degradation of the bound substrate another player, the CHIP protein, is required.

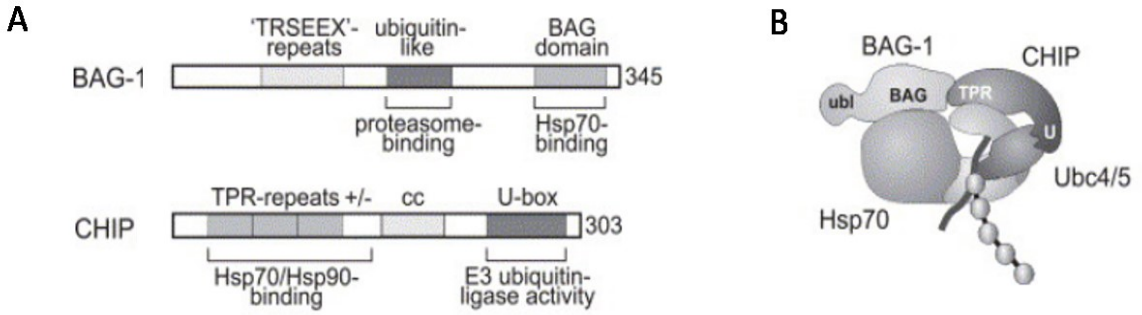

Figure 6: Structural domain of BAG1 and CHIP and Hsp70-BAG1-CHIP complex.
A) BAG1 presents in the central part a ubiquitin like domain to bind the proteasome and in the carboxyl-terminus is characterised by the BAG domain to link at ATPase site of chaperone Hsp70. CHIP possesses at amino terminus a TPR-repeats domain to bind chaperones (Hsp70, Hsp90) and U-box at its carboxyl terminus. U-box is necessary for binding to E2 ubiquitin-conjugating enzymes of the Ubc4/5 family and acts as an E3 ubiquitin ligase during the ubiquitination of known chaperone substrates. B) BAG1 through binding to the ATPase domain of Hsp70 and direct contacts with CHIP, promotes the ubiquitination on client protein. The ubiquitin-like domain (ubl) of BAG-1 remains exposed in the formed complex and serves as a proteasomal sorting signal to determine the final degradation. (Published in Esser C. et al, Cooperation of molecular chaperones with the ubiquitin/proteasome system. Biochimica et Biophysica Acta-Molecular Cell Research, 2004).

CHIP (C-terminus of Hsc70-interacting protein) is a cofactor characterised by a tetratricopeptide repeat (TRP) domain that binds chaperones such as Hsp70 (figure 6A). CHIP has a U-box that is structurally related to RING-finger domains found in many ubiquitin ligases, which suggests that CHIP may work as a ubiquitin conjugator (figure 6A). In agreement, experimental evidence demonstrated that CHIP cooperates directly with E2 and acts as E3 ubiquitin ligase and add Ub or Ub-chains to the BAG1-Hsc70/Hsp70-bound substrates (Demand et al, 2001). The cooperation between BAG1, CHIP and Hsc70/Hsp70 promote the proteasomal degradation of the ubiquitinated clients (figure 6B) (Demand et al, 2001; Alberti et al, 2002).

Besides the BAG1-CHIP-Hsp70 complex, other chaperones promote the degradation of misfolded proteins by the UPS system. Another example is represented by the DNAJ/Hsp70 complexes, as well as by the cooperation between the small heat shock protein Hsp27/HSPB1 and Hsp70.

Autophagy:

Autophagy is an intracellular conserved degradation system that maintains cellular nutrient levels and intracellular organelle homeostasis. Moreover, it is essential to remove potentially harmful aggregated proteins that are not degraded by the UPS. Autophagy can be distinguished in macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Yang and Klionsky, 2009) (figure 7).

Macroautophagy and microautophagy are conserved by yeast to humans instead, chaperone-mediated autophagy has been found only in mammals.

The macroautophagy can degrade different types of cargoes, including soluble molecules, defective organelles and aberrant protein aggregates. Macroautophagy, often referred to as autophagy, is a complex process that consists of several sequential steps: sequestration of the cargo inside a newly formed double membrane–bound vesicle, called the autophagic vacuole or autophagosome, transport of the vesicle towards the lysosomes and fusion with lysosomes, degradation of the cargo and recycling of digested products (figure 7) (Mizushima et al, 2008).

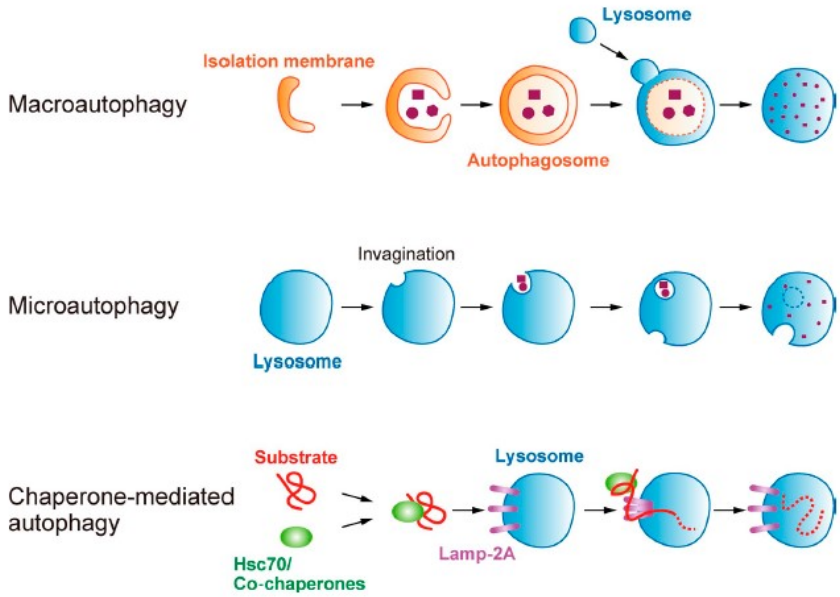

Figure 7: autophagy pathways.
Three distinct models of autophagy: macroautophagy, microautophagy and chaperone mediated autophagy (Published in Okamoto K. Organellophagy: Eliminating cellular building blocks via selective autophagy. Journal of Cell Biology, 2014).

For a long time macroautophagy was considered a non-selective bulk degradation process, but recent evidence underlines that it can be a selective degradation process. For example,

several types of autophagy have been described that selectively degrades mitochondria (mitophagy), lipid droplets (lipophagy), peroxisomes (pexophagy), ribosomes (ribophagy) or protein aggregates (aggrephagy) (Yang and Klionsky, 2009). Macroautophagy specificity in selective protein degradation is achieved by selective cargo receptors/adaptors that are ubiquitin-like modifiers localized on autophagosome membranes (Cohen-Kaplan et al, 2016). Cargo receptors have specialized domains that mediate the interaction between target proteins and the autophagy machinery. One of these receptors is p62/SQSTM1 (sequestosome 1), which contains a ubiquitin associated domain that binds ubiquitinated protein substrates and a LC3-interacting region that brings the cargo to the autophagosomes (Kirkin et al, 2009; Lamark et al, 2009). Furthermore, p62 can interact with the HSPB8-BAG3-Hsp70 chaperone complex.

BAG3 is characterised by two important domains: one is the BAG domain that binds the ATPase site of Hsp70, the other is the IPV (Ile-Pro-Val motif) domain that is responsible for the interaction with small Heat Shock Proteins such as HspB8 and HspB6 (Fuchs et al, 2009). So, IPV and BAG domains allow BAG3 to assemble a multichaperone complex with HspB8 and Hsp70 (Gamerdinger et al, 2011). BAG3 can also interact with the macroautophagy receptor p62/SQSTM1. BAG3 recruits Hsp70 to p62/SQSTM1 and induces the nucleotide exchange to promote the transfer of cargo proteins from Hsp70 to the autophagic p62/SQSTM1 receptor. Finally, p62/SQSTM1 controls the autophagic degradation of specific proteins by binding simultaneously the autophagosome protein LC3 and the polyUb-proteins (Waters et al, 2009). The cooperation between these co-chaperones, chaperones and autophagic receptors ensures the targeting of an Hsp70-bound substrate to the autophagy machinery, rather than to the UPS system (as it occur for the BAG1-Hsp70-CHIP complex described before).

Besides p62/SQSTM1, other autophagic receptors have been described. Examples are NBR1, which similarly to p62 promotes the autophagic degradation of ubiquitinated substrates (Kirkin et al, 2009), Nix, which functions as a receptor protein for mitophagy (Novak et al, 2010), and HDAC6, which regulates the ubiquitin-selective quality-control autophagy (Lee et al, 2010).

In contrast to macroautophagy, during the microautophagy a small portion of the cytoplasm, such as proteins and organelles, is engulfed via direct invagination or

protrusion, or septation of the lysosomal membrane (Okamoto, 2014). Similarly, to macroautophagy, the microautophagy can be a non-specific process or it can degrade specific cargo organelles such as, mitochondria, peroxisomes and nuclear membrane (Reggiori et al, 2013).

Concerning chaperone-mediated autophagy, it degrades only specific proteins and not organelles. CMA is a highly selective mechanism for the degradation of cytosolic molecules that contain in their amino acid sequence a specific pentapeptide motif such as KFERQ or KFERQ-like motif (Arias and Cuervo, 2011). These motifs are present approximately on the 30% of cytosolic proteins and are recognized by a group of chaperones and co-chaperones, the predominant member is Hsc70. These factors target the substrate to the lysosome surface by binding to the lysosome-associated membrane protein type-2A (LAMP-2A), which acts as specific CMA receptor. Once bound to LAMP-2A, the substrate is denaturated in a process that is mediated by the membrane associated Hsc70 and in complex with Bag1, hop, and Hsp40 (Orenstein and Cuervo, 2010). The unfolded substrate is then translocated across the lysosomal membrane into the lysosome lumen where it is digested by lysosomal proteases (figure 7) (Wang et al, 2013).

3. Ribosome Quality Control system (RQC)

As previously mentioned, molecular chaperones assist protein folding both co-translationally and post-translationally. In particular, the co-translational quality control system senses the folding state of nascent polypeptides during their translation. When this PQC system detects intrinsic defects, it cooperates with the degradation systems to clear defective and potentially toxic species. In addition, cells evolved the ribosome-associated quality control system (RQC), which senses defects at the level of the mRNAs, rather than of the nascent polypeptides. In fact, damaged mRNAs alert the RQC on plausible defects, thus resulting in the translational stalling (ribosome stalling) and consequent targeting of mRNA and nascent polypeptide to degradation.

3.1 The RQC pathway

RQC senses the state of translation rather than the state of the nascent chain. It is activated when the translation stalls, for example because of genetic mutations, truncated mRNAs, damaged mRNAs, mRNAs that form a secondary structure such as a hairpin loop. It is also activated in case of insufficient amounts of amino acids or charged-tRNA and when the poly(A) tail is translated as a consequence of the lack of the STOP codon on a given mRNA (Brandman and Hegde, 2016). Under all these conditions, the 80S ribosome stalls and protein factors such as Pelota-Hbs1 and Dom34 are recruited. These factors are able to recognise the stalled ribosome and promote the dissociation of two ribosomal subunits from the ribosome nascent-chains 80S complex and target the aberrant transcript to degradation via cytoplasmic exosome (Van Hoof et al, 2002; Doma and Parker, 2006).

After the ribosomal dissociation, the 60S subunit contains an aberrant nascent-chains bound to tRNA that must be degraded to avoid the accumulation of harmful products. These are detected by RQC, a complex composed of the nuclear export mediator factor (NEMF), E3 ubiquitin ligase Listerin (Ltn1), Rqc1, the AAA+ ATPase valosin containing protein (VCP) and its co-factors (figure 8) (Brandman and Hegde, 2016; Defenouillere et al, 2013).

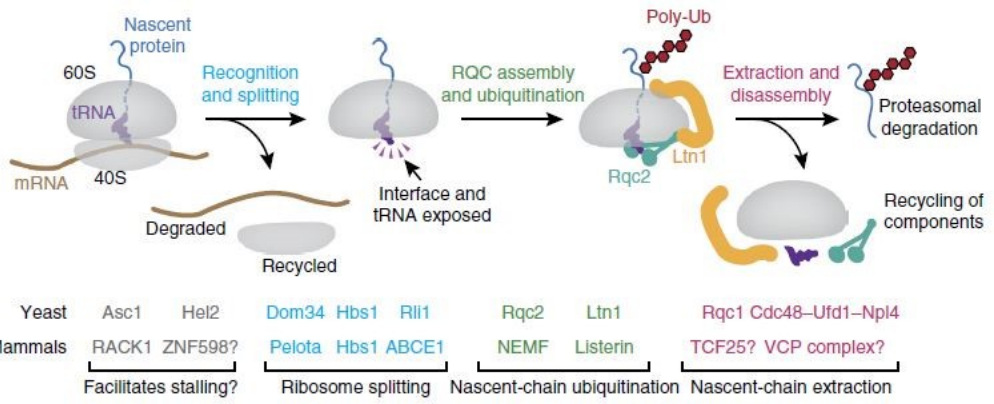

Figure 8: Primary steps and factors of ribosome-associated quality control.
The principal steps are the recognition and splitting of the ribosome, then the assembly of RQC complex on the 60S ribosomal subunit. RQC ubiquitinates the aberrant-nascent-chains that will be extract and degraded meanwhile the other components will be recycled. (Published in Brandman O. and Hegde R. Ribosome-associated protein quality control. Nature structural & molecular biology, 2016).

Upon removal of the small subunit from a ribosome-nascent chain complex, the interface of the nascent chain–60S complex is exposed (Shao et al, 2015). In this way, the peptidyl-tRNA and interface of the 60S subunit are subjected to the surrounding environment and these features promote the recruitment of the first RQC player, NEMF (figure 8).

NEMF consists of N-terminal and C-terminal lobes connected by a middle region. The N and C-terminal lobes are positioned at the ribosomal P-site of 60S. Here, NEMF has a double function: it prevents the reassociation of 60S with the 40S subunits and it concomitantly senses the tRNA in the P-site of the stalled 60S (Figure 9) (Lyumkis et al, 2014).

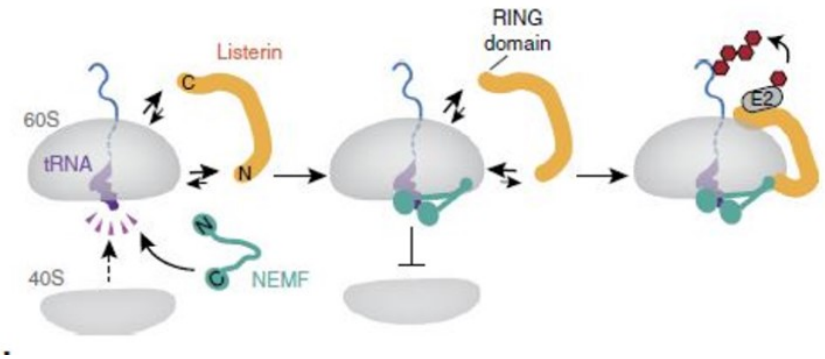

Figure 9: NEMF- Listerin interaction on 60S–peptidyl-tRNA complexes.
NEMF (represented in green in the scheme) binding the tRNA and 60S interface effectively precludes 40S reassociation through C and N domains. Its middle part facilitates the binding of Listerin (represented in yellow). Listerin's RING domain is positioned near the polypeptide exit tunnel, thus facilitating nascent-chain ubiquitination. (Published in Brandman O. and Hegde R. Ribosome-associated protein quality control. Nature structural & molecular biology, 2016).

NEMF coordinates the recruitment on the A-site of specific alanyl and threonyl-tRNAs, so it promotes the supplement bond at C-terminal of the nascent-chain (that is on the P-site) of a not templated alanine-threonine tail, called CAT-tail. CAT-tail is essential to activate the heat shock factor 1 in response to translational stress (Defenouillere et al, 2016). An additional function of NEMF is the recruitment of Listerin. In particular, the middle region of NEMF is precisely where the N terminus of Listerin interacts with the 60S, so NEMF stabilises Listerin.

Listerin is an E3 ubiquitin ligase and presents, on the C-terminal part, the ring ligase domain that is very close to the E-site and it is essential to add ubiquitin on the N-terminal of the nascent chains (Brandman and Hegde, 2016). Subsequently, NEMF interacts with 60S and acts as an adaptor to facilitate the binding of VCP and its co-factors, Npl4 and Ufd1, on 60S (Defenouillere et al, 2016). This allows the VCP complex to extract the polyubiquitinated

nascent-chains from the stalled 60S (figure 8). Thus, the ubiquitinated nascent-chains can be targeted to the proteasome degradation or to autophagic clearance to avoid the aggregation of these harmful peptidyl-chains.

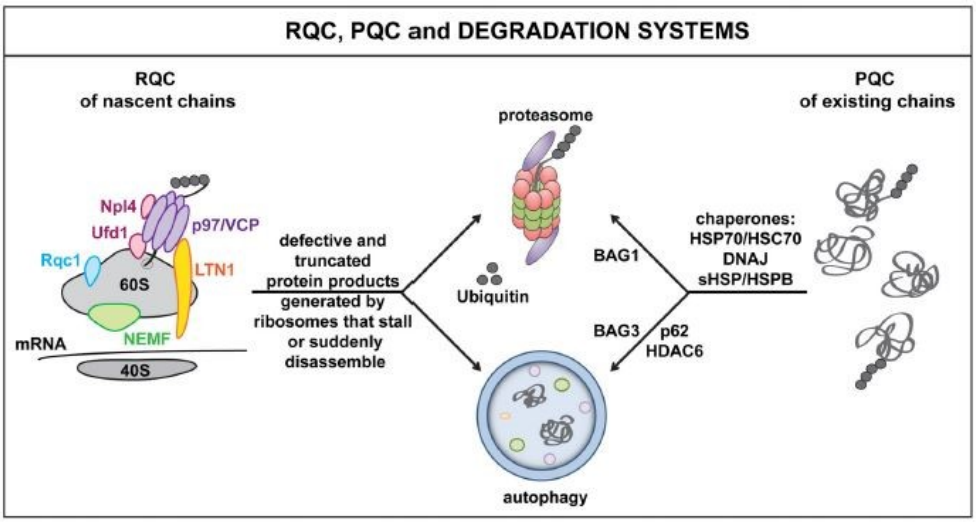

Figure 10: ribosome quality control (RQC), protein quality control (PQC) and cellular degradation systems. Defects at the level of the mRNA activate RQC mechanisms. This leads to the selective elimination of aberrant nascent polypeptides via the proteasome and/or autophagy. Key players of the RQC system include, e.g., LTN1, nuclear export mediator factor (NEMF), valosin containing protein (VCP) and co-factors Npl4 or Ufd1. Similarly, when misfolded proteins accumulate, for example because of proteotoxic stress, molecular chaperones, such as DNAJs and HSPBs, act in concert with HSP70 and specific co-chaperones to target misfolded proteins to the proteasome (BAG1) or autophagy (BAG3) for disposal. Autophagy receptors and adaptor molecules, such as p62 and HDAC6, participate in targeting (poly)ubiquitinated substrates to autophagosomes (Published in: Alberti S., Mateju D., Mediani L., Carra S. Granulostasis: Protein Quality Control of RNP Granules. Frontiers in molecular neuroscience 2017).

There are similarities between PQC and RQC, as both complexes recognise aberrant products and act to prevent their damage effects. The former, recognises unfolded-misfolded proteins during their synthesis or their translation while, the latter recognises mRNA alterations during the translation that cause aberrant proteins synthesis. Both complexes work to maintain protein homeostasis.

4. Protein aggregation: is it always bad?

4.1. Intrinsic disordered regions and proteins with prion like domains.

In 1960 Christian Anfinsen proposed a well-known paradigm: each protein has a unique three-dimensional-structure determined by its amino acid sequence and the structure is directly associated to protein function.

In the last couple of decades, the scenario has changed because of the discovery of a new class of proteins that constitutes more than 40% of the eukaryotic proteome. These proteins contain intrinsically disordered regions (IDR) that do not fold spontaneously into stable, well-defined tertiary structures (Oates et al, 2013).

IDRs can adopt different conformational states, such as unstructured, partially-structured or disordered and switch from one state to another (Babu, 2016). These regions are well characterised and present a peculiar amino acid composition. Indeed, they have a low content of hydrophobic residues and high content in polar or charged amino acids (arginine, glutamate, lysine, glutamine, serine) (Malinovska et al, 2013). The combination of low hydrophobicity and high charge are the determinant factors for the disorder. The first one causes the loss of co-operative folding that normally is mediated by hydrophobic amino acids, while the second leads to electrostatic repulsion that results from equally charged residues present inside the sites (Banani et al, 2017; Babu, 2016). For these reasons, IDRs lack the rigid secondary and tertiary structure and acquire a flexible conformational state. Yet, these intrinsically disordered proteins exert biological functions and the presence of these IDRs provides several advantages and new functions to proteins. The continuous fluctuation between different conformational states leads to several consequences: (a) exposure of short linear motifs that mediate interactions with a large number of other proteins; (b) the flexibility of IDRs facilitate exposure for proteins that through post-translation modifications regulate the protein expression-repression (for example the post-translation modification such as acetylation on the histone proteins tails, is associated with activation of gene expression); (c) IDRs adopt different conformations when bound to different proteins (figure 11). These properties of intrinsic disordered regions make them well suited to perform signalling and regulatory functions (Babu, 2016).

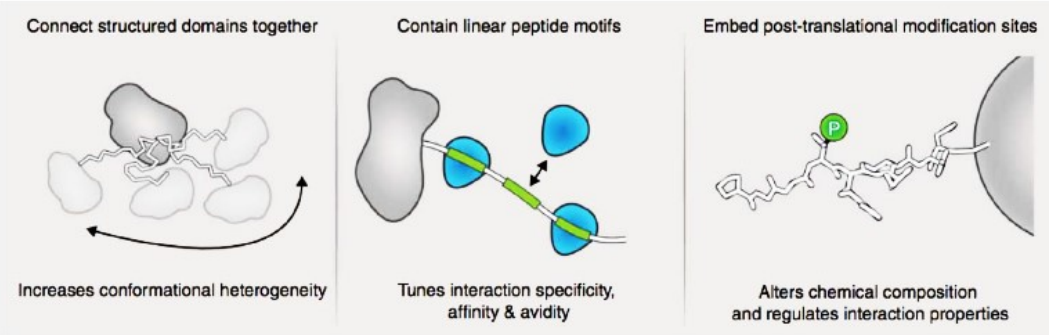

Figure 11: Function mediated by IDRs.
IDRs can link structured domains, where their flexibility permits the protein to adopt multiple conformations; linear motifs within IDRs mediate protein interactions; posttranslational modification of residues within IDRs permits encoding and decoding of information (Published in: Babu M. The contribution of intrinsically disordered regions to protein function, cellular complexity, and human Disease Biochemical Society Transactions, 2016).

A specific class of intrinsically disordered proteins is characterized by the presence of low complexity sequences (LCS). These contain single amino acid repeats or amino acid short motifs. These low complexity proteins can be enriched in glycine and uncharged polar amino acid such as glutamine, asparagine, tyrosine and serine (Alberti et al, 2009). These sequences are also known as prion-like domains (PrLDs) and the proteins containing these domains are referred to as prion-like proteins, due to several analogies with yeast prions (Malinovska et al, 2013). Prions are yeast proteins that contain domains enriched for asparagine and glutamine (N and Q) and form fibrillar, beta-sheet enriched assemblies that are called amyloid. Prions are transmissible states of proteins and have been implicated in a large variety of functions in yeast, spanning from propagation of heritable phenotypes, to gene regulation and disease (An et al, 2016). Due to their amino acid composition, mammalian prion-like proteins are highly aggregation-prone and tend to self-associate forming assemblies via a process known as liquid-liquid phase separations (LLPS).

4.2. Liquid-Liquid phase separation (LLPS): a process that organizes the cytoplasm and nucleoplasm

Liquid–liquid phase separation (LLPS) is a special form of organization relevant for living organisms. LLPS is commonly compared to the separation of oil drops in water. LLPS consists of a phase transition, in which a homogenous solution of molecules (oil and water)

spontaneously separates into two portions: the first is a dense phase (oil phase) and it is enriched for these molecules and the second is depleted (water) (Figure 12) (Alberti, 2017). This phenomenon is guided by supersaturation conditions, since the concentration of proteins with IDRs is one of the critical factors that can trigger LLPS (Ciryam et al, 2012).

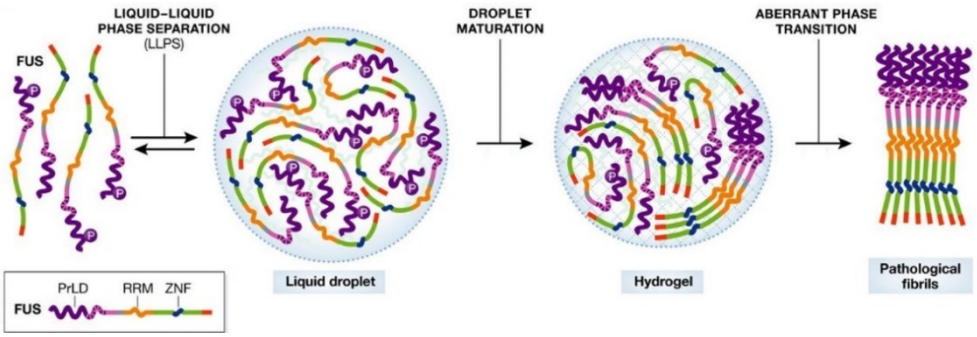

Figure 12: Liquid Phase separation transition.
In the figure is represented FUS protein as example of protein with PrLDs. The uncharged, polar PrLD enables FUS to undergo liquid–liquid phase separation (LLPS). In these condensed liquid droplets, the FUS PrLD initially remains intrinsically disordered, but the high local concentration of PrLDs promotes their nucleation into cross-β fibrils. As stable cross-β fibrils begin to dominate the droplet there is a shift from the liquid state to a hydrogel state and ultimately to a solid aggregate comprised of pathological fibrils (Published in: Shorter J. Liquidizing FUS via prion-like domain phosphorylation, Embo journal, 2018).

LLPS drives the formation of molecular condensates inside the cell, which have several biological functions. In the simplest case, the condensates increase the local concentration of resident chemical species, such as substrates and enzymes. Thus, the condensation of these species can accelerate and regulate the specificity of enzymatic reactions. For example, assuming that an enzyme has two alternative substrates and only one is sequestrated inside the condensate, the consequence of condensate formation would be that only one reaction can occur and is facilitated by LLPS. Consequently, only one of the two possible pathways that depends on the activity of these two different putative enzymes will be activated. Alternatively, the condensate can concentrate molecules that act in one particular biological pathway and exclude components of another one (Banani et al, 2017). Condensates can also sequester molecules from the environment. By sequestering molecules, they can prevent interactions, thereby inhibiting their activity outside the structure. In this way, via LLPS cells can control substrate flux through several pathways (Banani et al, 2017). An important advantage of phase-separated structure is that all functions can be activated or repressed very rapidly by simply controlling the formation

and dissolution of these condensates, whose composition can differ greatly, producing different population of liquid-like structure (Alberti and Carra, 2018). Example of condensates or membrane-less organelles (MLOs) that form in mammalian cells are: cytoplasmic stress granules, processing bodies, Balbiani body, nuclear PML bodies, speckles and paraspeckles, nucleoli (Figure 13) (Banani et al, 2017).

Based on the fact that the condensates are already present in resting cells or are induced upon a specific change in the environmental condition (or in response to a specific signal), we can distinguish constitutive condensates and inducible condensates. Examples of condensates that can be assembled in cells in resting conditions are P-bodies, nucleoli and PML bodies. Stress granules are, instead, examples of condensates that are induced upon specific stress conditions. Ultimately, some condensates can be induced during specific developmental stages, such as the Balbiani body which is found in the early ooplasm (Lei and Spradling, 2016). In both cases, their composition, size and number are highly dynamic and influenced by the cellular state, as well as by environmental insults. Therefore, also the number, composition and size/shape of constitutively present condensates can largely differ in response to stress, like in the case of PML nuclear bodies or speckles. These condensates or MLOs can exert many different functions and are required to enable cell response and adaptation to stressors.

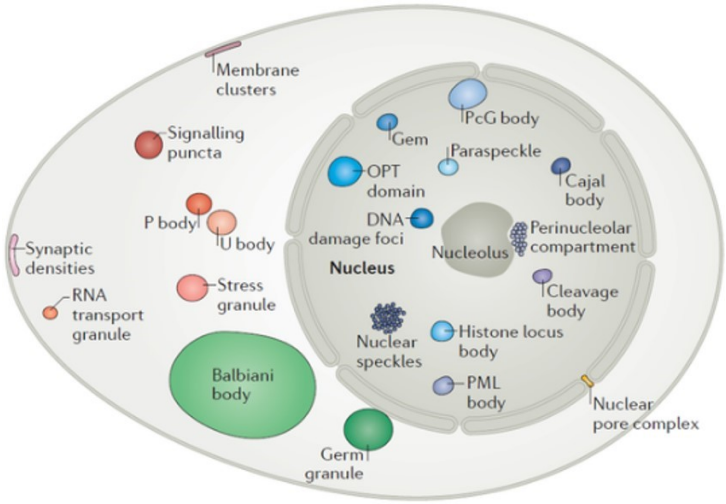

Figure 13: Membraneless organelles in eukaryotic cells.

Schematic representation of MLO in the nucleus, cytoplasm. Some of these MLO are present only in specific cells as Balbani body that is in the germ cell (Published in: Banani et al, Biomolecular condensate: organizers of cellular biochemistry. Molecular Cell Biology 2017).

Thus, the discovery of the principle governing LLPS enabled the understanding of how cells can compartmentalize their content without the need of membranes. In fact, in contrast to endoplasmic reticulum, Golgi or mitochondria, all these MLOs are not defined by surrounding lipid bilayer membranes. Thus, not only the principle of formation of these organelles (membrane-less and membrane-bound) are different; but also, the principle governing the exchange of molecules with the surrounding environment is different. In the case of membrane-bound organelles, lipid membranes not only separate the interior and exterior part of the organelle, but they also regulate the transport of biological molecules (inside or outside) with specific transport machinery.

Recent studies started to uncover the principle governing the formation and dissolution of MLOs, as well as their biological functions. Often, MLOs contain nucleic acid such as RNA and/or DNA and it has been shown that the interaction between proteins, which can directly bind to RNA (or DNA) and nucleic acid is important to regulate MLO assembly. However, the assembly of MLOs is driven by many cooperative interactions and it involves conventional interaction between mRNA-RNA-binding proteins and protein-protein, but also unconventional interaction between mRNA-mRNA and hetero-homotypic intrinsic disordered regions (Lin et al, 2015). Two important events for promoting the LLPS it has been identified: the first is the increase of the local concentration of RNA and RNA-binding proteins, the second is the post translational modification (PTM) inside IDRs (Bah and Forman-Kay, 2016). Indeed, the PTMs modulate the conformational properties and functions of IDPs. For example, the phosphorylation provides novel possibilities for intra-intermolecular electrostatic interactions allowing the phosphorylated residues to form strong salt bridges with adjacent amino-acid of other IDPs. These events promote the liquid-liquid phase separation and the subsequent formation of a subclass of MLO also called ribonucleoprotein (RNP) granules, because they contain both proteins and RNA. Examples of RNPs are cytoplasmic processing bodies, also called P-bodies, and stress granules. P-bodies contain untranslated mRNAs and proteins, such as enzyme involved in mRNA turnover. They are present in physiological condition and are in equilibrium with the polysomes, which are a complex of ribosomes that synthesize polypeptide chains from mRNAs (Parker and Sheth, 2007). In acute stress conditions, when polysomes disassemble and release untranslated mRNA molecules, the number of P-bodies increases (Decker and

Parker, 2012). From P-bodies, mRNAs can either be degraded or returned to translation (Brengues et al, 2005). P-bodies (PBs) are closely correlated with stress granules (SGs). This PhD thesis focuses on the factors that regulate the dissolution of SGs, which have received increased attention in the past decade due to their implication in neurodegenerative diseases.

4.3. Stress granules

Exposure to environmental stress (e.g. oxidative stress, heat shock and UV irradiation) alters normal cell function and homeostasis. Cells have developed specific responses to reallocate resources during stress and restore cellular homeostasis in order to survive (Anderson and Kedersha, 2009). One of these responses consist in the integrated stress response (ISR). This is a specific intracellular signalling pathway that couples stress detection with the activation of kinase proteins in order to control and adjust the translation rate in response to stress conditions, optimizing the cellular response to stress and regulating cell fate under diverse types of stress (Sidrauski et al, 2015).

Indeed, upon stress conditions via ISR the translation is inhibited and the polysome disassemble leading to the release of mRNAs, RNA-binding proteins (RBPs), translation factors, premature polypeptides (or defective ribosomal products), and ribosomal subunits. Many RBPs contain low complexity domains that promote their self-assembly via LLPS. Thus, after polysome disassembly, the release of mRNAs and RBPs leads to their local enrichment and promotes the formation, via LLPS, of cytoplasmic foci of 0.1-2.0μm size, known as SGs (Molliex et al, 2015). These structures have heterogeneous composition and contain translationally silent mRNAs, RBPs (e.g. TIA, G3BP, TDP43, FUS), the small but not the large ribosomal subunits, early initiation factors (such as eIF4E, eIF3, eIF4A, eIF4G) and many other signalling proteins (Anderson and Kedersha, 2009) (figure 14).

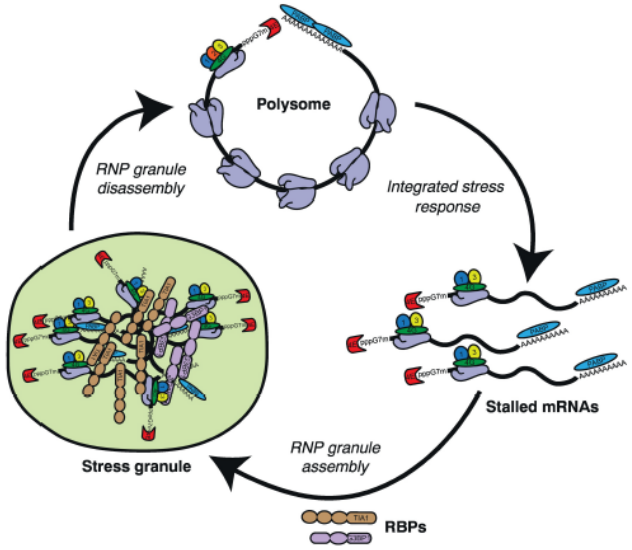

Figure 14: Schematic model of stress granules formation.
Upon stress, the activation of stress response leads to the polysome disassembly. mRNAs together with initiation factors (that in the picture are signed as 1: eIF1; 2: eIF2;3: eIF3; 4G: eIF4G; 4E: eIF4E), RNA-binding proteins such as TIA1 and G3BP, the small ribosome subunit and some proteins (not shown) are sequestered into stress granules (SGs). After stress, SGs are disassembled and mRNAs can be translated (Published in: Alberti and Carra, quality control of membraneless organelle, Journal of molecular biology, 2018).

SGs assembly is induced by two different pathways: one pathway depends on the phosphorylation of Eukaryotic Initiation Factor 2 (eIF2), while the second one is independent on eIF2. In the first pathway, the stress induces the activation of specific kinases, such as the protein kinase RNA-like endoplasmic reticulum kinase (PERK) or protein kinase R (PKR). These kinases, in turn, phosphorylate the regulatory serine residue of eIF2α. eIF2α is a component of eIF2 (formed by α, β, γ subunits) -GTP-tRNAi^{Met} ternary complex. This complex is part of the 43S complex (40S ribosomal subunit, eIF3, and ternary complex) that is recruited on mRNA by the eIF4F complex (eIF4E, eIF4G, and eIF4A) during cap-dependent translation. The tRNAi^{Met} recognizes the start codon on mRNA prior to the joining of small and large ribosomal subunits. The joining of the two ribosomal subunits determines the release of initiation factors via GTP hydrolysis of the ternary complex (Dang et al, 2006). So, the eIF2α phosphorylation alters the functionality of the ternary complex leading to accumulation of stalled translation preinitiation complexes. This promotes polysome disassembly and the release of all components (Anderson and Kedersha, 2009). This is followed by the assembly of mRNAs and stalled preinitiation complexes into SGs.
The second pathway that regulate SG formation is eIF2α independent. In this mechanism the translation factors eIF4A or eIF4G that recruit the ternary complex on mRNA, are

inactivated; this, in turn, results in polysome disassembly and SG assembly (Mokas et al, 2009).

SGs are highly dynamic and characterized by rapid trafficking of RNA-proteins in and outwards. This is important to regulate key signalling pathway in response to stress, influencing the cell fate. For example, molecules that regulate signalling pathways involved in cell metabolism and apoptosis, such as mTOR (mammalian target of rapamycin) complex 1 (mTORC1) and astrin are sequestered inside SGs. Targeting of mTORC1 to SGs occurs via the interaction with astrin that it is a negative regulator of the mTORC1 complex. When entrapped inside SGs, mTORC1 cannot be available for interaction with target proteins. Thus, during stress conditions, the SG-dependent sequestration of mTORC1 prevents the hyper-activation of this pathway and inhibits apoptosis (Mahboubi and Stochaj, 2017; Thedieck et al, 2013; Wippich et al, 2013). Once the stress subsides, SGs disassemble releasing signalling molecules, such as mTORC1, but also ribosomal subunits, mRNAs and RBPs. Polysome reassembles and the mTOR pathway is reactivated, promoting translation restoration. Being dynamic, SGs are suitable for storing and protecting mRNA from degradation. However, SGs can also regulate the fate of mRNAs directing their degradation through P-bodies (Anderson and Kedersha, 2009). Initially, the role of SGs was mainly associated with the protection of mRNAs during stress; however, in the last years, SGs have emerged being a key player for human health. Many neurodegenerative diseases are associated to alteration in the SGs composition and behaviour that trigger the pathological state (Mahboubi and Stochaj, 2017).

4.3.1 Stress granules and age-related neurodegenerative diseases

In the last years, several genetic and experimental evidence supports the interpretation that deregulated SG dynamics and RNA metabolism are linked to neurodegenerative diseases (NDs) such as Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD), Alzheimer`s disease (AD) and inclusion body myopathy (IBM) (Maziuk et al, 2017; Vance et al, 2009; Neumann et al, 2006). The central pathological hallmark of these diseases is the presence of cytosolic and nuclear inclusions in affected tissues and cells

(neuronal or muscle cells). These cytosolic inclusions contain several SG-resident proteins, such as TDP-43, FUS, TIA-1 and translation initiation factors. Importantly, several pathogenic mutations have been documented in genes encoding for SG components comprising mutations in TARDBP, FUS, HNRNPA1. Many of these mutations promote the conversion of these proteins from a dynamic liquid-like state into amyloid-like fibrils, *in vitro*; in cells, the expression of these mutated proteins leads to the formation of SGs that show a defect in their disassembly kinetic, with important consequences for mRNA metabolism (Lin et al, 2015; Molliex et al, 2015; Patel et al, 2015). Many of these RBPs are mainly nuclear in healthy cells and in response to stress translocate into the cytoplasm, where they are recruited inside SGs. Several mutations of these RBPs decrease their intranuclear localization and favour their accumulation inside the cytoplasm, which is considered to be a triggering event that mediates their toxicity. In fact, mutations in the nuclear localization sequence (NLS) of FUS alter its nuclear-cytoplasmic shuttling, promoting the formation of cytotoxic aggregates that have been related to ALS and FTD (Lagier-Tourenne et al, 2010). Similarly, ALS-linked truncated forms of TDP-43 accumulate in the cytoplasm, where they form aggregates. In addition to mutations in the genes encoding for TDP-43, FUS, hnRNPA1, the pathogenic expansions of a hexanucleotide repeat in C9orf72 gene has been reported as the most common cause of ALS and FTD (Renton et al, 2011). C9orf72 causes the generation of toxic dipeptide repeat (DPR) via a non-canonical form of translation referred to as RAN translation. C9orf72-DPRs can interact with the low complexity domains of RBPs and accumulate inside SGs. This, in turn, is associated with a decrease of SG dynamic behaviour (Lee et al, 2016). Further, cells that express C9orf72 repeat form foci of RNA that sequester important RBPs involved in RNA metabolism (Lee et al, 2016), alterating SG dynamics and contributing to dysregulate RNA metabolism. The observation that DPRs alter SG dynamics in a similar manner as mutated or dysfunctional SG-components suggest that the deregulation of SGs is a central pathogenic event in many age-related neurodegenerative diseases, such as ALS and FTD. The fact that SGs lose their dynamic properties either due to the accumulation of aggregation-prone DPRs inside SGs, or to the increased aggregation propensity of mutated RPBs support the notion that deregulated protein aggregation may be detrimental for the functionality of SGs. Altogether, these observations obtained from genetic and biochemical experiments have supported the hypothesis that: 1) the pathological aggregates that accumulate in the

patients affected by ALS, AD, FTD, may result from the maturation of SGs from a liquid-like state into an aggregated-like state; 2) the decrease in the functionality of SGs and RBPs are crucial players in ALS and FTD pathogenesis.

4.3.2. Molecular chaperones regulate stress granule disassembly: granulostasis

SGs are very dynamic condensates, formed upon stress exposure, which disassemble within few hours after stress relief. When cells are subjected to prolonged stress conditions, SGs are still able to disassemble, as observed for prolonged treatment with the proteasome inhibitor MG132 (Marzoui et al, 2007; Ganassi et al, 2016). SG dissolution is fundamental for cell functionality enabling the recycling of SG components. This avoids the *de novo* synthesis of SGs components and it allows the restoration of translation and reactivation of signalling pathways. Recent evidences from independent research groups show that, in order to orchestrate SG dissolution, cells need a functional PQC system (Ganassi et al, 2016; Mateju et al, 2017; Mogk and Bukau, 2004). One of the master regulators of SG dissolution is Hsp70, which, as described before, binds misfolded proteins and specifically targets them to the degradative pathways with the help of chaperones and co-chaperones like BAG1 and BAG3. Proteotoxic stress conditions lead to the formation of SGs and, in addition, to the accumulation of misfolded proteins. One of the main sources of misfolded proteins in mammalian cells are defective ribosomal products (DRiPs), which results from the misincorporation of amino acids, premature translation initiation, damaged mRNA or DNA mutations (Schubert et al, 2000). DRiPs and misfolded proteins can accumulate inside SGs, affecting their biochemical properties: SGs that accumulate misfolded proteins show decreased dynamism, delayed dissolution and are defined as aberrant SGs (Figure 15) (Ganassi et al, 2016).

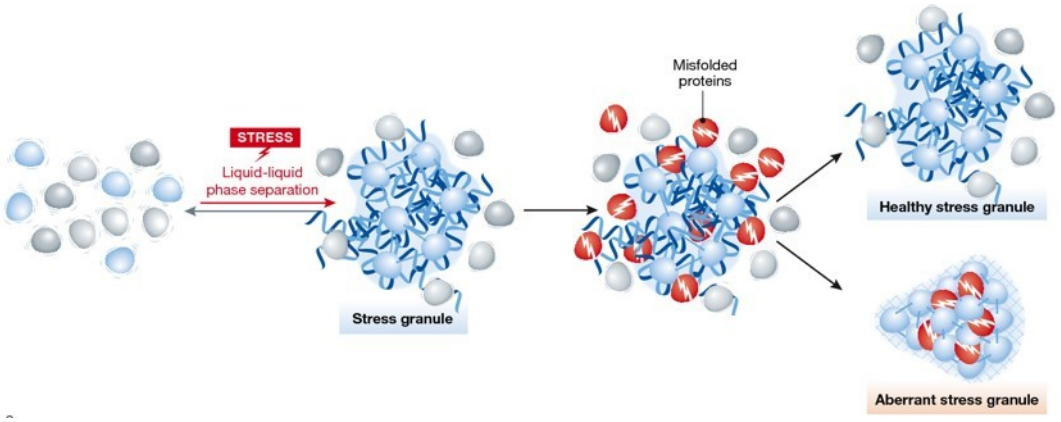

Figure 15: Physiological and Aberrant.
Stress causes the mRNA, RBPs and other macromolecules to phase separate into liquid-like stress granules. The scheme shows that the healthy or physiologic SGs do not contain misfolded proteins and are liquid-like, differently the aberrant one contains misfolded proteins that alters the composition and the chemical-physical properties (Published in: Siwach and Kaganovich, Getting stress out of stressed-out stress granules, Embo Journal 2017).

In addition, misfolded protein-enriched SGs become resistant to RNase digestion (Ganassi et al, 2016). This is in line with recent findings, where it has been reported that SGs are composed of an RNase-sensitive and dynamic shell and a protein-rich RNase resistant core (Jain et al, 2016). These data support the interpretation that DRiPs and misfolded proteins can lead to the formation of an RNase-resistant core inside SGs and promote their irreversible aggregation.

SGs contain proteins involved in the maintenance of protein homeostasis, like chaperones, small heat shock proteins, and components of the UPS and autophagy systems. This suggests an interplay between PQC factors and SGs.

Recently, it has been found that the HspB8-BAG3-Hsp70 complex (Figure 17) (Ganassi et al, 2016) limits the accumulation of DRiPs inside SGs promoting their targeting to degradative systems. This, in turn, helps maintaining the liquid-like properties of SGs, which disassemble upon stress relief (Ganassi et al, 2016). In detail, HspB8, which is a chaperone holdase, can be recruited inside SGs and it avoids promiscuous interaction between DRiPs and SG components. In addition, when SGs become enriched for misfolded proteins like DRiPs, HspB8 recruits the BAG3-Hsp70 subcomplex to extract and process them (Ganassi et al, 2016). This surveillance function at the level of SGs is referred to as granulostasis (Ganassi et al, 2016). Even if the HspB8-BAG3-Hsp70 complex plays a crucial role in granulostasis, other chaperones are recruited at SGs and participate to the preservation of

SG dynamics. These chaperones are recruited preferentially when SGs accumulate additional misfolding proteins at later time points; for this reason, they are considered as second line of defence (Ganassi et al, 2016). One example is HspB1, a chaperone holdase like HspB8 (Ganassi et al, 2016; Mateju et al, 2017). Thus, the chaperone machinery is crucial for the regulation of SG composition and for the reversion of their aberrant aggregation, when occurring.

A different approach that cells may adopt to clear SGs is through the autophagy pathway. While SGs that lack misfolded proteins are generally not recognized by the autophagy pathway, aberrant SGs attract also players of the autophagy machinery, such as BAG3 and p62/SQSTM1 (Ganassi et al, 2016). This suggests that cells can use two different mechanisms to dissolve SGs: they can be disassembled using chaperone-assisted pathway or they can be targeted and cleared by autophagy machinery. It is important to note that only a minor fraction of aberrant SGs is targeted to autophagosome for clearance (Figure 17). In fact, live-cell imaging studies show that also aberrant SGs that are targeted to the microtubule-organizing center (MTOC) for autophagy-mediated clearance tend to decrease in size, suggesting that chaperone-mediated disassembly is the preferred pathway (Mateju et al, 2017).

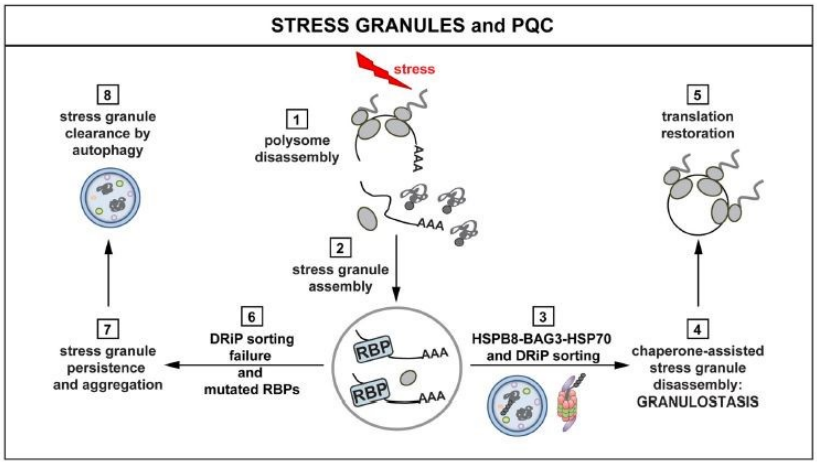

Figure 16: Interplay between stress granules (SGs) and PQC.
Polysomes disassemble themselves when cells are exposed to stress, releasing all the components (1). mRNAs are packaged into SGs (2). The concerted action of the HSPB8-BAG3-HSP70 complex limits the accumulation of misfolded proteins and DRiPs inside SGs (3). The monitoring function of HSPB8-BAG3-HSP70 ensures dynamic SG behaviour and their disassembly (granulostasis; 4), with subsequent translation restoration (5). Impairment of the complex or/and disease-linked mutations in RBPs leads to the formation of SGs that increasingly accumulate DRiPs (6) and, consequently, become less dynamic and more aggregate-like (7). Persisting SGs can be degraded via autophagy (8). Published in: Alberti et al, Granulostasis: Protein Quality Control of RNP Granules, Frontiers in Molecular Neuroscience, 2017).

Aim of the thesis:

The two main aims of this thesis were:

1) to investigate the fate of stress granules that accumulate misfolded and mutated proteins linked to disease, focusing on SG degradation by autophagy versus SG disassembly with the assistance of chaperones; 2) to further elucidate the interplay between protein quality control and SGs, identifying new molecular players of the granulostasis system.

In this thesis I will discuss data supporting the interpretation that SGs, also when aberrantly enriched for misfolded proteins, are not preferentially cleared by autophagy but they are rather disassembled with the assistance of chaperones. I will also provide evidence supporting a role for the Hsp90 chaperone as key regulator of SG turnover. Finally, I will show functional implications of deregulated SG disassembly on key signalling pathways that regulate cell metabolism and cell growth.

Material and methods:

Cell lines

Commercially available HeLa (human cervical cancer) cells, HeLa Kyoto or HEK293 cell lines stably expressing fluorescently tagged proteins generated by our colleagues Dr. Simon Alberti (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) and Dr. Daniel Kaganovich (Hebrew University of Jerusalem, Israel). In particular, HeLa Kyoto cells stably expressing G3BP2-GFP and HeLa Kyoto cells stably expressing G3BP1-mCherry were a kind gift from Dr. Alberti and were previously described (Poser et al, 2008) and HEK293 cells expressing endogenously tagged PABPC1-dendra2 were generated by Triana Amen (Dr. Kaganovich group) using the Crispr/Cas9 technology. We used also HeLa cells with tetracycline-inducible expression of V5-tagged Hsp70 (Flip-in V5-Hsp70) generated in Professor Serena Carra laboratory using Flp-in System technology.

Cell lines were cultured in DMEM (ECB7501L; EuroClone, Milan, Italy) supplemented with 2mM L-glutamine (ECB3000D; EuroClone, Milan, Italy), 100 U/mL penicillin/streptomycin (ECB3001D; EuroClone, Milan, Italy) and 10% Fetal Bovine Serum (FBS) (F7524; Sigma-Aldrich, Milan, Italy) in a humidified atmosphere at 37°C incubator with 5% CO2. HeLa Kyoto cells stably expressing G3BP2-GFP were cultured in presence of Geneticin (Gibco, 250 µg/mL), HeLa Kyoto cells stably expressing G3BP1-mCherry were cultured in presence of Blasticidin (Sigma, 10mg/mL) and HeLa Flip-in V5-Hsp70 were grown in presence of Blasticidine (Sigma,10mg/mL) and Hygromycin B (Invitrogen, 50mg/mL).

Cell treatments

Cells were grown for 24hours on petri dish or on poly-L-lysine coated coverslip and then treated with the following drugs at the concentrations indicated here: sodium arsenite 0.5 mM (or 0.05mM for live imaging experiments); Z-Leu-Leu-Leu-al (MG132) 20 μM; E64d 10µg/mL; Leupeptin 200µM; Bortezomib 100nM; ammonium chloride (NH_4Cl) 20 mM; VER-155008 40 μM, 17-demethoxy-17-(2-propenylamino)-geldanamycin (17-AAG) and Geldanamycin (GA) 5 μM, or when specified 0.5, 1μM; O-Propargyl-puromycin (OP-puro) 25 μM, puromycin dihydrochloride (puro) 5µg/mL, tetracycline 1µg/mL.

The reagents used in this study are as follows: Sodium arsenite (Carlo Erba Reagents), MG132 (C2211; Sigma), E64d (E8640; Sigma-Aldrich), Leupeptin (L5793; Sigma-Aldrich) Bortezomib (Sc-217785; Santa Cruz Biotech), ammonium chloride (A9434), VER-155008 (S0271; Sigma), 17-AAG (D5193; Sigma), Geldanamycin (EL 280-0001 Enzo), OP-puro (NU-931; Jena Bioscence), Puromycin dihydrochloride (P8833; Sigma-Aldrich, Tetracyclin hydrocloride (T7660; Sigma-Aldrich).

Transfection

HeLa-Kyoto cells stably expressing G3BP2-GFP were transiently transfected for 24 hours with 25 ng of a cDNa encoding for DCP1mRFP, HeLa-Kyoto cells stably expressing G3BP1-mCherry were transiently transfected for 24 hours with 250 ng of a cDNa encoding for myc-Raptor and HeLa cells were transiently transfected for 24 hours with 250ng of a cDNA encoding for GFP-NS. For all the transfections we used lipofectamine 2000 (Life Technologies, Monza, Italy) according to the manufacturer's instructions. After 24hours of transfection, the cells were either further incubated at 37°C with 5% CO2, treated, or processed for confocal microscopy and live-cell microscopy.

The cDNA encoding for mRFP-DCP1A was a kind gift from Dr. Anderson (Kerdesha et.,al 2008), the cDNA encoding for myc-Raptor was a kind gift from Pr.Pelkman (Wippich et al, 2013) and the cDNA encoding for GFP-NS was a kind gift from Dr. Sherman.

Small Interfering RNA (siRNA)

HeLa Cells were transfected using lipofectamine 2000 (Life Technologies, Monza, Italy) according to the manufacturer's instructions, with siRNA ON-TARGETplus smart pool, purchased from Dharmacon GE Healthcare (Milan, Italy).
siRNAs were used at the following concentrations: 5 nM siGENOME non-targeting control siRNA, 100 nM NEMF siRNA and 75 nM LTN1 siRNA.

Small interfering RNA	TARGET SEQUENCE
NEMF	CGUUAGAGGGAAAGGAUAA CCUACAAAUAGUUGACAGA GAAAAUGGAUUCUCGGGUA AAUUAUAGGUGGACGAGAU
LTN1	GGGUGUAUCUAACCUAUUA AAACAACAAUGCUUCCAUA GCGAAAGGAUGCUUGCUAA GAUUGAAGGCUGGGAAUUA
NON-TARGETING	UAGCGACUAAACACAUCAA UAAGGCUAUGAAGAGAUAC AUGUAUUGGCCUGUAUUAG AUGAACGUGAAUUGCUCAA

After 24hours of transfection, the medium was replaced. 72hours after transfection, the cells were treated or processed for analysis such as: immunofluorescence or western blot.

Immunofluorescence Microscopy

Cells (HeLa, HeLa-Kyoto G3BP2-GFP, HeLa-Kyoto G3BP1-mCherry and HEK293 PABPC1-dendra2) were grown on poly-L-lysine (P8920; Sigma) coated glass-coverslip. Following treatment, cells were fixed with 3.7% formaldehyde in PBS 1x for 9 minutes at room temperature, followed by permeabilization with ice-cold acetone 100% for 5 minutes at -20°C. Alternatively, cells were fixed with ice-cold methanol for 10 minutes at -20°C. Coverslips were blocked with blocking solution (PBS 1x containing 3% BSA and 0.1% Triton X-100) for 1hours at room temperature and then incubated overnight with primary antibodies at 4°C. Following PBS 1x wash, cells were incubated with fluorescent secondary antibodies for 1 hour at room temperature in the dark.

Primary and secondary antibodies were diluted in blocking solution. Primary and secondary antibodies used are listed below. Cells were then washed with PBS 1x and the coverslips were mounted on glass microscope slides with mounting solution (PBS pH 7,4, Mowiol 20%, 1.4-diazabiciclico-[2,2,2]-ottano DABCO as antifading agent). Slides were stored at 4°C.

Analysis of the cells was done by confocal imaging using a Leica SP2 AOBS system (Leica Microsystems) and a 63x oil-immersion lens.

The primary antibodies used in this study were:
mouse anti-G3BP (BD Biosciences); goat anti-TIA-1(C-20) and rabbit anti-EIF4G (11373) (Santa Cruz); mouse anti-HSP70 (SMC-100), mouse anti-HSC70 (SMC-151), mouse anti-HSP90 alpha (SMC-147A) and mouse anti-HSP90 beta (SMC-107) (StressMarq); mouse anti-DCP1A (186-285) (Abnova); mouse anti-c-MYC (SC-40) (Santa Cruz); rabbit anti-DYRK3 (P050) (Aviva System Biology).

The secondary antibodies used were:
Alexa-conjugated secondary antibodies (Life Technologies): donkey-anti-goat-Alexa488, donkey-anti-mouse-Alexa594, donkey-anti-rabbit-Alexa594, donkey-anti-rabbit-Alexa488 and donkey-anti-mouse-Alexa488.

OP-puro labelling of cultured cells

Cells were fixed with methanol as previously described; the protocol for OP-puro labeling was adapted from Liu et al, 2012. Briefly, the cells were incubated with 25 μM OP-puro for 45 min; to detect the OP-puro-labelled nascent proteins, after fixation, the cells were incubated with CuAAC and then with Alexa Fluor 594 azide (A10270, Life Technologies). Cells were subsequently processed for immunofluorescence microscopy as described above.

Protein lysate preparation and western blot

Cells were harvested in lysis buffer: Laemmli buffer (2%) or where indicated the buffer was added with PhosSTOP Phosphatase Inhibitor Cocktail 1X (P8340; Sigma) and homogenized by sonication for 5 seconds. Protein samples were reduced with β-mercaptoetanol (final 3-5%) and boiled for 3 minutes at 100 °C. Protein samples were electrophoretically separated by SDS-PAGE (Laemmli et al. 1970) followed by blotting of the proteins onto a nitrocellulose membrane.

Membranes were blocked with PBS-T (137mM NaCl, 2.7mM KCl, 10mM Sodium Phosphate dibasic, 2mM Potassium Phosphate monobasic, 0.1% Tween-20 Bio-Rad, pH 7,4) and 5% dried non-fat milk for 1hour at room temperature. Primary antibodies diluted in PBS-T containing 3% BSA and 0.02% Na-azide were added 1hour after blocking; cells were then incubated overnight at 4°C with primaries antibodies. HRP-conjugated secondary antibodies (GE Healtcare) were prepared in PBS-T and 3% dried non-fat milk and incubated for at least 1hour at room temperature. Western Blot was developed through the induction of chemiluminescence using ECL kit Westar Eta C Ultra 2.0 (Steroglass).

The primary antibodies used in western blotting were:
mouse anti-Tubulin (T6074; Sigma-Aldrich, Milan, Italy), rabbit anti p70 s6k (9202S; cell signalling), mouse anti-Puromycin (12D10; Merkel), rabbit anti-ubiquitin (Z0458; Dako Cytomation), mouse anti-LC3 (NB-100-2220; Novus Biologicals), mouse anti-HSP70 (SMC-100), mouse anti-HSC70 (SMC-151).

The secondary antibodies used were:
mouse and rabbit HRP-conjugated secondary antibodies for western blot were from GE Healthcare Europe GmbH (Milan, Italy).

Live–cell microscopy

For live imaging experiments, the cells (HEK293 PABPC1-dendra2 and HeLa-Kyoto G3BP2-GFP) were grown on 35X10 mm, CELLview Glass Bottom Cell Culture Dish, Four Compartments (Greiner Bio-One) in DMEM complete medium, as described in the cell line section.
To induce stress granules, cells were stressed with sodium arsenite for 45 minutes; during this period, we studied stress granule assembly. Then, to study stress granule disassembly kinetics, cells were washed with warm PBS (37°C) for 3 times and incubated at 37 ˚ C in complete DMEM. Inhibitors of the HSP70 or HSP90 ATPase activity, ammonium chloride were added during this period, whenever indicated in the text and figure legend (recovery control, without drugs or with drugs: VER-155008 40 µM, 17AAG 5 µM, Geldanamycin 5

μM, NH_4Cl 20mM). Live cell imaging was performed for 4h, where stable temperature (37˚C) and CO_2 (5%) were maintained, and a frame was taken every 5/10 minutes.

HEK293 PABPC1-dendra2:

Time-lapse images were acquired (in the laboratory of Dr. Daniel Kaganovich at the Hebrew University of Jerusalem, Israel) with a NIKON A1 confocal microscope, using a 63X oil objective. Movies were acquired in resonant-scanning mode and imaging was performed using a 488-nm laser. Fiji was used to obtain the maximum intensity projections from collected *Z*-stacks.

HeLa-Kyoto G3BP2-GFP:

Time-lapse images were acquired (at CIGS in Modena, Italy) with a white light laser Leica SP8 confocal microscope, using a 63X oil objective. Movies imaging was performed using a 488-nm laser and 555nm. Fiji was used to obtain the maximum intensity projections from collected *Z*-stacks.

RNA extraction, RT-PCR and Real-Time PCR

Total RNA was isolated using KIT PROMEGA (A500) according to the manufacturer's instructions. Subsequently 1 μg of extracted RNA was treated with DNase I (AM2222; Life Technologies) according to the manufacturer's instructions, prior to reverse transcription-PCR (RT-PCR).

First-strand cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. In detail the 2x Reverse Transcription Master Mix (10x RT BUFFER; 25x dNTP Mix; 10x RT random primers; Multiscribe Reverse Transcriptase; RNase inhibitor Nuclease free H2O) was added to the DNase treated RNA. The samples were incubated at 25°C for 10 minutes followed by a step of 37°C for 120 minutes, and then 85°C for 5 minutes. The cDNA was stored at -20°C or used immediately for real-time PCR (Q-PCR).

Relative changes in transcription levels of hNEMF (also called SDCCAG1) and hListerin (hLTN1 or ZNF 294) and hRPL0 were determined using CFX96 Touch Thermal cycler (Bio-Rad, Hercules, CA, USA) in combination with SYBR green master mix. The primers used are listed below.

hNEMF rev	5`TCTGTTGCTGATCTCGTCCA3`
hNEMF for	5`CAGCAGAAAAGAAAACAAAGCA3`
hLTN1 rev	5`TTCATCAACTGTGGAATGCG3`
hLTN1 for	5`AATGAGCTTGATTCTCTGGAGG3`
hRPL0 rev	5`TCCAAATCCTCGGCATAATGA3`
hRPL0 for	5`CGTCGTGATTAGCGATGATGA3`

The real-time PCR was performed as follow: one cycle of denaturation (95°C for 3 minutes) followed by 40 cycles of amplification (95°C for 10 seconds, 60°C for 30 seconds). Each reaction was monitored by the use of a negative control (no template). All the analyses were performed in triplicate, and hRPL0 used as housekeeping gene. Data were analyzed with Bio-rad CFX Manager 3.1 (Windows 7.0).

Statistical analysis and SGs persistence analysis

One-way ANOVA followed by Bonferroni–Holm post-hoc test was used for comparisons between three or more groups using Daniel's XL Toolbox. Student's t-test was used for comparisons between two groups. *P<0.05; **P<0.01; ***P<0.001.

Fiji was used to analyze and count the SG persistence during the time laps experiments, while the automated quantitation of DRiPs enrichment in SGs was performed using Olympus Scan^R (in Dr. Simon Alberti lab, Dresden, Germany).

Results:

Autophagy is not the preferred clearance mechanism for both physiological and aberrant SGs

Carra lab previously published that the majority of physiological SGs disassemble with time and only a minor fraction of circa 5-10% is targeted to autophagy for clearance (Ganassi et al, 2016). Here, we addressed the question if physiological and aberrant SGs, the latter forming in cells with dysfunctional Hsp70, are preferentially disassembled with the assistance of chaperones or are instead degraded via autophagy by the cells.

We used HEK293 cells that express the SG-resident protein PABPC1 endogenously tagged with the fluorescent probe Dendra2 (Dendra2-PABPC1) and that were generated using the Cripsr/Cas9 technology in the laboratory directed by Dr. Daniel Kaganovich (unpublished tool). In these cells, we performed live-cell imaging studies to monitor SG formation and dissolution.

When cells were treated with sodium arsenite, they rapidly formed SGs containing Dendra2-PABPC1 (Gray et al, 2015), which are referred to as physiological SGs. During the recovery phase, when sodium arsenite was removed from the medium, these physiological SGs rapidly disassembled and typically disappeared within 1 hour (Fig. 17A, blue line). We then incubated the cells during the recovery phase with ammonium chloride, which increases the pH of lysosomes therefore halting degradation of enclosed proteins and inhibiting lysosome degradative function. Addition of ammonium chloride slightly delayed the disassembly of Dendra2-PABPC1 SGs (Fig. 17A, red line). These data are in line with previous findings obtained using HeLa Kyoto cells expressing G3BP2-GFP (Ganassi et al, 2016) and confirm that only a minor fraction of physiological SGs is cleared by autophagy.

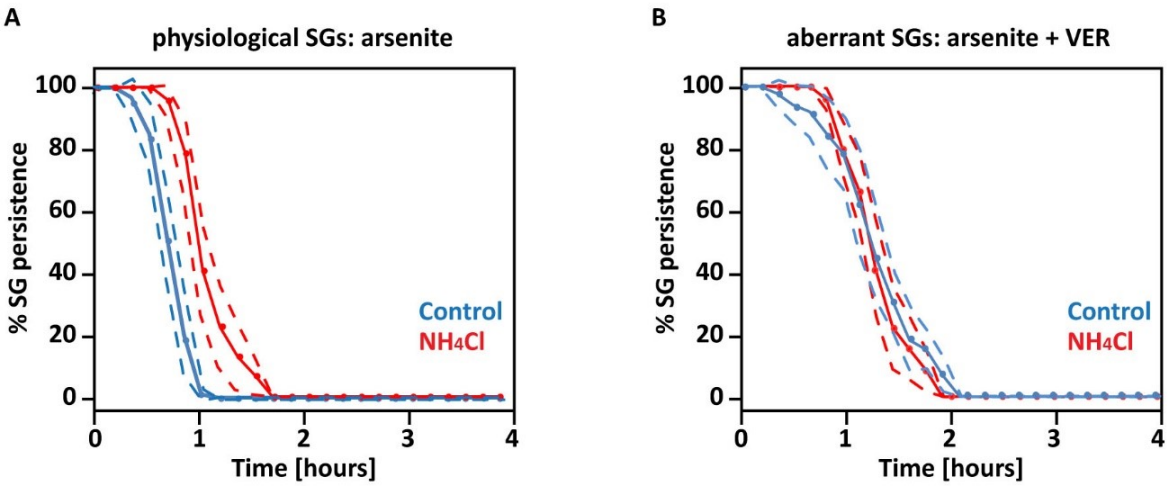

Figure 17: SGs disassembly in presence of ammonium chloride.
A) HEK293 cells expressing Dendra2-PABPC1 were treated for 45 min with sodium arsenite 50µM and recovered for 4 hrs in drug free medium (81 cells, blue line) or medium containing NH_4Cl 20mM (85 cells, red line). B) HEK293 cells expressing Dendra2-PABPC1cells were treated for 45 min with sodium arsenite 50µM in co-treatment with VER 40µM, and recovered for 4 hrs in drug free medium (97 cells, blue line) or medium containing NH_4Cl 20mM (81 cells, red line). Quantification of SGs` persistence analysed from live-cell imaging. Dashed lines = 95% confidence intervals.

We then induced aberrant SGs by co-treating the cells with sodium arsenite and VER, a specific inhibitor of Hsp70 ATPase activity; then, we allowed the cells to recover in drug-free medium and we followed SG disassembly for up to 4 hours. First, we compared the disassembly kinetics of physiological and aberrant SGs in drug-free medium (Fig. 17A and B, blue lines) and we found that the inhibition of Hsp70 doubled the disassembly time of SGs from 1 hour (Fig. 17A, blue line) to 2 hours (Fig. 17B, blue line). Thus, accumulation of misfolded proteins inside SGs delays their dissolution. Next, to test if aberrant SGs are degraded by autophagolysosomes, we added ammonium chloride to the medium during the recovery phase. We found that the disassembly kinetics of aberrant SGs in absence or presence of ammonium chloride were overlapping (Fig. 17B, blue and red lines). These data suggest that, like physiological SGs, also aberrant SGs are not preferentially cleared by autophagy.

Physiological and aberrant SGs are preferentially disassembled by chaperone-mediated pathways

Considering that autophagy is not the main pathway used by cells to clear SGs, the next step was to investigate the role of chaperone-assisted disassembly, testing the implication of the Hsp70 chaperone. We treated cells with sodium arsenite alone or combined with VER, to form physiological or aberrant SGs and then we allowed the cells to recover in drug-free medium or in presence of VER. We followed the cellular SG` dissolution by live-cell imaging. The presence of VER during the recovery phase severely delayed the disassembly kinetics of both physiological (Fig. 18A) and aberrant SGs (Fig. 18B). We found that after 4 hours of recovery in presence of VER the disassembly kinetics of aberrant SGs were lower compared to the ones of physiological SGs (Fig. 18A and 18B, red lines). These results confirm that the chaperone-assisted disassembly is the preferred pathway used by the cells to clear SGs.

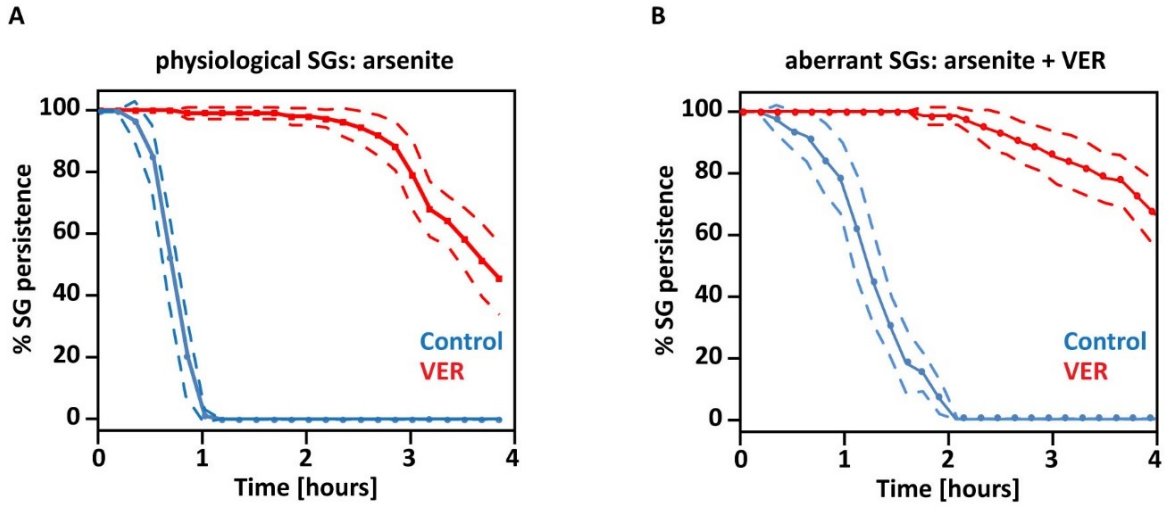

Figure 18: SGs disassembly in presence of HSP70 inhibitor.
A) HEK293 cells expressing Dendra2-PABPC1were treated for 45 min with sodium arsenite 50µM and recovered for 4 hrs in drug free medium (81 cells, blue line) or VER 40µM medium (92cells, red line). B) HEK293 cells expressing Dendra2-PABPC1 were treated for 45 min with sodium arsenite 50µM in co-treatment with VER 40µM, and recovered for 4 hrs in drug free medium (97 cells, blue line) or VER 40µM medium (136 cells). Quantification of SG` persistence analysed from live-cell imaging. Dashed lines = 95% confidence intervals.

We noticed that, during the recovery time, after 4 hours of incubation with VER, circa 50% of cells could disassemble physiological SGs and circa 30% of cells could disassemble aberrant SGs (Fig.18A, red line and 18B, red line). Considering that all physiological and aberrant SGs were disassembled within 2 hours when autophagolysosome activity was

blocked (Fig.17A, red line and 18B, red line), our data suggest that other chaperones may participate with Hsp70 to regulate SG turnover.

Functional Hsp90 required for SGs disassembly

Recent mass spectrometry analysis identified a new SG-resident chaperone, Hsp90 (Jain et al, 2016; Markmiller et al, 2018). Hsp90 is an abundant chaperone, constituting about 1–2 % of total proteins (Jakob and Buchner, 1994). Hsp90 fulfills housekeeping functions by assisting the folding, maturation and the maintenance of the structural integrity of a large variety of proteins, referred to as Hsp90 clients. Many of these clients are proteins involved in important signalling pathway that regulates, for instance, the cell cycle. These include also several proteins that were identified as SG components by mass spectrometry, such as argonaute protein AGO1, the La related RNA binding protein LARP4B, the apolipoprotein B mRNA editing enzyme APOBEC3C, the decapping enzyme Dcp1, the negative elongation factor NELF-E, the dynein complex regulator NUDC and the Zinc Finger CCCH-Type protein ZC3H7B (Jain et al, 2016; Markmiller et al, 2018). These data open the possibility that Hsp90 is important to maintain the proper folding of these SG-resident clients; this, in turn, may also participate in the regulation of SG turnover.

To test the hypothesis that Hsp90 may regulate SG turnover, we treated HEK293 cells expressing Dendra2-PABPC1 with sodium arsenite, alone or combined with VER, to form physiological and aberrant SGs, respectively. Then, we allowed the cells to recover in drug-free medium or in presence of Hsp90 inhibitors. We tested two drugs that inhibit the ATPase activity of Hsp90: Geldanamycin (GA) and 17-Allylamino-17-demethoxygeldanamycin (17AAG), which is a less toxic derivative of GA (Chiosis et al, 2003; Roe et al, 1999; Schulte et al, 1998). Analysis of SG disassembly kinetics showed that GA delayed the dissolution of both physiological and aberrant SGs (Fig.19A, red line and Fig.19B, red line). Concerning 17AAG, it delayed SG disassembly, similarly to GA (Fig.19C red and green lines). Importantly, we verified that treatment of HEK293 cells expressing Dendra2-PABPC1 with VER, GA or 17AAG alone for up to 4 hours did not induce SGs (Fig. 20B). The results obtained using GA and 17AAG demonstrate that also Hsp90 is important for the regolation of SGs turnover.

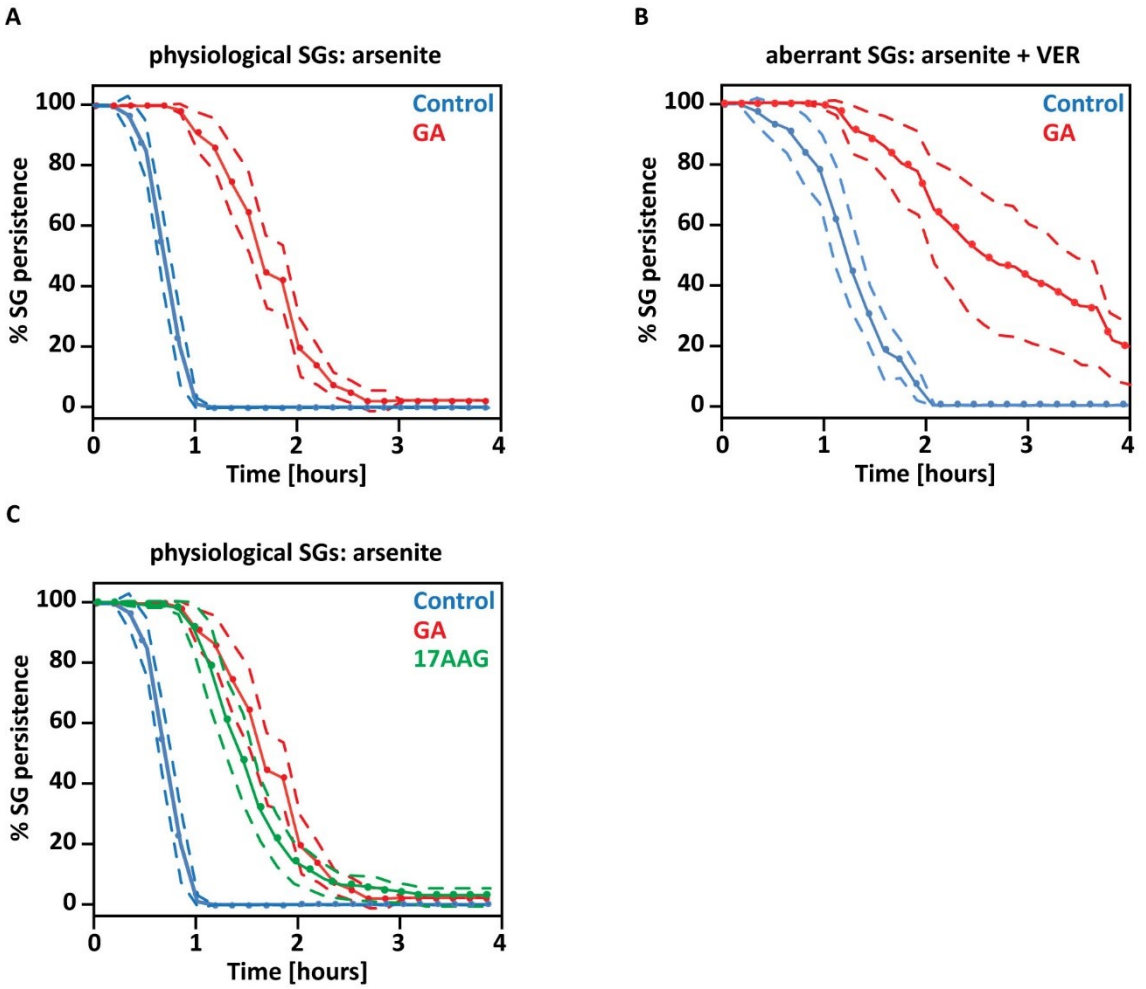

Figure 19: SGs disassembly in presence of HSP90 inhibitors.
A) HEK293 cells expressing Dendra2-PABPC1 were treated for 45 min with sodium arsenite 50µM and recovered for 4 hrs in drug free medium (81 cells, blue line) or Geldanamycin 5µM medium (166 cells, red line). B) HEK293 cells expressing Dendra2-PABPC1 were treated for 45 min with sodium arsenite 50µM in co-treatment with VER 40µM and then, cells were allowed to recover for 4 hrs in drug free medium (97 cells, blue line) or with Geldanamycin 5µM (153 cells, red line). C) HEK293 cells expressing Dendra2-PABPC1 were treated for 45 min with sodium arsenite 50µM and recovered for 4 hrs in drug free medium (81 cells, blue line) or Geldanamycin 5µM medium (166 cells, red line) or 17AAG 5µM medium (193 cells, green line). Quantification of SG persistence from live-cell imaging. Dashed lines = 95% confidence intervals.

We then asked if the effects observed with the Hsp70 and Hsp90 inhibitors were specific to Hek293 cells or if Hsp70 and Hsp90 participate in the regulation of SG turnover also in other mammalian cells. We thus used HeLa Kyoto cells stably expressing G3BP2-GFP (Ganassi et al, 2016). First of all, we evaluated if the treatment with the inhibitors VER, GA or 17AAG alone for up to 4 hours could promote the formation of SGs. We found that also in HeLa Kyoto G3BP2-GFP cells the inhibitors per se do not induce SGs (Fig.20A). Compared to untreated cells, we observed only a minor induction of SGs in few HeLa-kyoto cells treated with the inhibitors, but this effect was not significant (Fig. 20A).

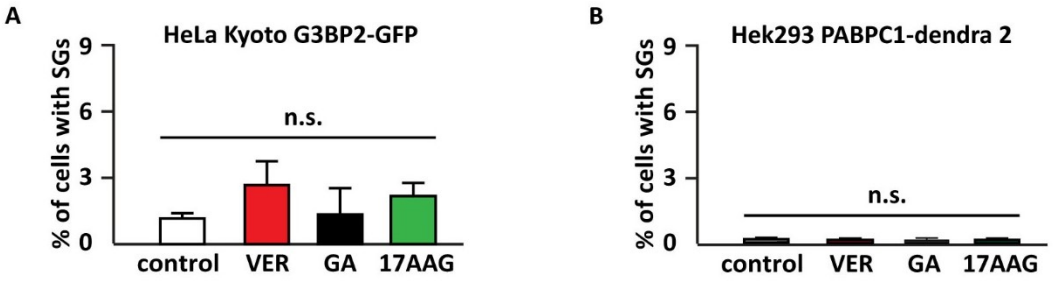

Figure 20: Inhibitors and SGs persistence.
24 hrs after seeding, cells were treated for 4 hrs with drug-free medium or VER 40 µM or GA 5 µM or 17AAG 5 µM. Cells were then fixed and nucleic acid was stained with DAPI. A) HeLa Kyoto cells stably expressing G3BP2-GFP. The number of cells analysed was: 450 cells (control: drug free medium, white column), 459 cells (inhibition of Hsp70 with VER, red column), 433 cells (inhibition of Hsp90 with 17AAG medium, green column), 452 cells (inhibition of Hsp90 with GA, black column). B) HEK293 cells expressing Dendra2-PABPC1. The number of cells analysed was: 494 cells (control: drug free medium, white column), 404 cells (inhibition of Hsp70 with VER, red column), 487 cells (inhibition of Hsp90 with17AAG, green column), 415 cells (inhibition of Hsp90 with GA, black column). The percentage of cells that contain SGs were quantified and are presented as histogram; ns= not significant.

Then, we investigated the effect of Hsp70 and Hsp90 inhibition of SG dissolution in HeLa Kyoto cells stably expressing G3BP2-GFP. Concerning Hsp70, physiological and aberrant SGs were induced by treating the cells with sodium arsenite alone or with VER, respectively. As previously described, we allowed cells to recover in drug free medium or in presence of VER for up to 4 hours and SG dissolution kinetics were studied by live-cell imaging (Fig.21). We confirmed that the inhibition of Hsp70, during the recovery phase, delayed the disassembly of both physiological and aberrant SGs (Fig.21 red lines).

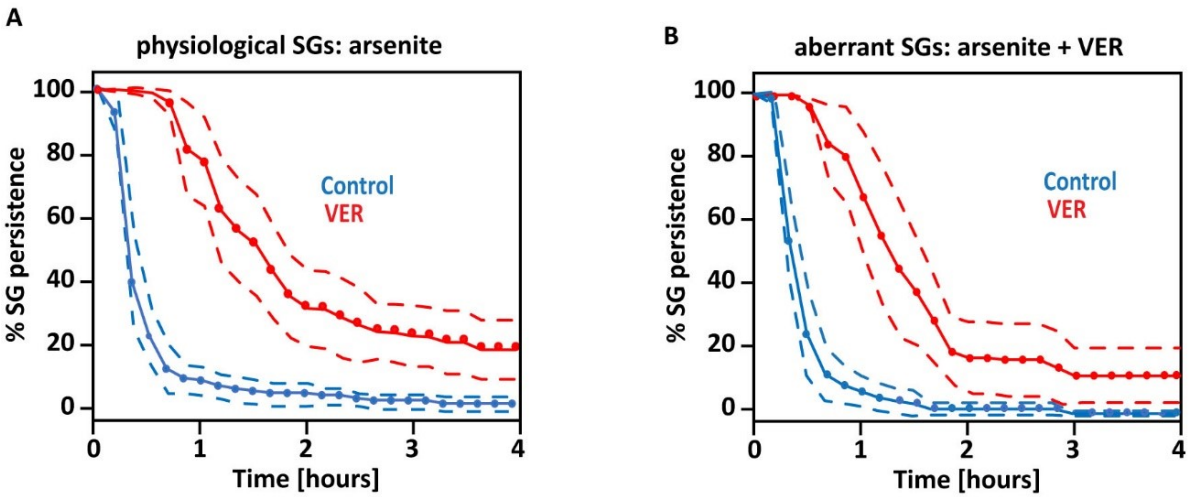

Figure 21: SGs disassembly in presence of HSP70 inhibitor.
A) HeLa Kyoto cells stably expressing G3BP2-GFP were treated for 45 min with sodium arsenite 50 µM and allowed to recover for 4 hrs in drug free medium (193 cells, blue line) or with VER 40µM (209 cells, red line). B) HeLa Kyoto cells stably expressing G3BP2-GFP were co-treated for 45 min with sodium arsenite 50 µM and VER 40µM and allowed to recover for 4 hrs in drug free medium (121 cells, blue line) or with VER 40 µM (139 cells, red line). Quantification of SG persistence from live-cell imaging. Dashed lines = 95% confidence intervals.

In parallel, we performed the same experiment in presence of the inhibitors of Hsp90 similarly to VER, also GA and 17AAG delayed the dissolution of both physiological and aberrant SGs (Fig. 22). 17AAG, in particular, had a strong inhibitory effect on the dissolution of both physiological and aberrant SGs, with more than 90% of the cells still presenting SGs after 4 hours of recovery-time (Fig. 22A, green line and Fig. 22B, green line).

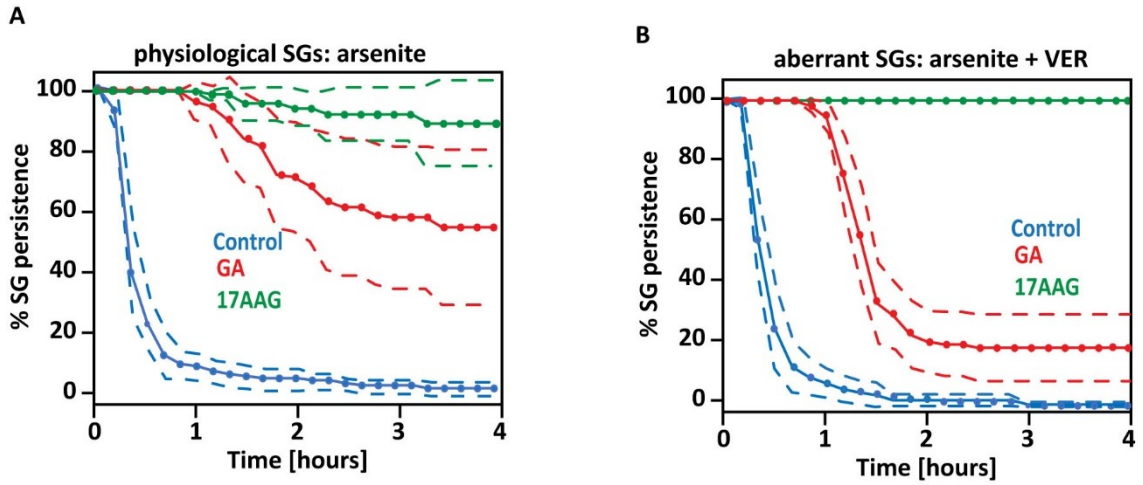

Figure 22: **SGs disassembly in presence of HSP90 inhibitors.**
A) HeLa Kyoto cells stably expressing G3BP2-GFP were treated for 45 min with sodium arsenite 50 µM and allowed to recover for 4 hrs in drug free medium (193 cells, blue line), or with GA 5µM (201 cells, red line) or 17AAG 5 µM (187 cells, green line). B) HeLa Kyoto cells stably expressing G3BP2-GFP were co-treated for 45 min with sodium arsenite 50 µM and VER 40µM, and allowed to recover for 4 hrs in drug free medium (121 cells, blue line), or with GA 5µM medium (151 cells, red line) or 17AAG 5µM (155 cells, green line). Quantification of SG persistence from live-cell imaging. Dashed lines = 95% confidence intervals.

We conclude that both Hsp70 and Hsp90 participate to the regulation of SG turnover.

We then asked if the effect observed was specific to arsenite-induced SGs or it could be generalized to SGs induced by other types of stressors. To this end, we induced SGs with temperature upshift. Impaired SG disassembly kinetics due to Hsp90 or Hsp70 inhibition were also observed on heat shock-induced SGs; this result demonstrates that the

chaperones Hsp70 and Hsp90 modulate the turnover of SGs that form in response to different stressors (Fig.23).

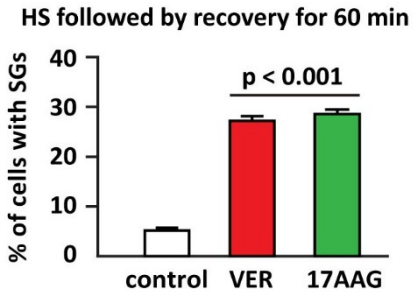

Figure 23: SGs persistence after HS in different recovery conditions.
HeLa Kyoto cells were subjected to HS by incubation at 43.5℃, for 1h. Immediately after the HS treatment, cells were allowed to recover for 60 min at 37℃ in drug-free medium (control) or in presence of VER or 17AAG. Cells were then fixed and labelled with α-TIA1 (SGs marker). We quantified the percentage of cells that contain SGs. The number of cells counted was: 605 cells (control: in drug-free medium, white column), 510 cells (VER, red column) and 468 (17AAG, green column).

Having established that also Hsp90 is required to dissolve SGs, regardless the accumulation of misfolded proteins (physiological and aberrant SGs), we then asked how mechanistically this occurs. Long-term treatments of the cells with GA and 17AAG leads to the activation of the HSF1 and induces the expression of Hsp70 and other HSF1-regulated chaperones (Zuo et al, 1998). We thus verified in HeLa Kyoto cells by immunoblotting the effect of Hsp90 inhibitors on Hsp70 expression levels. We confirmed that treatment of HeLa Kyoto cells with GA or 17AAG for 12-24 hours strongly upregulated Hsp70, while not affecting the expression of the cognate form Hsc70 (Fig. 24A). Two different concentrations of these drugs were used, with similar effects: 1µM to 5µM. Instead, treatment of HeLa Kyoto cells for 60-90 minutes with 17AAG and GA, but also with VER, did not influence the expression of Hsp70 (Fig. 24B).

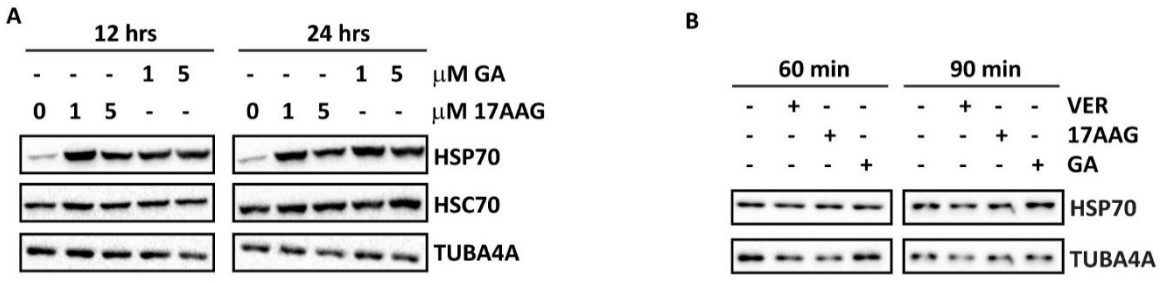

Figure 24: Hsp70 inductions in long-short treatment.
A) HeLa Kyoto cells were treated for 12-24 hrs with GA or 17AAG (1 or 5 µM) or left untreated. Total protein extracts were prepared and analysed by immunoblotting to measure the expression levels of Hsp70 and Hsc70. TUBA4A was used as loading control. B) HeLa Kyoto cells were left untreated (-) or treated (+) for 60-90min with VER 40 µM, or GA-17AAG 5µM. Total protein extracts were prepared and analysed by immunoblotting to measure the expression levels of Hsp70 and Hsc70. TUBA4A was used as loading control.

Then, we verified the expression levels of Hsp70 in cells treated with sodium arsenite. Hsp70 expression was only mildly induced in HeLa Kyoto cells subjected to treatment with sodium arsenite (to induce SGs), followed by recovery for up to 4 hours in absence and presence of the Hsp90 inhibitors 17AAG or GA (Fig. 25).

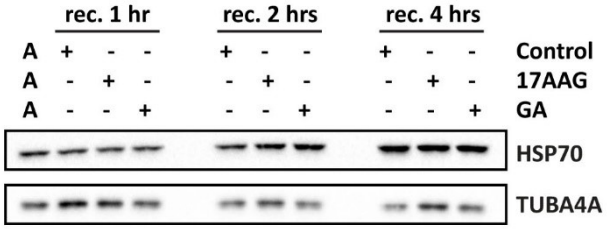

Figure 25: Induction of Hsp70 in SGs disassembly recovery conditions.
HeLa Kyoto cells were treated with sodium arsenite 50 µM for 45 min and allowed to recover for 1h-2hrs-4hrs in drug-free medium, or in presence of 17AAG 5 µM or GA 5 µM. Total protein extracts were prepared and analysed by immunoblotting to measure the expression levels of Hsp70 and Hsc70. TUBA4A was used as loading control.

Thus, Hsp90 inhibition affects SG turnover and the heat shock response with different kinetics, ranging from less than 2 hours (SGs) to up to 12-24 hours (HSF1), suggesting that the effects of Hsp90 inhibition on SG turnover and the heat-shock response are occur via distinct mechanisms. Based on these findings, we conclude that both Hsp70 and Hsp90 ATPase activities regulate SG turnover in mammalian cells and that the inhibitory effect of Hsp90 on SG dissolution does not correlate with Hsp70 expression levels.

Delayed SG dissolution upon Hsp90 inhibition does not correlate with enrichment for DRiPs

We previously showed that inhibition of Hsp70 leads to the accumulation of DRiPs inside SGs; this, in turn changes their liquid-like behaviour and convert them into an aberrant persistent state (Ganassi et al, 2016). Considering the strong impact of DRiPs on SG

dissolution kinetics, we then verified if Hsp90 inhibition may also lead to the accumulation of DRiPs inside SGs. Like Hsc70 and Hsp70, also Hsp90 binds to nascent proteins. However, while Hsc70 and Hsp70 play a well-established role in targeting DRiPs to clearance (Hartl and Hayer-Hartl 2002; Ganassi et al, 2016), Hsp90 has a more prominent role in assisting the proper folding and maturation of its newly synthesized clients (Schopf et al, 2017). Accordingly, by immunostaining, we found that constitutively expressed Hsc70 and inducible Hsp70 colocalize with DRiPs accumulating in form of cytoplasmic foci; in contrast, constitutively expressed Hsp90 beta and the inducible form Hsp90 alpha did not colocalize with DRiPs (Fig. 26A). Then, we quantified the enrichment for DRiPs in SGs induced by heat shock alone or in presence of the inhibitors 17AAG, GA and VER, the latter used as positive control (Fig. 26B). In contrast to Hsp70 inhibition, which leads to a strong enrichment of DRiPs inside SGs (Fig. 26B red column) (Ganassi et al, 2016), Hsp90 inhibition led to a mild accumulation of DRiPs inside SGs (Fig. 26B black and green columns). Nevertheless, both Hsp90 inhibitors (GA and 17AAG) had a stronger impact on SG dissolution compared to Hsp70 inhibition (Fig. 21A and Fig. 22A).

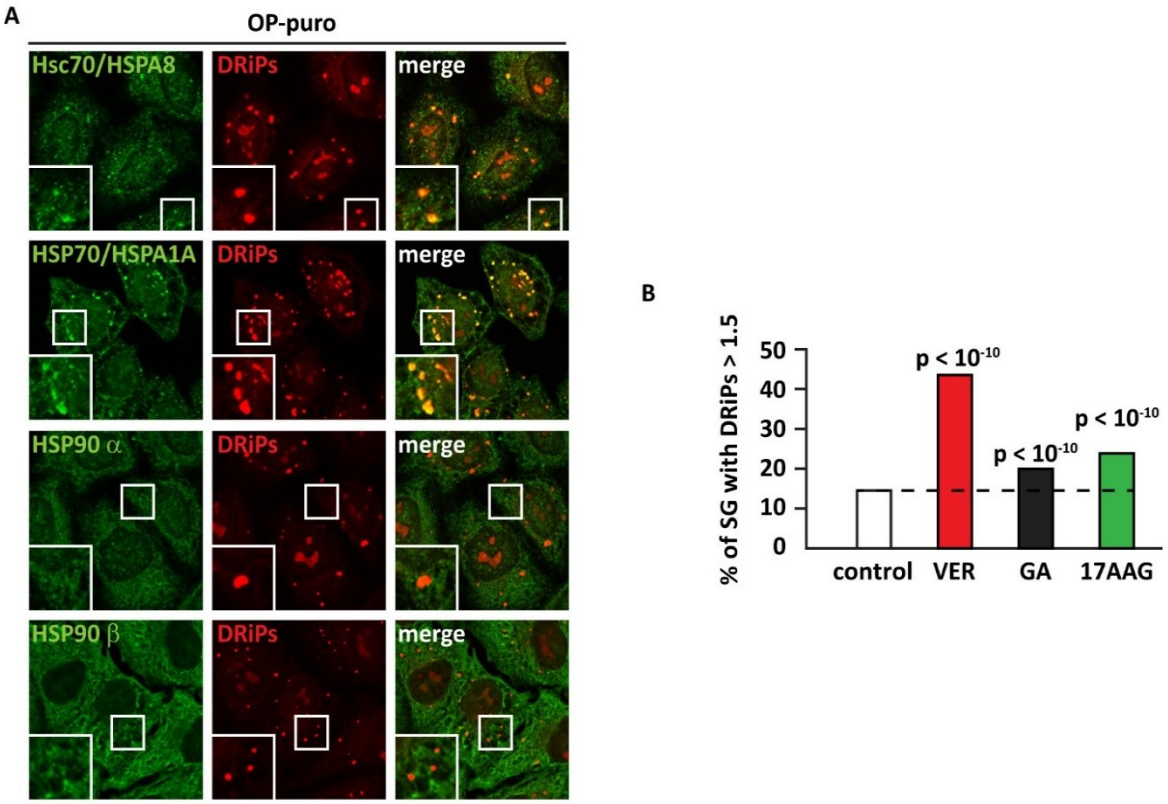

Figure 26: DRiPs and Hsp90.
A) HeLa Kyoto cells were treated for 1h with op-PURO 25 µM to label DRiPs. Cells were fixed and DRiPs, Hsc70, Hsp70, Hsp90α and Hsp90β were visualized by confocal microscopy. B) SGs were induced in Hela Kyoto cell by exposure to heat shock at 43,5 ̊C for 1h in presence of op-PURO 25 µM. Where indicated cells were co-treated with VER 40 µM, GA 5 µM and 17AAG 5 µM. Cells were then fixed and labelled for DRiPs and SGs using an antibody specific for TIA-1. Quantitation of DRiP enrichment in SGs. $p < 10^{-10}$. Automated imaging and SG segmentation are based on TIA-1 signal. Data are presented as histogram.

Next, we employed HeLa cells with tetracycline-inducible expression of V5-tagged Hsp70 (Fig. 27B) and we asked if upregulation of Hsp70, which assists DRiP degradation (Ganassi, 2016), could rescue SG turnover in cells treated with the HSP90 inhibitors. We found that the induction of Hsp70 (Fig. 27A white columns) could not rescue SG turnover in cells treated with 17AAG or GA, as well as in cells treated with VER, used as a control (Fig. 27A). Combined these data strongly suggest that other mechanisms rather than accumulation of DRiPs inside SGs and malfunction of Hsp70 are responsible for impaired SG disassembly upon Hsp90 inhibition.

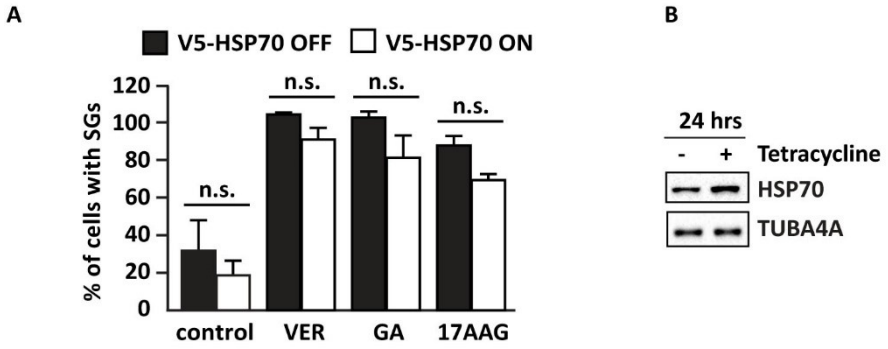

Figure 27:Induction of HSP70 and rescue on SG dissolution kinetics.
A) Hela cells with tetracycline-inducible expression of V5-tagged HSP70 were left untreated or treated with tetracycline 1µg/mL [ON (white column) and OFF (black column)]. After 24 hrs, cells were treated with sodium arsenite 500 µM for 45 min, to induce SGs. Then, we allowed cells to recover for 90 min in drug-free medium or in presence of VER 40 µM, GA 5 µM or 17AAG 5 µM. Cells were fixed and labelled using an antibody specific for the SG marker G3BP; SG persistence was quantified. Data of three independent experiments are presented as histogram; ns= not significant. B) Hela with tetracycline-inducible expression of V5-tagged Hsp70 were left untreated or treated with tetracycline 1µg/mL, in order to switch on the Hsp70 expression. After 24 hrs, total protein extracts were prepared and analysed by immunoblotting to measure the expression levels of Hsp70. TUBA4A was used as loading control.

Short-term inhibition of HSP90 does not affect the dynamics of P-bodies

Processing bodies (PBs) are cytoplasmic mRNP granules that are already present in normally growing cells and can be induced upon stress conditions that inhibit translation initiation (Kerdesha et al, 2005). Like SGs, PBs contain mRNAs that are not engaged in

translation and recent evidence suggests that both SGs and PBs are involved in miRNA-induced translational silencing pathways (Emde and Hornstein, 2014). However, in contrast to SGs, PBs have been also involved in mRNA decapping and nonsense-mediated decay (Parker and Sheth, 2007). SGs and PBs are intimately connected: they share several components such as e.g. AGO1/2, FAST (Fas-activated serine/threonine kinase), XRN1 (5'–3' exoribonuclease 1), eukaryotic translation initiation factor 4E (eIF4E), tristetraprolin (TTP) (Kedersha et al, 2005). PBs and SGs are dynamic structures that intermittently and transiently interact one each other, allowing the transfer of specific mRNPs; in fact, it is though that mRNPs continuously shuttle from polysomes, where mRNAs are translationally active, to PBs and SGs were it is un-translated (Parker and Sheth 2007; Karginov and Hannon, 2013).

Interestingly, long-term treatment with GA was reported to decrease the number of PBs (Suzuki et al, 2009). Moreover, recruitment of AGO2 to SGs was shown to depend, at least in part, on Hsp90 activity (Pare et al, 2009; Johnston et al, 2010). Based on these data we asked if inhibition of Hsp90 may perturb the equilibrium between SGs and PBs, thereby affecting both SG and PB dynamics.

To visualize PBs we used an antibody specific for the PB-resident protein DCP1A. According to the literature, we found that treatment of HeLa Kyoto cells with GA or 17AAG for 24 hours led to the disappearance of PBs (Fig. 28A and Fig. 28B). In contrast, treatment of HeLa cells with VER for 24 hrs did not affect the percentage of cells with PBs (Fig. 28A and Fig. 28B red column).

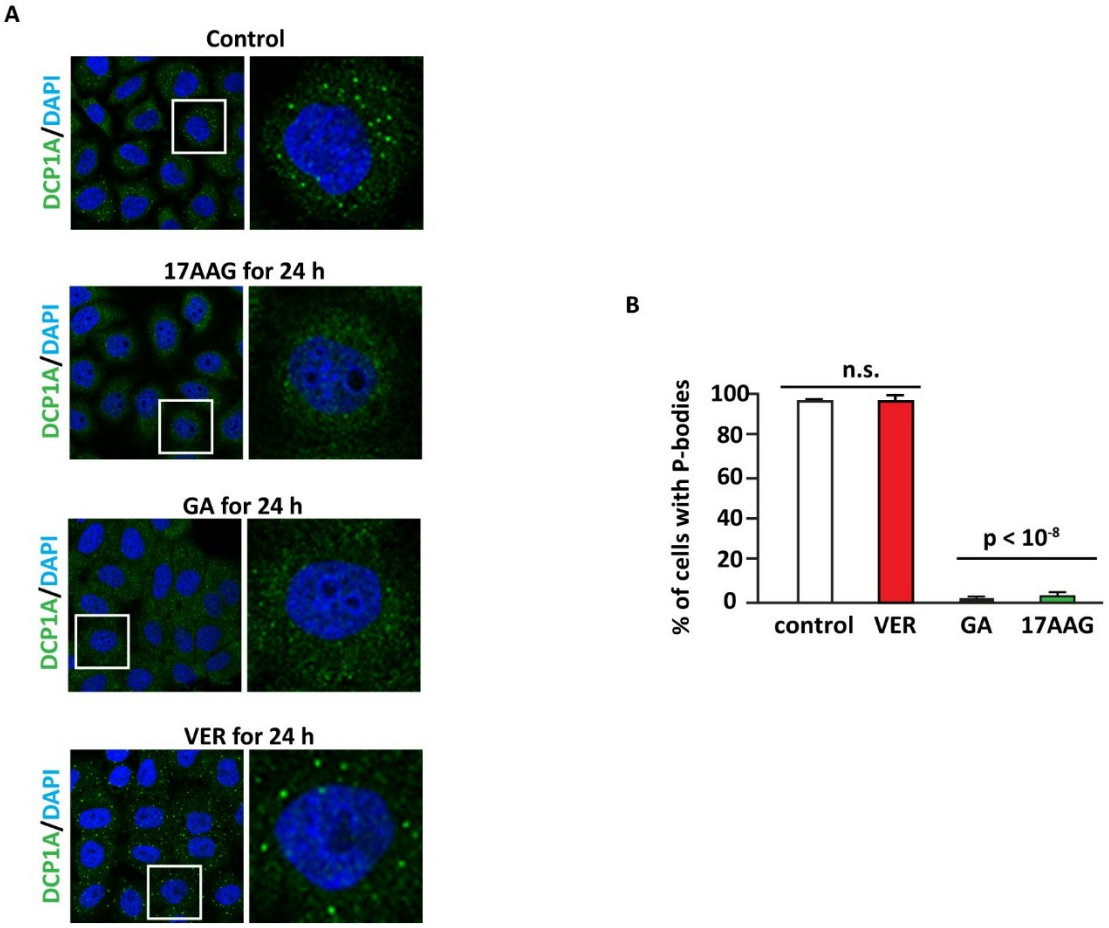

Figure 28: PBs and Hsp90 inhibition for 24 hrs.
A) HeLa Kyoto cells were treated for 24 hrs with drug-free medium or medium containing 17AAG 5 µM, GA 5 µM or VER 40 µM. Then, cells were fixed and stained with anti-DCP1A to label PBs and DAPI to label nuclei, followed by confocal microscopy analysis. B) Quantification of A: three independent experiments; the percentage of cells that contain PBs after 24 hrs of treatment is shown. Data are presented as histogram; ns= not significant.

We then studied whether short-term treatment of the cells with the Hsp90 inhibitors affects PB number. In contrast to long-term treatment, we found that treatment of HeLa Kyoto cells with GA or 17AAG for 4 hours only mildly affected the percentage of cells with PBs (Fig. 29A and Fig. 29B black and green column). Also, in this case inhibition of Hsp70 had no effect on PB number (Fig. 29A and Fig. 29B red column).

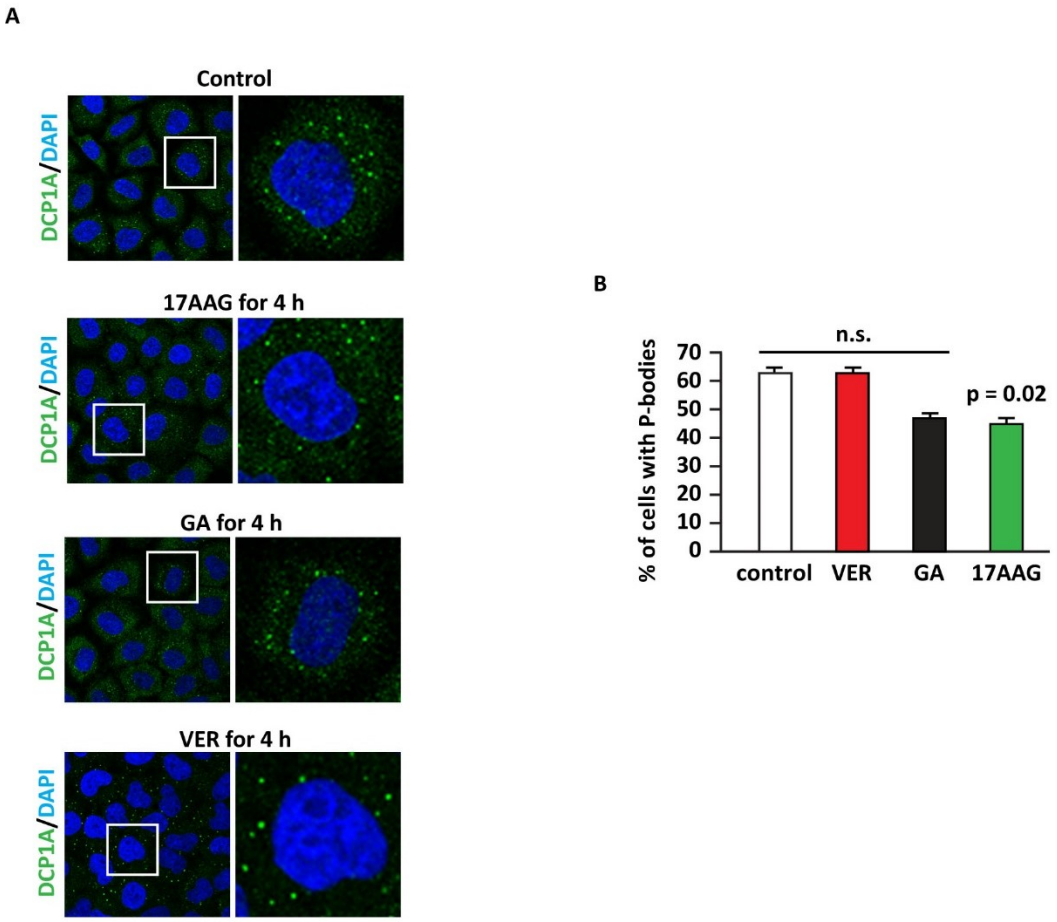

Figure 29: PBs and Hsp90 inhibition for 4 hrs.
A) HeLa Kyoto cells were treated for 4 hrs with drug-free medium or medium containing 17AAG 5µM, GA 5µM or VER 40 µM. Subsequently, cells were fixed and stained with anti-DCP1A to label PBs and DAPI, followed by confocal microscopy analysis. B) Quantification of A: three independent experiments; the percentage of cells that contain PBs after 4 hrs of treatment is shown. Data are presented as histogram; ns= not significant.

Considering that Hsp90 inhibition affects SG dynamics very rapidly, within 1 hour of treatment, while leaving unaffected PBs, our results suggest that GA and 17AAG perturb SG turnover independently on PB dynamics. To further test this hypothesis, we treated HeLa Kyoto cells with heat shock to induce SGs and after 2 hours when the cellular SGs response was maximal, we allowed the cells to recover at physiological temperature in drug-free medium or in presence of VER, GA or 17AAG for 2 hours. At this time-point, we visualized both SGs and PBs by immunostaining, focusing on presence or absence and their distribution (Fig. 30). We found that all cells contained PBs, regardless of the fact that SGs dissolved (recovery in drug-free medium) or persisted due to the inhibition of Hsp70 or Hsp90 ATPase activity. This result further suggests that the effects of Hsp90 inhibition on SG dissolution and PB formation are distinct events that occur with different kinetics.

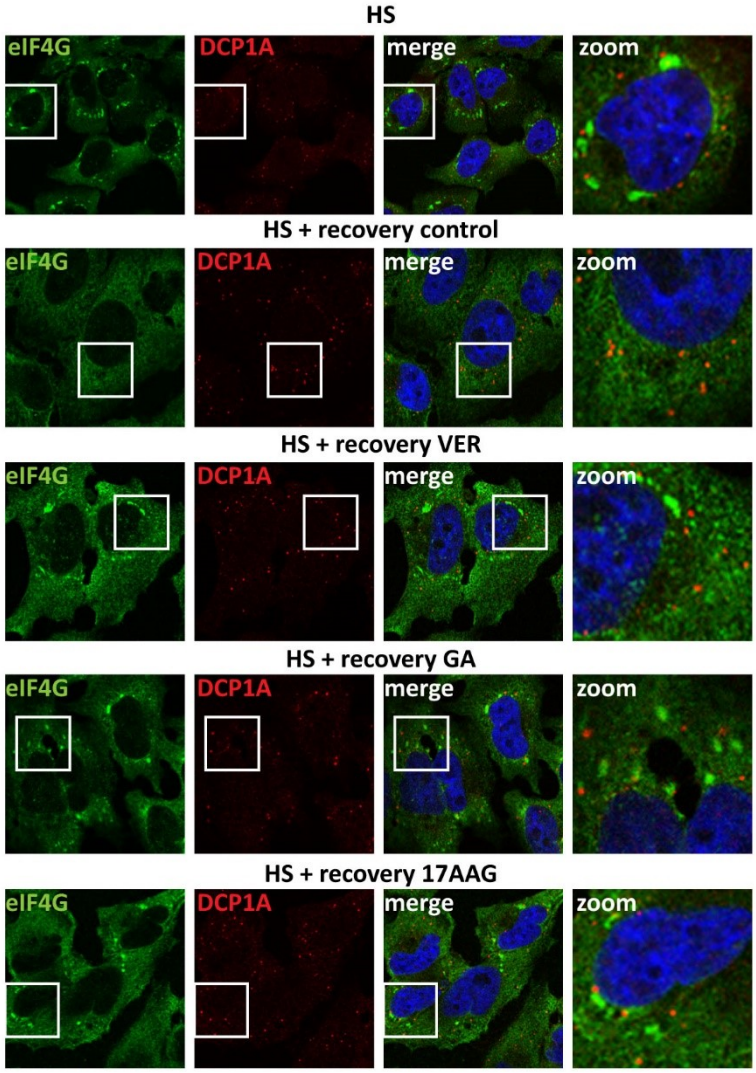

Figure 30: PBs and SGs.
HeLa Kyoto cells were treated for 2 hrs at 42 ℃, then cells were allowed to recover at physiological temperature in drug-free medium or in medium containing 17AAG 5 µM, GA 5 µM or VER 40 µM. Cells were fixed and stained with anti-eIF4G (to label SG), anti-DCP1A (to label PBs) and DAPI, followed by confocal microscopy analysis.

In mammalian cells, SGs and PBs show different movements; while SGs constantly fuse and divide, changing shape, PBs maintain their size and shape and move rapidly, often docking at SGs (Kedersha et al, 2005). To better investigate if the Hsp90 inhibition alters the dynamic behaviour of SGs and PBs, we performed live-cell imaging studies in HeLa Kyoto cells stably expressing G3BP2-GFP that were transiently transfected with a cDNA encoding for mRFP-DCP1A (Kedersha et al, 2008). SGs were induced by treating the cells with sodium arsenite, followed by recovery in drug-free medium or in presence of Hsp70 or Hsp90 inhibitors (Fig. 31). We observed dynamic behaviour of PBs and kissing events between PBs and SGs in all conditions tested, regardless of Hsp90 inhibition (Fig. 31).

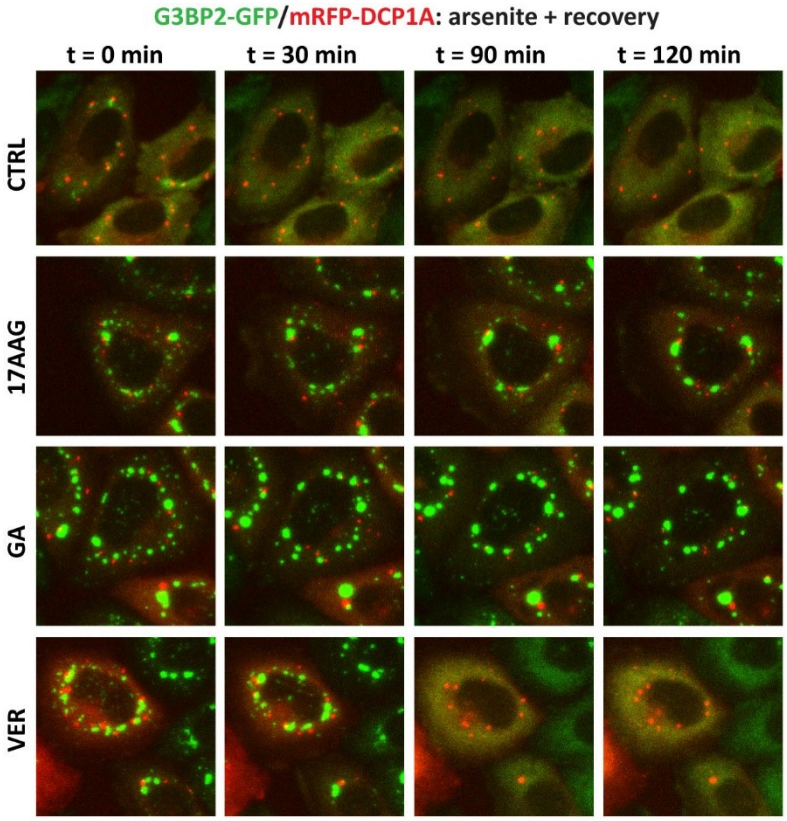

Figure 31: Dynamism of SGs and PBs.
HeLa Kyoto cells stably expressing G3BP2-GFP were lipofected with cDNA encoding for mRFP-DCP1A. 24 hrs after transfection, cells were stressed with sodium arsenite 50 µM for 45 min, followed by recovery in drug-free medium or in medium containing VER 40 µM, 17AAG 5 µM or GA 5 µM for 4 hrs. Images were extracted from live-imaging experiment movies each 30 min.

Combined these data support the interpretation that the delayed SG dissolution observed in presence of Hsp90 inhibitors is not due to altered PBs turnover. Future studies will be required to understand how mechanistically Hsp90 inhibition affects SG turnover.

The RQC member NEMF participates to the regulation of SG turnover

To further understand the role of the PQC machinery in the regulation of SG turnover, we focused on the ribosomal quality control (RQC), which, as mentioned in the introduction, senses defects at the levels of RNAs that lead to ribosome stalling and to the production of defective peptides (Brandman et al, 2012). Amongst the key players of the RQC are NEMF and Listerin (Brandman et al, 2012). We therefore silenced the expression of NEMF and Listerin and we verified if they affect SG formation and dissolution.

HeLa cells were transiently transfected with siRNA targeting specifically Listerin or NEMF. Because antibodies that specifically recognize these proteins in human protein extracts are still lacking, we relied on the mRNA expression levels of Listerin and NEMF in order to assess silencing efficiency. By qPCR analysis of Listerin and NEMF expression, we found that both siRNA significantly decreased the expression of their target mRNA (Fig. 32A and B). Thus, we used these siRNAs to silence NEMF and Listerin and therefore impair the RQC system.

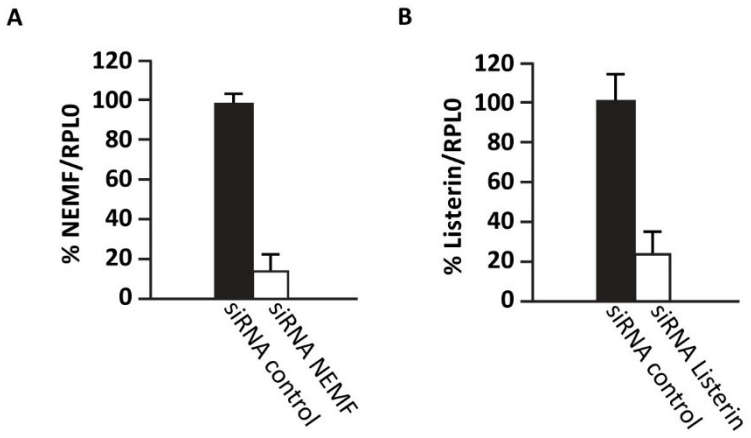

Figure 32: Silencing efficiency of Listerine and NEMF depletion.
A) HeLa cells were lipofected with control or NEMF specific siRNA for 72 hrs and then total RNA was extracted. The transcript levels of NEMF and RPL0 were measured by qPCR. B) HeLa cells were lipofected with control or Listerin specific siRNA for 72 hrs and then total RNA was extracted. The transcript levels of Listerin and RPL0 were measured by qPCR. RPL0 is a housekeeping gene and was used to normalize the expression levels of NEMF and Listerin.

Next, to validate the functional consequences of Listerin and NEMF depletion, we monitored the degradation of a typical Ribosome Quality Protein-model substrate, namely GFP lacking the stop codon (GFP-NS). The translation of this mRNA results in the addition of a C-terminal poly-lysine tract, encoded by the poly(A) tail. This causes the stalling of the

non-stop protein inside the ribosome (Ito-Harashima et al, 2007). GFP-NS is recognized and degraded with the assistance of the ribosomal quality control factors NEMF and Listerin and by proteasomes (Brandman et al, 2012). In agreement, we found that the expression levels of GFP-NS in control cells were extremely low, below detection levels. Instead, GFP-NS accumulated in cells after inhibition of the proteasome-mediated degradation (Fig. 33, lanes 2-4).

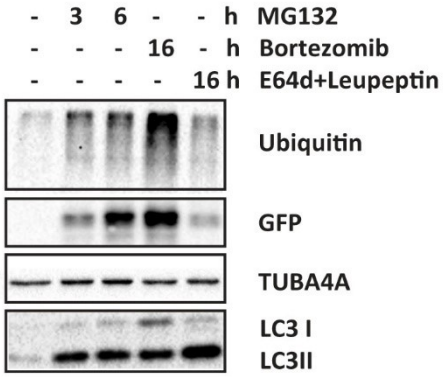

Figure 33: GFP-NS is mainly degraded by the proteasome.
HeLa cells were lipofected with a cDNA encoding for GFP-NS. 24 hrs post-transfection, cells were left untreated or treated for 3-6 hrs with MG132 20 µM or for 16 hrs with bortezomib 100nM in order to block the proteasome. To test the autophagy degradation, we inhibit it using for 16 hrs E64d 10 µg/mL in cotreatment with Leupeptin 200 µM. Total protein extracts were prepared and analysed by immunoblotting to measure the expression levels of GFP-NS, ubiquitinated proteins and LC3I and LC3II. TUBA4A was used as loading control.

Then, we measured the expression levels of GFP-NS in control or Listerin or NEMF depleted cells and we found that depletion of these RQC factors leads to the accumulation of GFP-NS (Fig.34).

Figure 34: GFP-NS accumulates in Listerin and NEMF depleted cells.
HeLa cells were silenced with control or NEMF or Listerin siRNAs for 72 hrs. In the last 24hrs, the cells were lipofected with a cDNA encoding for GFP-NS. Total protein extracts were then prepared and analysed by immunoblotting to detect the expression levels of GFP-NS. TUBA4A was used as loading control.

At this point, we investigated if depletion of Listerin and NEMF, which affect RQC, has an impact on SG formation or dissolution. First, we focused on SG assembly; we thus silenced HeLa cells for NEMF or Listerin and then we treated cells with MG132 for 3 hours to induce SGs (Fig. 35A). We found that the depletion of Listerin and NEMF did not alter the formation of SGs. Then, to study NEMF and Listerin implication in SG dissolution, we counted the percentage of cells with persistent SGs after treatment for 7 hours with MG132 (Fig. 35B). In fact, we previously published that, upon prolonged treatment with MG132, SGs tend to disassemble and completely disappear after 7-8 hours of treatment (Ganassi et al, 2016). after 7 hours of treatment with MG132, SGs persisted in less than 20% of the cells lipofected with a non-targeting control siRNA or with a Listerin specific siRNA (Fig. 35B, black column and white column). In contrast, more than 60% of the NEMF-depleted cells depleted in NEMF showed persistent SGs (Fig. 35B, grey column).

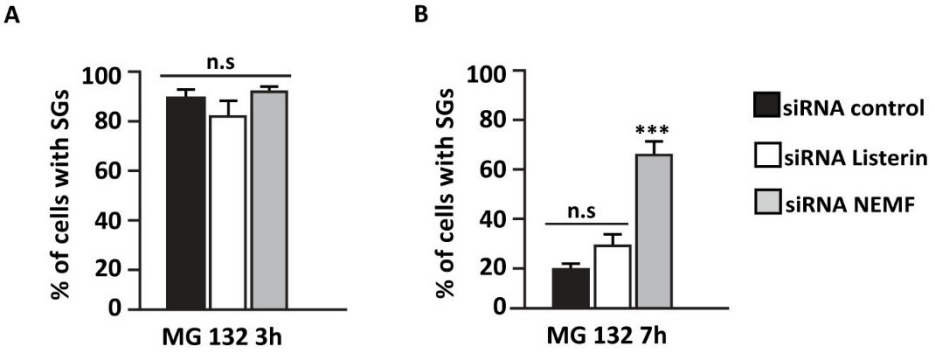

Figure 35: Depletion of NEMF severely delays the disassembly of SGs.
A) HeLa cells were treated with control or NEMF or Listerin siRNA. The cells were stressed with MG 132 20µM for 3 hrs. At this time point, the cells were fixed and immunostained with antibodies to label SGs (G3BP and TIA-1), followed by confocal microscopy analysis. B) HeLa cells were treated with control or NEMF, Listerin siRNA. The cells were stressed with MG 132 20µM for 7 hrs. At this time point cells were fixed and immunostained with antibodies to label SGs (G3BP and TIA-1), followed by confocal microscopy analysis. We quantified the percentage of cells that contain SGs; n = three independent experiments. Final data are presented as histogram; ns= not significant, ***P<0.001.

Combined these data suggest that Listerin is not involved in the regulation of SG turnover. Nevertheless, these results should be confirmed in the future when antibodies specific for Listerin will be available in order to verify the expression levels of the Listerin protein in our experimental conditions. In fact, we cannot presently exclude that the amount of Listerin proteins that is still present in Listerin -depleted cells may be sufficient to exert its essential functions. By contrast, NEMF seems to play a role in SG dissolution, with yet unclear mechanisms. Future studies should address the hypothesis that GFP-NS and RQC-substrates may accumulate inside SGs, thereby delaying their turnover.

Study the functional consequences of delayed SG dissolution in mammalian cells: focus on the mTORC1 signalling pathway

As previously mentioned, SGs contain RNAs, RNA-binding proteins, translation initiation factors and a number of signalling molecules, including protein kinases. Recently, SG assembly has been coupled to the regulation of important signalling pathways upon stress conditions, including the mammalian target of rapamycin (mTOR) signalling pathway (Fournier et al, 2013; Thedieck et al, 2013; Wippich et al, 2013). The mTOR signalling pathway comprises the mTORC1 and mTORC2 complexes and regulates many biological processes, including cellular growth, protein synthesis, autophagy and mRNA biogenesis. The mTORC1 and mTORC2 complexes contain a unique subset of components and, as such, they exert different functions (Laplante and Sabatini, 2012). mTORC1 complex contains RAPTOR, mammalian lethal with Sec13 protein 8 (mLST8), proline-rich Akt substrate of 40 kDa (PRAS40) and DEP domain containing mTOR-interacting protein (DEPTOR) (Hara et al, 2002; Yang et al, 2013; Sancak et al, 2007; Peterson et al, 2009). Instead, mTORC2 complex contains RICTOR, SIN1, PROTOR, XPLN, NBS1, IKKα and IKKβ21. Concerning the properties and function, mTORC1 complex is sensitive to the immunosuppressive drug rapamycin and it promotes protein and lipid synthesis by integrating several extracellular signals, such as growth factors and amino acid availability. Therefore, mTORC1 complex is essential for cell growth and survival. By contrast, mTORC2 complex activation and functions are less understood compared to mTORC1. mTORC2 complex phosphorylates and activates members of the AGC kinase family, including PKB, SGK1, and cPKCs and acts as a positive regulator of the organization and polarization of the actin cytoskeletal; however, like mTORC1 complex, also mTORC2 complex can regulate cell growth, proliferation, survival and differentiation.

In particular, it has been recently shown that the activity of the mTORC1 complex, which includes the component Raptor, is regulated via its partitioning in SGs, both in yeast and mammalian cells (Thedieck et al, 2013; Wippich et al, 2013). Upon stress, a pool of mTORC1, including Raptor, is sequestered inside SGs and, thus, it is not available to phosphorylate and activate its target proteins, such as e.g. the Ribosomal protein S6 kinase (p70S6K); this,

in turn, contributes to protein synthesis inhibition upon stress. In addition, during stress, the sequestration of Raptor inside SGs prevents the hyperactivation of mTORC1 and the induction of apoptosis (Thedieck et al, 2013). Instead, during the recovery phase after stress, mTORC1 is released from disassembling SGs and this pool becomes available for the reactivation of its substrates, stimulating translation and cell metabolism (Thedieck et al, 2013; Wippich et al, 2013).

Intriguingly, Hsp90 binds to Raptor and facilitates the phosphorylation of p70S6K mediated by mTORC1 (Ohji et al, 2006). Moreover, p70S6K itself has been suggested to be a client of Hsp90, since prolonged inhibition of Hsp90 ATPase activity with GA suppresses the phosphorylation of p70S6K mediated by mTORC1 and decreases p70S6K total levels (Ohji et al, 2006; Qian et al, 2010). Based on these findings we studied whether inhibition of Hsp90 during the recovery phase after stress specifically prevents the re-activation of the mTORC1 pathway or if this is a more general consequence linked to sequestration of mTORC1 players inside aberrant SGs. To address this point, we compared the effects of the Hsp70 and Hsp90 inhibitors on the release of Raptor from aberrant SGs and the subsequent activation of the mTORC1 pathway. In fact, since SGs can exchange components with the surrounding environment, we cannot exclude the possibility that SG that mature with time into an aberrant state change progressively their composition, becoming enriched for misfolded proteins and releasing other factors, such as e.g. Raptor. Thus, we transiently transfected myc-tagged Raptor in HeLa cells stably expressing G3BP1-mCherry, used as SG marker. Cells were either left untreated or treated with sodium arsenite to induce SGs. We found that myc-Raptor is specifically recruited inside arsenite-induced SGs (Fig. 36A), in agreement with previous findings (Thedieck et al, 2013). Next, we allowed the cells to recover in drug-free medium or in presence of the Hsp90 and Hsp70 inhibitors for 2 hours. Interestingly, myc-Raptor is sequestered inside aberrant SGs that persist in cells treated with GA, 17AAG (used both at a low and high concentration) and with VER (Fig. 36B). This result supports the idea that cells that show a defect in SG disassembly may experience metabolic stress.

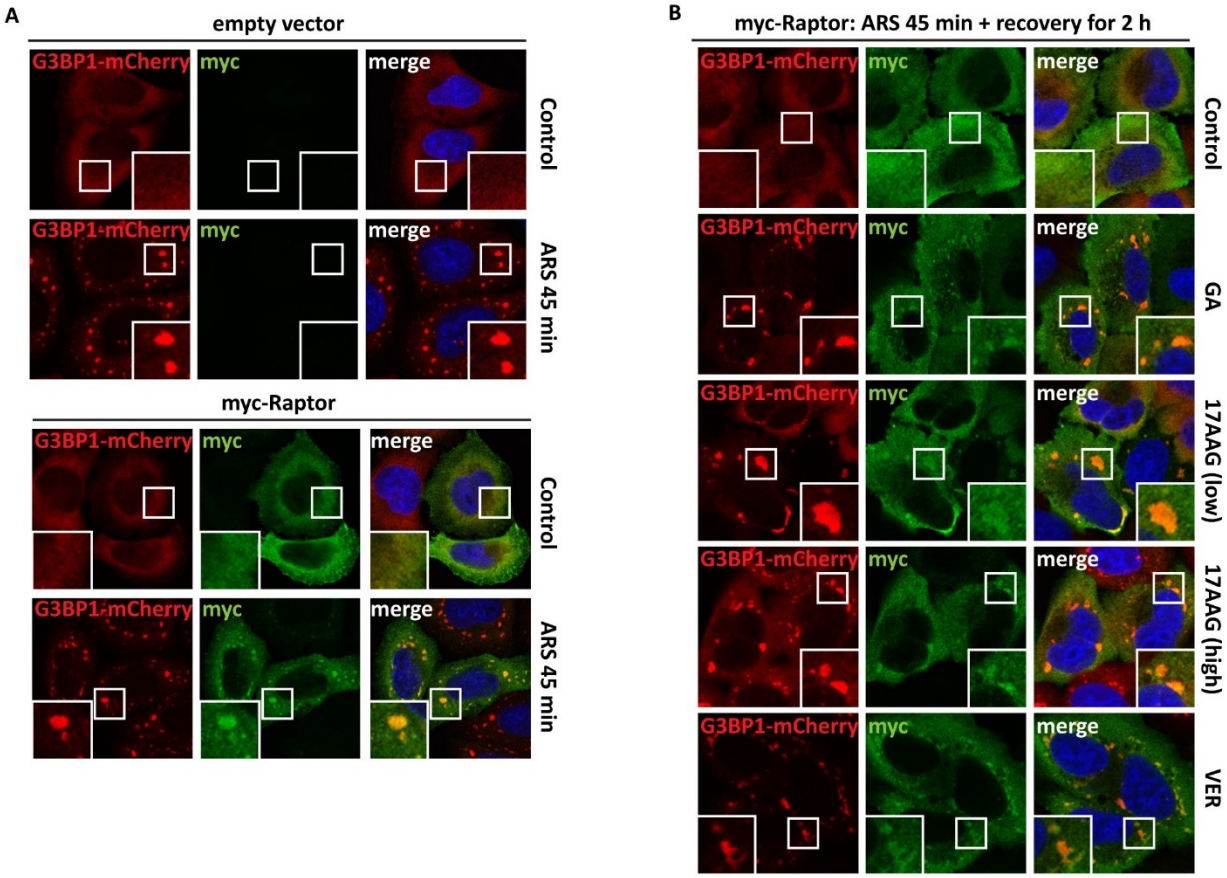

Figure 36: Raptor is sequestered inside aberrant SGs in cells with impaired Hsp90 and Hsp70 ATPase activity.
A) HeLa Kyoto cells stably expressing G3BP1-mCherry were lipofected with an empty vector or a cDNA encoding for myc-Raptor. 24 hrs after transfection, cells were either left untreated or treated for 45 min with sodium arsenite 50 µM. B) After arsenite treatment, cells transfected as described in A were allowed to recover for 2 hrs in drug-free medium or in presence of GA 5 µM, 17AAG 0.5 or 5 µM, low and high, respectively or VER 40 µM. Cells were fixed and stained with anti-myc and DAPI, followed by confocal microscopy analysis.

SG dissolution kinetics correlate with p70S6K phosphorylation during the stress recovery phase

We next asked what the functional consequence of Raptor sequestration inside aberrant SGs is, focusing on the activation or the mTORC1 pathway during the recovery phase after stress. First, we verified the phosphorylation of p70S6K during the recovery phase after exposure of the cells with sodium arsenite, in absence and presence of the Hsp90 inhibitors GA and 17AAG. p70S6K phosphorylation at threonine 389 by mTORC1 can be monitored both using a specific antibody for the phosphorylated form, as well as by judging the increased molecular weight and the subsequent upshift of P-p70S6K using an antibody that recognizes the total protein (Jung et al, 2003). Here, we mainly used the MW upshift of

total p70S6K to follow both changes in the total levels of the protein, as well as its phosphorylation.

It was previously reported that treatment of the cells with sodium arsenite rapidly induces the phosphorylation of Raptor and activates mTORC1, leading to the transient phosphorylation of p70S6K. This effect is mediated by the p38-β MAP kinase (Wu et al, 2011). In line, we observed a marked upshift of total p70S6K after 45 minutes of treatment with sodium arsenite, compared to untreated cells (Fig. 37 first and second lanes). These results indicate that, although a pool of mTORC1 is recruited inside SGs, this is not sufficient to prevent the phosphorylation of p70S6K after exposure to sodium arsenite. Concerning the recovery phase after stress, we found that the upshift of p70S6K was maintained after 4 hours of recovery in drug free-medium (control) (Fig. 37 third lane). This result suggests that, during the recovery phase, the pool of mTORC1 that is released from disassembled SGs can further phosphorylate p70S6K, thereby promoting the restoration of translation. If this interpretation is correct, conditions that prevent the dissolution of SGs should decrease the ability of the cells to sustain, during the stress recovery phase, the phosphorylation of p70S6K that was triggered by the sodium arsenite treatment via the p38-β MAP kinase/mTORC1 pathway. In line with this idea, we found that p70S6K upshift was lost in cells treated with 17AAG (5 μM) for 4 hours (Fig. 37 fourth lane). Importantly, nearly 100% of the cells exposed to 17AAG (5 μM) still contained persistent SGs after 4 hours of recovery time. Instead, p70S6K upshift was maintained during the recovery phase in cells that were treated with GA, similarly to control cells (Fig. 37 fifth and third lanes). Of note, although with a slower kinetic compared to control cells, SGs could disassemble in nearly 70 % of the cells exposed to GA for 4 hours, as previously showed. These data suggest that, during the recovery phase after stress, a direct correlation exists between the ability of the cells to sustain the arsenite-induced phosphorylation of p70S6K and the disassembly kinetic of SGs.

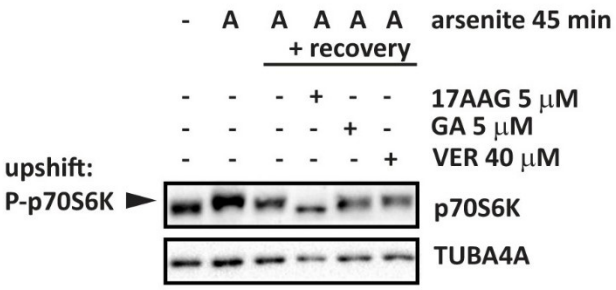

Figure 37: phosphorylation of p70S6K during the recovery phase.
Hela Kyoto cells stably expressing G3BP2-GFP were left untreated (-) or treated (A) with sodium arsenite 50 µM for 45 min and allowed to recover in drug-free medium or medium containing 17AAG 5 µM, GA 5 µM or VER 40 µM until 4 hrs. From each sample was prepared a total protein extract and was analysed by immunoblotting to detect p70S6K, TUBA4A was used as loading control. Phosphatase inhibitors were used to preserve protein phosphorylation.

To corroborate this interpretation, we verified p70S6K upshift in cells exposed to a lower concentration of 17AAG (0.5 µM instead of 5 µM). The reason for choosing this lower concentration of 17AAG (0.5 µM) is that we found that under these conditions, SG disassemble during the recovery time with a kinetic that is similar to the one observed for control, 4 hours after the recovery period, SGs are disassembled (Fig. 38A, yellow line). In line with our interpretation, p70S6K upshift was maintained during the recovery phase after stress in presence of the lower concentration of 17AAG (Fig. 38B fifth lane); this p70S6K upshift was similar to the one found in cells that were allowed to recover in drug-free medium or in presence of GA or of VER, where circa 70% of the cells dissolved their SGs at the 4 hours' time-point (Fig. 38B).

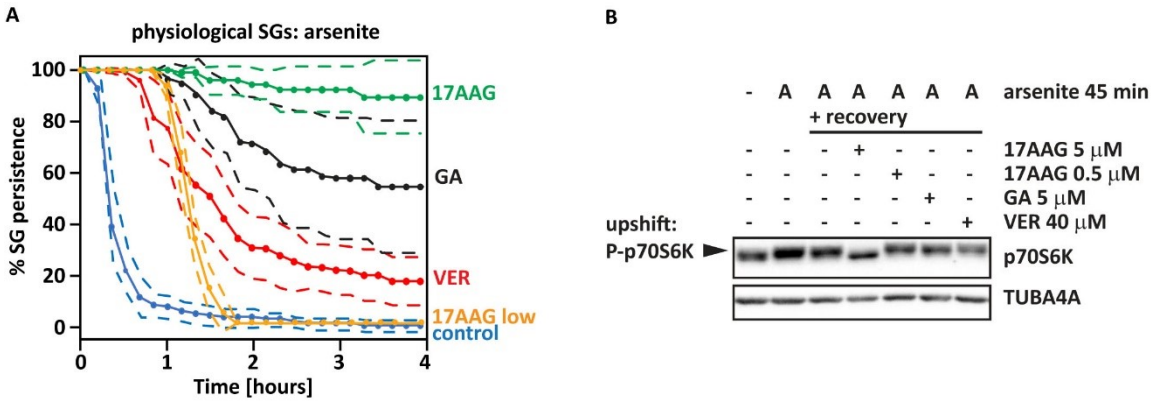

Figure 38: correlation between SGs disassembly and up-shift of p70S6K
A) Hela Kyoto cells stably expressing G3BP2-GFP were treated with sodium arsenite 50 µM for 45 min and allowed to recover in drug-free medium (193 cells, blue line) or medium containing 17AAG 5 µM (187 cells, green line) or 0.5µM (190 cells, yellow line), GA 5 µM (201 cells, black line) and VER 40 µM (209 cells, red line)

until 4 hrs. Quantification of SG persistence analysed from live-cell imaging. Dashed lines = 95% confidence intervals. B) Hela Kyoto cells stably expressing G3BP2-GFP were untreated (-) or treated (A) with sodium arsenite 50 µM for 45 min and allowed to recover in drug-free medium or medium containing 17AAG 5 µM or 0.5 µM, GA 5 µM and VER 40 µM until 4 hrs. Total lysates from each condition were analysed by western blot for p70S6K and TUBA4A was used as loading control. Phosphatase inhibitors were used to preserve protein phosphorylation.

To further test the functional consequences of delayed SG dissolution and sequestration of Raptor inside aberrant SGs, we monitored the translation rate, using puromycin, in samples subjected to treatment with arsenite alone or combined with severe inhibition of Hsp90. Puromycin is a structural analogue of tRNA that can be incorporated into elongating peptide chains. As a consequence, the chain elongation stops, and puromycin-marked peptides are released from the ribosome. Puromycin-labelled peptides thus reflect the overall rate of protein synthesis at a given time-point. Puromycin was added only during the last 15 minutes of treatment. We found that untreated cells incorporate puromycin, witnessing the ongoing protein synthesis (Fig. 39 second lane). As expected, treatment with sodium arsenite for 30 minutes suppresses puromycin incorporation (Fig. 39 third lane), which is instead partly restored during the recovery phase in drug-free medium, when SG disassemble from all cells (Fig. 39 fourth lane). Treatment of the cells with 17AAG at the higher concentration (5 µM), which leads to SG persistency after 4 hours in the majority of the cells, also strongly impairs puromycin incorporation (Fig. 39 fifth lane). This correlates with the lack of upshift of P-p70S6K, which is shown in Figures 37 and 38. Combined the data presented in Figure 39 support the interpretation that sequestration of Raptor inside SGs, which is important during stress to avoid hyperactivation of mTORC1 and apoptosis (Thedieck et al, 2013), can exert a detrimental effect on the ability of the cell to restore translation and metabolism when SG disassembly is impaired. This effect is directly linked to Raptor sequestration and defective activation of the mTORC1 pathway. Thus, we conclude that conditions that severely impair the dissolution of SGs compromise the mTORC1 pathway, with functional consequences on cell metabolism and growth (translation).

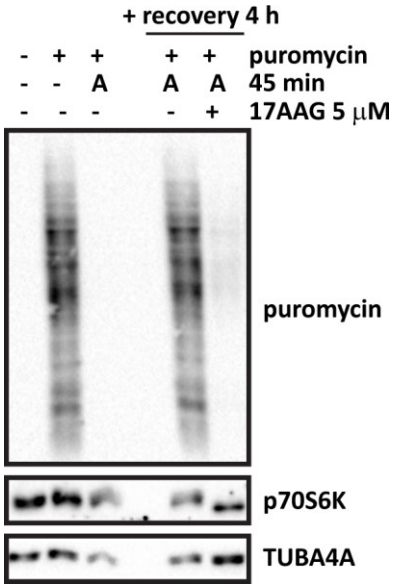

Figure 39: Rate of protein synthesis.
HeLa cells were either left untreated (-) or treated for 45 min with sodium arsenite (A). When indicated cells were allowed to recover for 4 hrs in drug-free medium (-) or in presence of 17AAG (5 μM). Puromycin was added (+) during the last 15 min of treatment. Total protein extracts were prepared and subject to immunoblotting using the antibodies indicated in the figure.

Like Raptor, also DYRK3 is sequestered inside aberrant SGs that show delayed disassembly kinetics

Dissolution of SGs and release of the signalling molecules that are transiently sequestered inside SGs can be regulated by several factors, amongst which is the dual specificity tyrosine-phosphorylation-regulated kinase 3 (DYRK3). During cellular stress, DYRK3 partitions between SGs and the cytosol via a low-complexity domain that is located in its N terminus. When DYRK3 is inactive, it prevents the dissolution of SGs (Wippich et al., 2013). As a result, mTORC1 is sequestered inside SGs and the pathway is inhibited. When DYRK3 is active, it promotes the dissolution of SGs and in parallel, it phosphorylates PRAS40, blocking its activity. Since PRAS40 has an inhibitory effect on mTORC1, phosphorylation and inhibition of PRAS40 by DYRK3 results in the activation of mTORC1. Thus, the partitioning of DYRK3 represents a mechanism by which cytoplasmic phase transition can compartmentalize specific cellular components, influencing cell signalling and ultimately cell metabolism (Wippich et al, 2013). These results lead to the following question: is DYRK3 sequestered inside aberrant SGs? Sequestration of DYRK3 inside aberrant SGs could contribute, together with sequestration of Raptor to the impaired activation of the mTORC1 pathway during the recovery phase after stress.

We thus verified if DYRK3 colocalizes with stress granules induced by sodium arsenite treatment and if it is sequestered inside the aberrant SGs that show impaired disassembly due to inhibition of Hsp90 and Hsp70 (Figure 40).

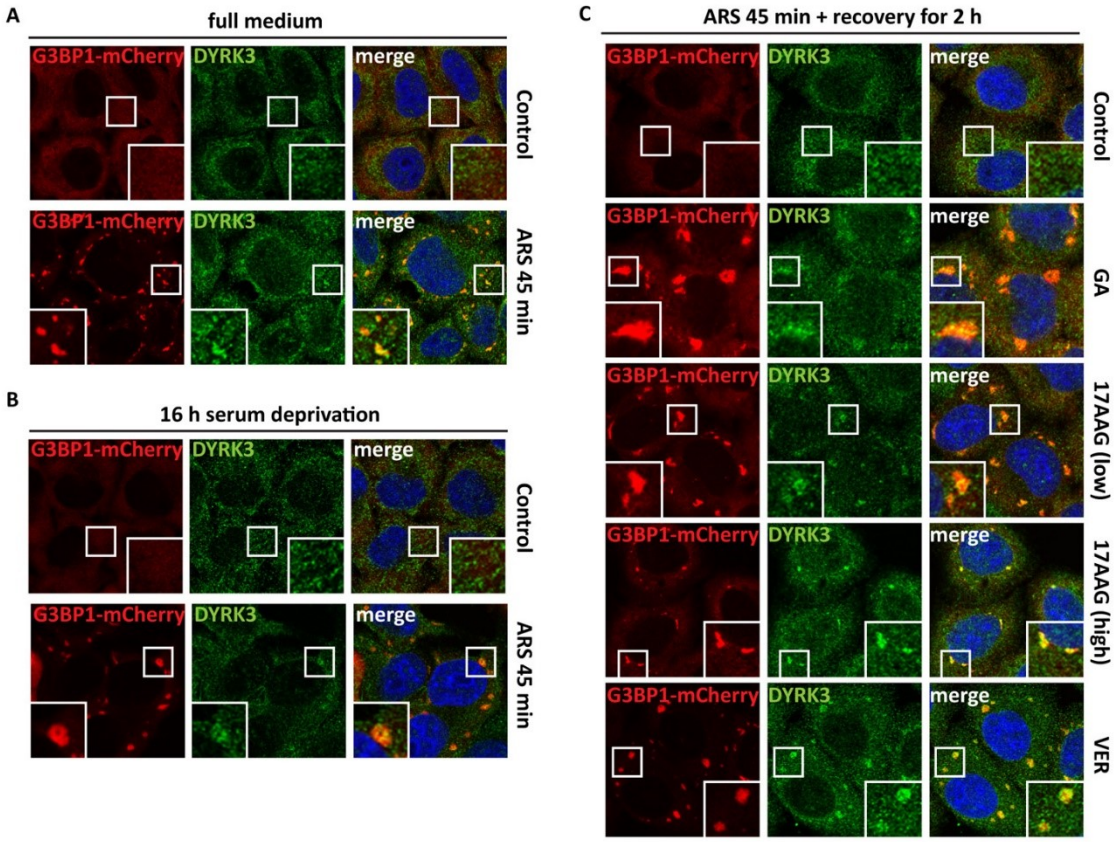

Figure 40: DYRK3 is sequestered inside SGs and also in SG of cells with impaired Hsp90 and Hsp70 ATPase activity.
A) HeLa Kyoto cells stably expressing G3BP1-mCherry were left untreated or treated with sodium arsenite 50 µM for 45 min. B) HeLa Kyoto cells stably expressing G3BP1-mCherry were grown for 16 hrs in a medium that lacks FBS (serum deprivation), prior to induction of SGs with sodium arsenite 50 µM for 45 min. C) HeLa Kyoto cells stably expressing G3BP1-mCherry were treated with sodium arsenite 50 µM for 45 min; then, cells were allowed to recover for 2 hrs in drug-free medium or in presence of GA 5 µM, 17AAG 0.5 or 5 µM, low and high, respectively or VER 40 µM. Cells were fixed and stained with anti-DYRK3 and DAPI, followed by confocal microscopy analysis.

DYRK3 is recruited inside SGs induced by sodium arsenite both in cells grown in full medium as well as after 16 hours of serum deprivation (Fig. 40A and B). When SGs disassemble, during the recovery time, DYRK3 is released and partitions into the cytoplasm. However, DYRK3 becomes sequestered inside SGs that show delayed disassembly kinetics due to inhibition of both Hsp70 and Hsp90 ATPase activity (Fig. 40C). These results suggest that DYRK3 persistence inside SGs is not a consequence of impaired Hsp90 activity, since we observe a similar effect with the Hsp70 inhibitor; they support the interpretation that

DYRK3 sequestration inside SGs is a consequence of their persistence; this, in turn, might be due to inhibition of the kinase activity of DYRK3 (which is so important to regulate SG unmixing). Thus, cells with persistent SGs may experience both impaired activation of mTORC1 and impaired activity of DYRK3, influencing their metabolism and functionality.

Discussion:

Stress granules are cytoplasmic membrane-less condensates that contain translationally stalled mRNAs, pre-initiation factors and RNA-binding proteins (RBPs) (Anderson and Kedersha, 2009). SGs are induced by various stress conditions and form via a process known as liquid-liquid phase separation, which is driven by SG-resident RBPs with intrinsically disordered regions (Banani et al, 2017). In mammalian cells, SGs appear as dynamic liquid-like condensates that rapidly dissolve upon stress relief. Recent evidence demonstrates that SGs can change their material properties and convert from a dynamic liquid-like state into a solid-like state, referred to as physiological and aberrant states, respectively.

The accumulation of inclusion bodies that contain components of SGs has been connected to the onset and development of age-related neurodegenerative diseases (Lagier-Tourenne et al, 2010; Bentmann et al, 2013; Alberti and Carra, 2018). Genetic and biochemical experimental evidence demonstrates that SG-resident RBPs are unstable and spontaneously convert from liquid and dynamic droplets into hydrogel and, finally into amyloid-like fibers that are reminiscent of the pathological aggregates found in the patient autopsy (Alberti, 2017). Importantly, the rate of conversion and maturation of these RBPs from a physiological liquid state into aggregates is accelerated by the disease-linked mutations, leading to the hypothesis that SGs can mature into pathological “aberrant” aggregates. Thus, understanding the molecular causes that trigger the formation of aberrant solid-like SGs and identifying rescuing factors have important therapeutic value.

Recent work from independent research groups, including the lab of prof. Carra, demonstrated that two main mechanisms that convert physiological SGs into aberrant SGs. First, genetic mutations in SG-resident RBPs increase their aggregation propensity and promote their “fibrillization” in test-tube, while promoting their irreversible aggregation in cells. The second mechanism is represented by the accumulation of misfolded proteins and defective translation products (DRiPs) inside SGs. Formation of aberrant SGs is prevented by specific protein quality control machineries: the HspB8-BAG3-Hsp70 complex and the ZFAND1/VCP/26S proteasome complex. Both complexes target misfolded proteins to clearance, via autophagy and the proteasome, respectively and prevent the accumulation of DRiPs inside SGs, thus ensuring timely SG dissolution and translation restoration (Ganassi

et al, 2016; Turakhiya et al, 2018). Combined these data demonstrate that a tight cross-talk between the PQC system and SG dynamics exists.

In this thesis, we investigated the fate of physiological and aberrant SGs and the interplay between SGs and the PQC system, with the goal of identifying other PQC players that may counteract the conversion of SGs into an aberrant persistent state.

Previous data published by Prof. Serena Carra showed that only a minor fraction (circa 10%) of physiological SGs are cleared by the cells through the autophagy pathway (Ganassi et al, 2016). Here, we demonstrate that in mammalian cells, both physiological and aberrant SGs are not preferentially degraded by autophagy (Figure 17). In any case, autophagy is an important degradative pathway for damaged organelle and aggregated proteins; indeed, it has been demonstrated that SGs containing aggregated proteins are transported along the microtubules and are targeted to aggresomes, where they can be degraded through autophagy (Mateju et al, 2017). However, live-cell imaging studies aimed at following SG fate demonstrated that during this transport process the size of SG progressively decreases, suggesting that cells try to disassemble SGs with the assistance of the above-mentioned molecular chaperones (Mateju et al, 2017). From an energetic point of view SG dissolution represents a more attractive choice, since it enables the recycling of SG components, which include ribosomes, mRNAs, translation factors and signalling molecules.

Then, we report the implication of another surveillance system in SG turnover: the RQC machinery. NEMF is a key player of the RQC and seems to play a role in SG dissolution; indeed, we found that NEMF-depleted cells showed a persistence of SGs (Figure 35), opening the possibility that also DRiPs generated from stalled ribosomes may affect SG turnover. Although this is an attractive hypothesis, for time restraint during my PhD I could not further address how mechanistically NEMF depletion delays SG disassembly. Nevertheless, taken together our data indicate that SGs are highly sensitive to protein misfolding and require the constant assistance of PQC and RQC to maintain their liquid-like dynamic behaviour. This, in turn, maintains the functionality of SGs and prevents the formation of protein-RNA aggregates, whose accumulation is a typical hallmark of aging and age-related neurodegenerative diseases. Thus, drugs that boost the function of these

granulostasis chaperones and boost the clearance of DRiPs and stalled peptides may hold promise for the treatment of neurodegenerative diseases.

Next, we identified Hsp90 as a novel player in the regulation of SG turnover. Detailed investigation of Hsp90 effect on SGs allowed us to demonstrate that the inhibition of its chaperone activity severely alters the ability of the cells to disassemble both physiological and aberrant SGs (Figure 19 and 22). Subsequent studies underlined that this effect is unlikely related to DRiP enrichment in SGs (Figure 26B). Since Hsp90 was shown to regulate PB number and formation and considering that PBs and SGs are in equilibrium and exchange components, we then went on investigating the possible impact of altered PB biogenesis on SG turnover in cells with defective Hsp90 function. We found that the kinetics of the inhibitory effect of Hsp90 on SGs and PBs are different and we excluded altered PB dynamics (at least upon short-term inhibition of Hsp90) as mechanism regulating SG dissolution (Figure 29, 30, 31).

In light of the role of Hsp90 in the proper folding and maturation of a large variety of clients (Jain et al, 2016; Markmiller et al, 2018), which also include SG-resident proteins, we then hypothesized that unfolding and loss of function of specific SG resident proteins that are clients of Hsp90 may be at the basis of the defective SG dissolution observed. Several possible candidates exist. This hypothesis and the identification of the candidate/s responsible for altered SG dynamics upon Hsp90 inhibition certainly will deserve future investigation.

Then, we studied the functional implications of persistent SGs, focusing on the activity of key signalling pathways. This because recently, SG assembly has been coupled to the regulation of one particular important signalling pathway upon stress conditions, the mTOR pathway that regulates cell metabolism and cell growth (Fournier et al, 2013; Thedieck et al, 2013; Wippich et al, 2013). In particular, it was shown that the activity of the mTORC1 complex that is formed by RAPTOR, mLST8, PRAS40, DEPTOR (Hara et al, 2002; Yang et al, 2013; Sancak et al, 2007; Peterson et al, 2009), activity is regulated via its partitioning in SG (Wippich et al, 2013). Here we demonstrated that aberrant SGs, which persist inside the cells, sequester the mTORC1 component Raptor (Figure 36). The segregation of Raptor can

exert a negative effect on the cellular ability to activate the mTORC1 pathway during the recovery phase after stress; this, in turn, can alter the cellular metabolism (Figure 37) and the translation resumption (Figure 39). In addition, we found that the kinase DYRK3 itself becomes sequestered inside aberrant SGs (Figure 40C). However, DYRK3 sequestration occurs both upon Hsp70 and Hsp90 inhibition. We therefore conclude that DYRK3 sequestration inside persistent SGs is not a specific consequence of Hsp90 inactivation, but rather a general consequence of defective SG dissolution. Thus, we demonstrate that cells with persistent SGs may experience impaired activation of both mTORC1 and DYRK3, influencing their metabolism and functionality. Collectively the results obtained during this PhD thesis show that aberrant SGs can exert harmful effects at least with two distinct mechanisms: first, by promoting the formation of aggregates, which may burden the PQC system, contributing to cell toxicity (previous work); second, sequestering during the recovery phase after stress signalling molecules, such as the mTORC1 complex and the DYRK3 kinase itself, thereby compromising cell growth and metabolism.

To summarize, our data demonstrate that a tight interplay between the PQC and SGs exists. The identification of the role of Hsp90 in SG regulation points to the need of a better understanding of how this mechanistically occurs. This specific aspect certainly deserves future investigations because it could provide a comprehensive understanding of the effects of Hsp90 on metabolic pathways, which could be mediated, at least in part, via the regulation of SG turnover. Understanding how Hsp90 regulates SG turnover and how this is linked to cell functionality and metabolism/growth may have important implications beyond neurodegenerative diseases. In fact, drugs that inhibit Hsp90 are currently used for the treatment of several types of cancers, such as the FDA-approved bortezomib (Velcade), which can induce SGs. Interestingly, while used for the treatment of myelomas and other hematological tumors, solid tumours are bortezomib resistant (Kane et al, 2003). Fournier and colleagues demonstrated that solid tumors are refractory to drug treatment and the cancer cells which present bortezomib resistance subsequently to pharmacological treatment generate SGs and survive to apoptosis. These results suggest that the formation of SGs might represent a survival mechanism by which cancer cells resist to bortezomib-mediated apoptosis (Fournier et al, 2010). If timely dissolution of SGs enables cell survival to chemotherapeutic treatments, one could wonder what the consequences of delayed SG

dissolution in these cells will be. Clinical trial that combine Velcade and Hsp90 inhibitors (GA or 17AAG) are currently ongoing (Walker et al, 2013). Combination of Hsp90 inhibitors, which delay SG dissolution and Velcade may have deleterious effects on cancerous cell ability to restore translation and metabolism, enhancing therapeutic efficacy. This speculative hypothesis may deserve future investigations.

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