

CHARACTERIZING THE ROLE OF RSP5 IN STRESS GRANULE CLEARANCE

by

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Abstract

Stress granules (SGs) are conserved cytoplasmic condensates of non-translating mRNA and associated RNA-binding proteins. SG assembly is induced by the accumulation of non-translating mRNAs during adaptation of cells to stressful environments. While SG assembly is relatively well understood, many mechanisms including chaperone function, post-translational modifications, proteasomal and autophagic (granulophagy) degradation have been reported to function in SG clearance. However, the mechanisms, utilization and integration of these clearance pathways is poorly understood. Understanding SG clearance is important as it likely impacts post-transcriptional gene expression, and possibly other SG-related functions. Studying SG clearance may also aid in furthering our understanding of diseases such as Amyotrophic Lateral Sclerosis (ALS) and Cancer, where aberrant persistence of SGs is theorized to contribute to disease pathology. In this work, we performed an unbiased genome wide screen in *Saccharomyces cerevisiae* (budding yeast) to identify regulators of SG clearance via granulophagy. This screen identified several genes involved in ubiquitin-related biology, including co-factors of the essential E3 ligase Rsp5, which adds K63-linked ubiquitin chains to substrates which facilitates vacuolar/lysosomal trafficking. Using conditional genetics, we find that Rsp5 strongly promotes SG clearance after heat shock (HS) and stationary phase stress (SP), but not sodium azide stress. Additionally, our preliminary data suggests that Rsp5 colocalizes with SGs during the initial phase of stress recovery. Rsp5-mediated SG clearance represents a novel stress specific clearance mechanism, which given the presence of a clear homolog in human cells (NEDD4), could ultimately be harnessed as a therapeutic target to counter SG-associated disease pathology.

Introduction:

In order to survive, divide, and maintain proper cellular function, cells must adapt to different stressful environmental cues. The stresses faced by cells are diverse and include oxidative stress, nutrient deprivation, osmotic stress and temperature shock. As a response to stress, cells rapidly activate the integrated stress response (ISR) as a means of stress adaptation by repressing bulk protein synthesis and upregulating the translation of various stress adaptive mRNAs [1].

Translation involves the association of the small ribosomal subunit (40S) with eukaryotic initiation factors (eIF)s 1, 1A, 3, and 5. Next, the “ternary complex”, consisting of eIF2-GTP-Met-tRNA also binds to 40S, forming the 43S preinitiation complex (PIC). The PIC is then recruited to an mRNA bound by the eIF4F complex, forming the 48S initiation complex. The eIF4F complex consists of the cap-binding protein eIF4E, the scaffold protein eIF4G and the RNA helicase eIF4A, which facilitates 5'UTR structure unwinding. This aids scanning of the small ribosomal subunit in search of a start codon cognate to Met-tRNA (most commonly AUG), though other RNA helicases (e.g. Ded1) may also aid in this function [2] [3]. As soon as a start codon is found, eIF5 promotes the hydrolysis of GTP to GDP on eIF2. Subsequent binding by eIF5B-GTP and the large ribosomal subunit (60S) results in displacement of most eIFs, and the formation of a mature 80S ribosome complex. A second GTP hydrolysis event by eIF5B leads to displacement of itself and other remaining eIFs, and the commencement of translation elongation [4] (**Figure 1**).

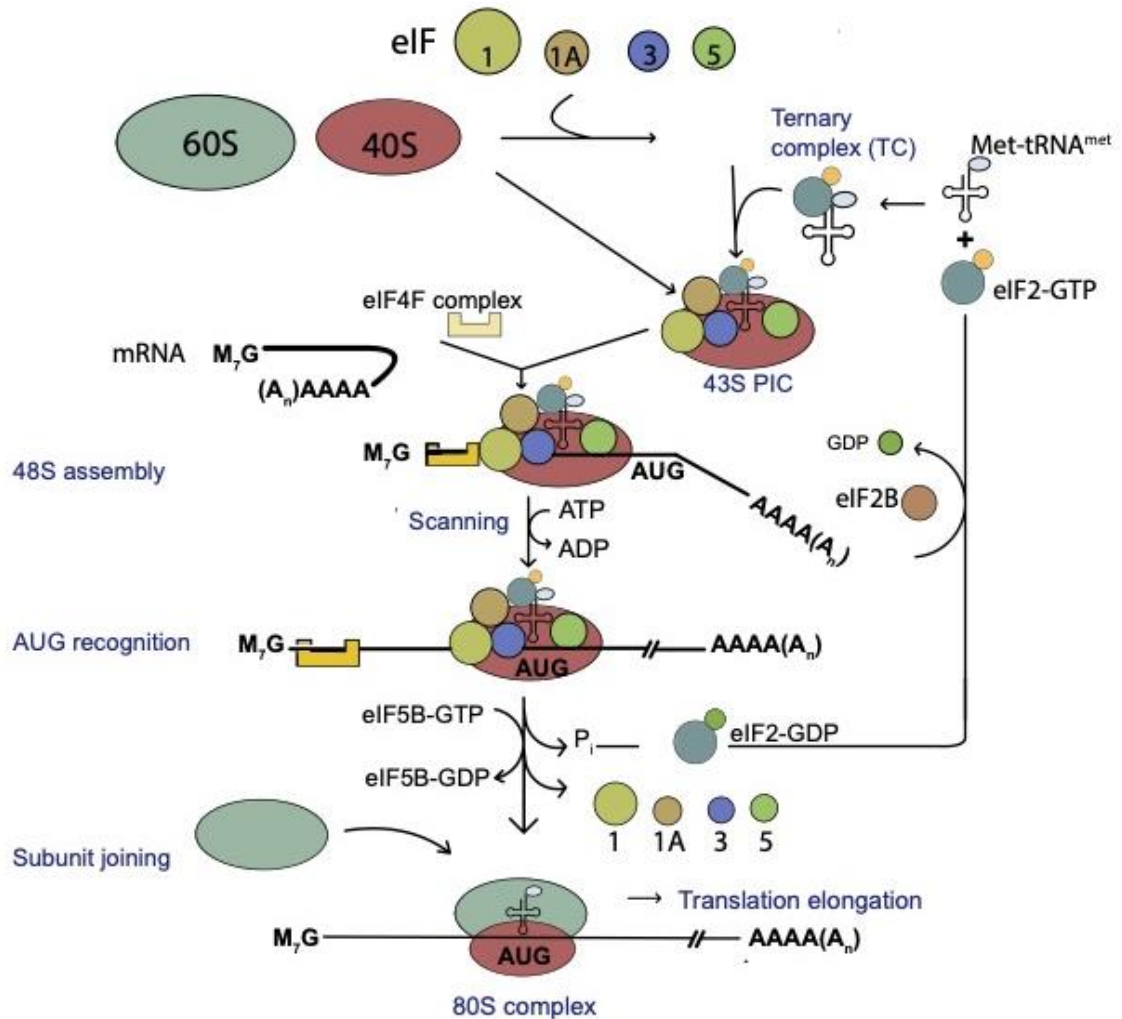


Figure 1: Translation initiation. In brief, (1) translation initiation begins with the 40S small ribosomal unit recruiting eIFs 1, 1A, 3. Once in complex with the 40S subunit, eIF5 and eIF2-GTP-Met-tRNA are recruited and assembled to form the 43S PIC. The eIF4F complex then interacts with mRNA via the 5' cap to facilitate mRNA unwinding, placement and scanning of mRNA for the start codon. Once the start codon is found, GTP hydrolysis of eIF5B mediates the disassociation of most eIF factors, allowing for the association of the large ribosomal subunit and initiation of translation elongation. (Figure adapted Sonenberg et al 2009,[5])

The key rate limiting step in translation initiation is the availability of ternary complex. In order to bring Met-tRNA to the small ribosomal subunit, eIF2-GDP must undergo GDP-GTP exchange, which is driven by its interaction with eIF2B, a guanine exchange factor. During most stress conditions, the ISR activates serine/threonine kinases that lead to phosphorylation of eIF2. This phosphorylation decreases translation initiation by disrupting the formation of the eIF2-GTP-Met-tRNA complex, specifically by preventing the exchange of GDP to GTP by eIF2B. An incomplete PIC, lacking the charged Met-tRNA necessary for translation initiation [5], leads to an ensemble of liquid-liquid phase separated anomalies driven by spontaneous multivalent interactions [6]. This results in the accumulation of translationally stalled messenger ribonucleoproteins (mRNP), which are the key component necessary for assembly of stress granules (SGs) [7-10].

SGs are cytoplasmic biomolecular condensates, which lack a limiting membrane, and which are enriched in non-translating mRNPs and numerous other proteins and small molecules [11-13]. Assembly of SGs is driven by RNA-RNA, RNA-protein, and protein-protein interactions, with protein assembly factors often harboring intrinsically disordered regions (IDRs) usually rich in Asp and Glu that are key for their assembly-promoting functions. Non-translating mRNA is also essential to SG assembly as studies in numerous models have demonstrated that trapping mRNAs in association with ribosomes (e.g., with cycloheximide, emetine) blocks or actively disassembles SGs, whereas disrupting polysomes (e.g., with puromycin) increases SGs [7, 9, 14]. mRNA isolated from yeast can even assemble into biomolecular condensates *in vitro* that strongly resemble SG transcriptomes *in vivo* [15].

SGs are highly dynamic, at least during the initial periods of a stress response. Fluorescence recovery after photobleaching (FRAP) experiments on multiple SG protein and RNA components indicate that most SG factors enter and exit SGs within seconds to minutes [8]. More recent data using super-resolution microscopy indicates that SGs harbor a substructure, with an outer shell that is more dynamic than inner “cores”, that are likely maintained via electrostatic and hydrophobic interactions based on their dissolution in the presence of high salt and 1,6 hexanediol [12].

Based on the type of stress, SGs can be assembled in a canonical eIF2 phosphorylation dependent or independent manner [9, 16]. The limit on translation initiation is not the only factor that influences the assembly of SGs. Many proteins that localize in SGs that support SG assembly and have been related to functions with translation initiation, translational repressors, RNA binding proteins (RBPs), mRNA stabilizers, RNA metabolism and many others. The over-expression of nucleating SG proteins such as G3BP1 and TIA1 in mammalian cells was shown to be sufficient to induce SGs in the absence of stress [17]. These factors show the diverse topology of SGs and their driving forces for assembly.

SGs have been implicated in cell survival during stress responses [18-20], which could reflect a regulatory role in apoptosis. Notably, various apoptotic factors are sequestered within SGs, which can limit apoptotic signaling. For example, RACK1 is sequestered in SGs under certain non-lethal stresses, which limits its ability to bind and activate the kinase MTK1, which plays a key role in many apoptotic responses [18].

Given a generally pro-survival function, it is perhaps unsurprising that SGs have been linked to cancer progression. A well-known SG assembly factor in human cells, G3BP1, is over-expressed in various types of cancer such as pancreatic cancer [21], sarcoma [22], and hepatocarcinoma [23] to name a few. Upregulation of SGs correlates with enhanced tumor fitness and metastasis. SG assembly is also thought to promote resistance to various chemotherapies [24]. Importantly, genetically impairing SG assembly can limit cancer metastasis in mouse lung tissue [22], suggesting the value of SGs as a cancer therapeutic target [25].

Aberrant, persistent SGs have also been implicated in the progression of Amyotrophic Lateral Sclerosis (ALS) [26]. A cellular hallmark of ALS is the cytoplasmic mis-localization and accumulation of TAR DNA binding protein 43 (TDP-43) in motor neurons. TDP-43 is an RNA binding protein that localizes in SGs under many stresses, and several mutations associated with ALS also promote SG assembly or impair their clearance. This has led to the theory that SGs may sequester and facilitate a pathological transition of TDP-43 within the cytoplasm, though SG independent means of TDP-43 pathological accumulation may also exist [27].

While SG assembly and composition are now well understood, and various disease-related connections established, **a key gap in understanding is how SGs are cleared from cells**. Disruption in this process may be key to disease progression as described above but could also impact mRNA regulation and that of other pathways whose factors accumulate in SGs. Multiple SG clearance mechanisms have recently been described including: chaperones [28], proteasomal function [29], post-translational modifications (PTM) [30], eIF4A and Ded1 helicase activity [3, 31], microtubule-based transport [32, 33] and autophagy/granulophagy-based clearance [34-36] (**Figure 2**). However, the relative importance of each of these SG clearance mechanisms, and whether they function cooperatively or only under specific stress conditions or cellular contexts is unknown. Below, I will discuss each of these reported SG clearance mechanisms.

The most common chaperones within cells are Heat Shock proteins (Hsp); of which Hsp70 and their specificity co-factors Hsp40 are the most abundant. Chaperones have been characterized by their different roles in preventing aggregation, responding to misfolded proteins and the disassembly of protein complexes [37, 38]. Mammalian cell data have shown that the overexpression of Hsp70 proteins prevent SGs from forming [17]. In yeast, inhibition of distinct Hsp40 proteins alters SG clearance fate. For example, Ydj1 knockout prevents translational recovery following oxidative stress, and results in SG clearance solely via granulophagy. In contrast, Sis1 knockout does not impact translational recovery, and instead results in the accumulation of SGs in the cytoplasm that would normally be granulophagy targets [28]. Thus, SGs are likely heterogenous in nature, and can be cleared by different mechanisms that chaperones may help regulate [39].

RNA helicases disrupt RNA-RNA and RNA-protein interactions [40], and thus are likely candidates for regulating SG clearance. Several helicases localize in SGs, including Ded1/DDX3 and eIF4A [12]. Recently, eIF4A helicase activity was demonstrated by genetic and chemical inhibitor means, both *in vitro* and *in vivo*, to suppress SG assembly, likely by disrupting RNA-RNA interactions [31]. Ded1 helicase activity has also been implicated in SG clearance [3]. The formation and clearance of SG may also be mediated by other helicases, but additional studies have yet to be conducted. In both assembly and disassembly of SGs, the microtubule network has been observed to play a critical role. Following microtubule disruption with nocodazole, SGs assembled more slowly, and formed much smaller foci [32, 33]. SGs also associate with and can seemingly translocate along tubulin to specific cellular locations [41]. Cells with depolymerized microtubules affected disassembly despite cycloheximide, a drug known to disassemble SGs, thereby maintaining foci/cell and percent of cells with granules [41]. Microtubule-associated motor proteins dynein and kinesin also colocalize with SGs, however the role of each motor protein in assembly and disassembly is still debated.

Many SG-localized proteins undergo various forms of PTM such as phosphorylation, methylation, acetylation or ubiquitination, which can either impair or upregulate the formation of SGs. While the underlying mechanisms of why such modifications impact SG assembly (or disassembly) are often not known, such events allow cells a rapid means to alter SG dynamics [30].

Ubiquitination is a 76 amino acid modification which is added to substrates via an E1-E2-E3 enzyme cascade. Ubiquitin's (Ub) are added to a lysine residue on a substrate either in a mono (singular), or poly (chain/branched chain) form. For poly-ubiquitinated proteins, Ubs are linked to each other via one of seven lysines (K6, K11, K27, K29, K33, K48, K63) present within Ub itself. The nature of ubiquitin linkages can dictate the consequences to the modified protein, with K63-linked substrates often targeted for vacuolar/lysosomal degradation but also necessary for DNA damage response [42], whereas K48-linked substrates are often targeted to the proteasome [43]. Notably, SGs are often highly ubiquitinated [13, 36]. Ubiquitination has also been identified as an essential post-translational modification needed to facilitate SG clearance after heat shock stress in mammalian cells [44]. In particular, G3BP1 ubiquitination was shown to be required for efficient SG clearance following 30-90min of heat shock stress [45]. What SG clearance mechanisms were elicited by such ubiquitination of G3BP1 was not clearly described, however.

Protein degradation by the proteasome has also been implicated in SG clearance. Specifically, chemical inhibition of the proteasome following oxidative stress in human cells slows SG clearance [29]. In addition, ZFAND1 aids in SG clearance following oxidative stress (but not other stresses) by recruiting the proteasome and the Ub-segregase chaperone VCP/p97 (Cdc48 in yeast) to SGs. VCP is well known to aid in the disassembly of protein complexes by using ATP to disturb interactions between targeted ubiquitylated substrate targets [46], though Ubiquitinated targets within SGs of VCP activity are unknown. Finally, blocking proteasomal function or knocking out ZFAND1 also promotes formation of aberrant less dynamic SGs that harbor misfolded proteins. What specific SG proteins may be recognized by ZFAND1, and degraded by the proteasome, also remain unclear.

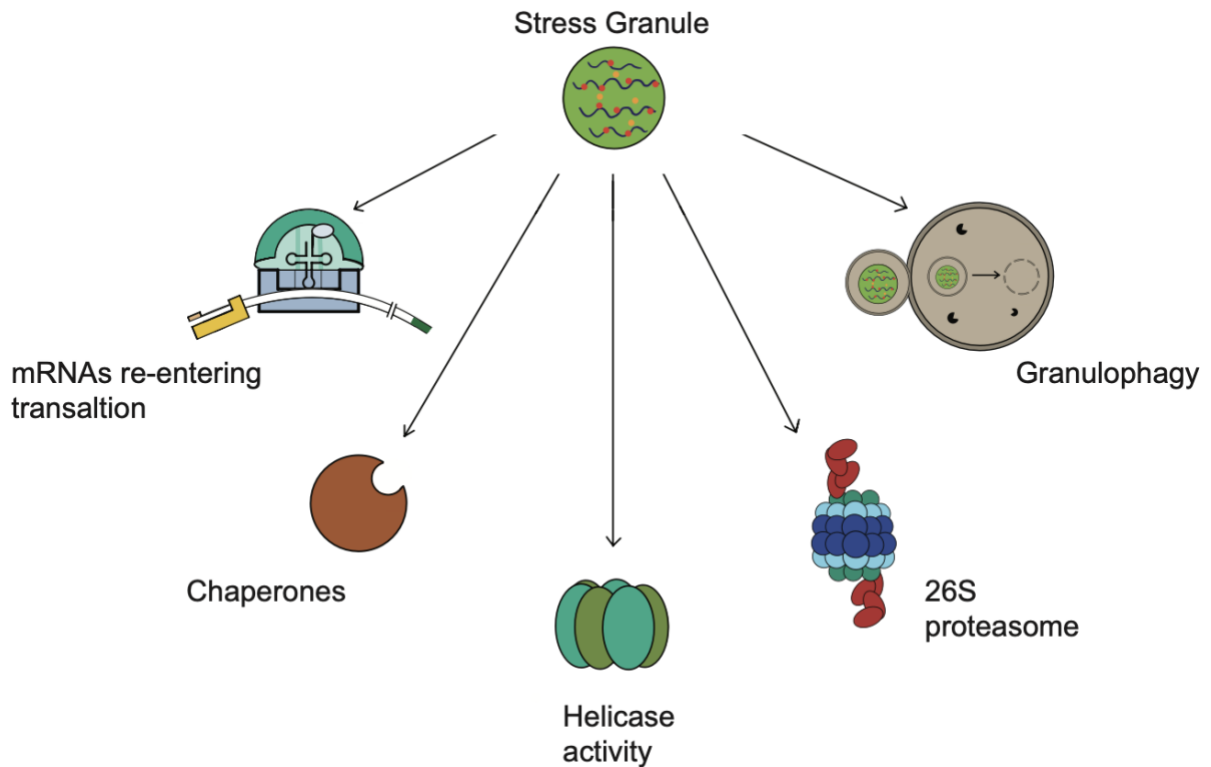


Figure 2: Reported pathways of SG clearance. These pathways include mRNAs re-entering translation, chaperone mediated clearance, helicase activity involving eIF4A and Ded1, the 26S proteasome, microtubules, and granulophagy. However, how these mechanisms work together to facilitate clearance is unknown.

Macroautophagy (henceforth termed autophagy) is a conserved pathway involving the engulfing of cellular material into a double membrane autophagosome for targeting and degradation in vacuoles (yeast) or lysosomes (higher eukaryotes). Autophagy can occur both non-selectively and selectively. Non-selective autophagy involves sequestering random cytosolic material for degradation. This process is usually a response to starvation conditions. Selective autophagy can happen regardless of nutrient conditions, where the cell selectively chooses what cargo needs to be degraded while excluding all other material. Examples of selective autophagic cargos include protein aggregates and damaged organelles (e.g., ribosomes, mitochondria). Selectivity is attributed to cargo binding autophagy “receptors”, which can bind directly to cargo proteins, which typically are ubiquitinated prior to recognition.

SGs are cleared by a selective autophagic pathway termed granulophagy [28, 34] (**Figure 3**). Granulophagy occurs in yeast following chronic nutrient deprivation [34], and heat shock, oxidative [28] and viral replication stress in human cells [47], with roles reported for the selective autophagy receptor p62 in human SG clearance. VCP/Cdc48 also plays a key role in this pathway, though much of the mechanism of granulophagy remains unknown. This motivated the design and conducting of a screen to identify granulophagy regulators in yeast which serves as the beginning point of my project.

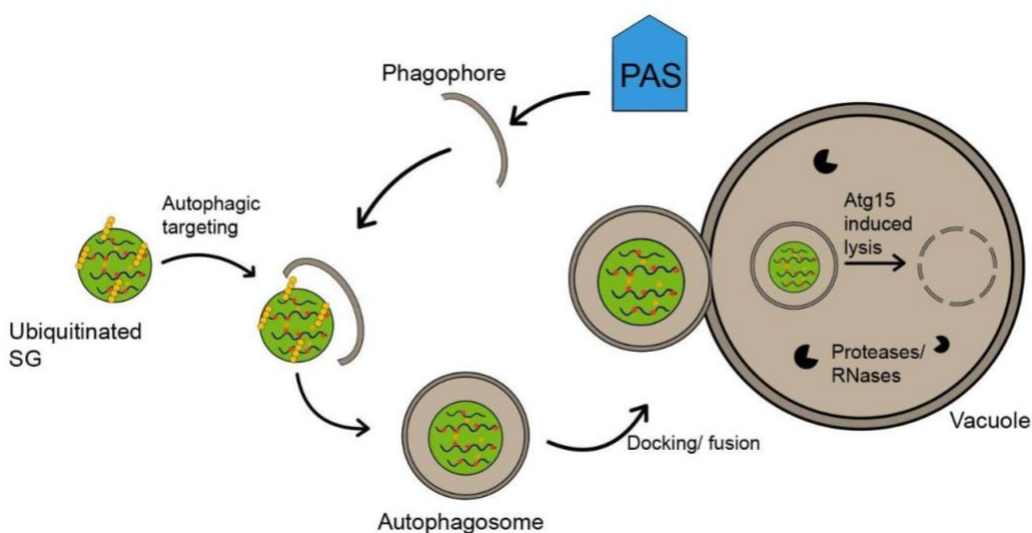


Figure 3: Overview of the granulophagy pathway. The autophagy pathway is mediated by a cascade of elements that aid in identification, placement and engulfment of targeted substrates for autophagic degradation. Targeted cargo (SGs) is recognized by special receptors that often bind Ub. Receptor-bound cargo is brought to phagophore assembly sites (PAS). Several ATG proteins are recruited to PAS, a specific perivacuolar location that serves as an organizing center. This leads to the formation of autophagosomes that engulf the targeted substrates, which then traffic to and fuse with vacuoles to initiate vacuolar degradation.

While many pathways have been observed as necessary for SG clearance, how all of these individual elements work to facilitate SG clearance is unknown. Particularly focusing on granulophagy, for substrates to be recognized by the autophagic machinery, they must first be tagged by ubiquitin. Ubiquitination is mediated by E3 ligases which in the case of SG ubiquitination, have largely yet to be characterized. Targets of interest from our granulophagy screen included adapters to the E3 ligase Rsp5. Seeming to be an ideal candidate, Rsp5 has been characterized to promote K63 polyubiquitination [48, 49]. This form of polyubiquitination has been characterized to preferentially tag and traffic membrane proteins for degradation by autophagy [48]. Rsp5/NEDD4 (human homolog) could ubiquitinate SG substrates, possibly facilitating SG clearance.

In this work, I have characterized the function of the E3 ligase Rsp5 and its role in SG clearance. Key findings include that Rsp5 greatly facilitates SG clearance under heat shock and stationary phase in yeast, but not after oxidative stress. I have also observed that during heat shock, Rsp5 colocalizes more readily to SGs during stress and in the initial phases of stress recovery. This identifies Rsp5/NEDD4 as a potentially important regulator of SG clearance in eukaryotic cells.

Results

An unbiased screen revealed adaptors associated with the E3 ubiquitin ligase Rsp5 as granulophagy effectors.

Using the model organism *Saccharomyces cerevisiae*, our lab characterized possible candidates for autophagy-based SG clearance. This was done by using a microscopy-based screening approach wherein double mutant yeast strains lacking the gene Autophagy Related 15 (*ATG15*), and another non-essential “gene X” from the yeast gene deletion library [50] were generated via Synthetic Genetic Array methods [51] (**Figure 4A**). All strains also expressed pRB1, a plasmid bearing Pab1-GFP (SG marker) and Edc3-mCh (PB marker). Atg15 is a vacuole-resident phospholipase that facilitates breaking open vesicular structures that enter vacuoles via various trafficking mechanisms (e.g., autophagy, endocytosis) [52]. *ATG15* deletion aids the visualization of autophagy-targeted SG material (Pab1-GFP) present inside vacuoles by impeding its degradation. Yeast were grown into early stationary phase, which is a known condition that induces granulophagy, prior to imaging [34]. *Atg15Δ* yeast served as a baseline control of granulophagy activity, and *atg15Δ geneXΔ* mutants with significant increases or decreases in vacuolar SG material were identified as hits (**Figure 4B**).

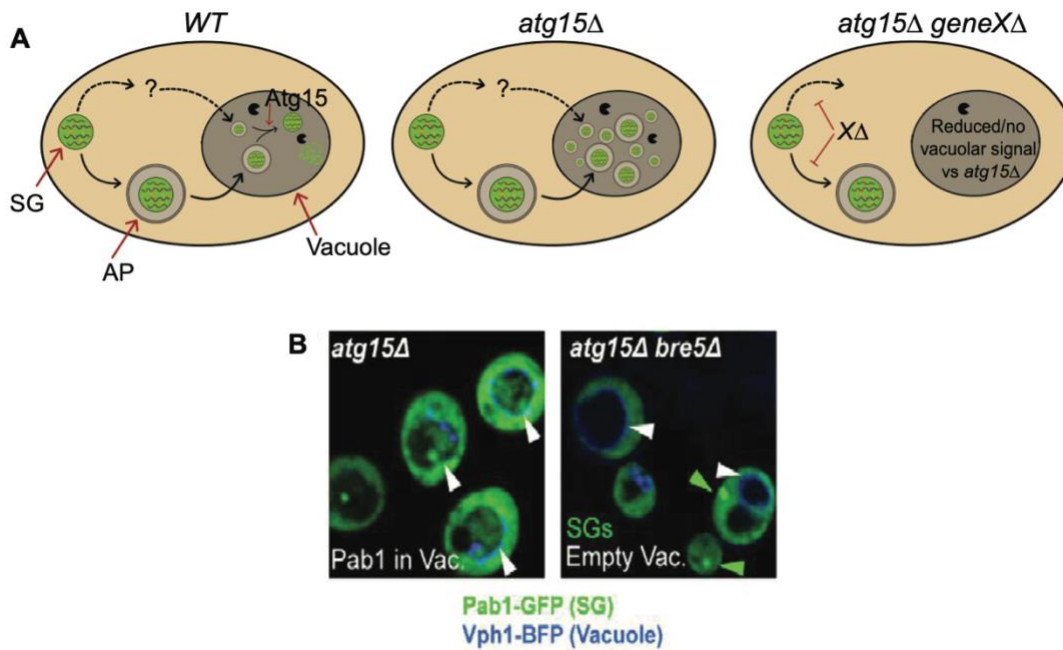


Figure 4: Unbiased screen suggest Rsp5 as a candidate for facilitating granulophagy. A) schematic representation of *atg15Δ* increasing visualization of SG vacuolar targeting **B)** Example images of a 'hit' that facilitates granulophagy. Left image: Pab1 accumulation in the vacuole (white arrowheads) under granulophagy inducing conditions (early stationary phase). Right image: *bre5Δ* decreases Pab1-GFP vacuolar content (white arrowheads) and SGs accumulate in cytoplasm (green arrowheads). Vph1-BFP marks the vacuolar membrane.

We identified several positive and negative regulators of granulophagy, including core autophagy genes, various E2s, E3 ligases, E3 ligase adaptors, kinases, chaperones and chaperone cofactors among others (**Table 1**). We identified several E3 adaptors including Art5, Rim8 and Tre1 which associate with the E3 ubiquitin (Ub) ligase, Rsp5 [48]. *RSP5* is an essential gene, and therefore was not a hit in our screen. Rsp5 has been characterized in the misfolded protein response, targeting substrates for proteasomal degradation [53]; several studies have indicated that misfolded proteins can accumulate within and potentially facilitate SG assembly [39, 54]. Additionally, given the abundance of Rsp5 specific adaptors, and the previously described connections between SGs and ubiquitination, we sought to characterize the role of Rsp5 in the SG clearance under different stress contexts, including some that may result in protein misfolding.

Protein category	Decreased Pab1 in Vacuole
Putative SG cargo	<i>TIF4631 (eIF4G)</i>
Autophagy receptors	<i>ATG19</i>
Autophagy genes	<i>ATG8, ATG9, ATG12, ATG13, ATG14, ATG17, ATG18, ATG20, ATG23, ATG27, ATG31</i>
E2 Ligases	<i>UBC5</i>
E3 Ligases	<i>APC9, BRE1, DOC1, HEL1, RMD5</i>
E3 adaptors	<i>ART5*, RIM8*, GRR1, RAV1, TRE1*</i>
DUBs	<i>BRE5, UBP3, UBP4, UBP12, UBP14</i>
Other trafficking factors	<i>VPS17, VPS27, VAM3</i>
Cdc48 co-factors	<i>DOA1</i>
Kinases	<i>SNF1, TPK2, TP3, KIN1, YAK1, KDX1</i>
Chaperones + cofactors	<i>AHA1, JJJ1, SBA1</i>
Helicases	<i>SUV3, MRH4, DHH1</i>

Table 1: Granulophagy hits identified in our screen. (* = identified Rsp5 adaptors)

Rsp5 mediates stress granule clearance following heat shock and stationary phase stress, but not NaN₃ stress.

To determine the role of Rsp5 in SG clearance, we initially utilized a temperature sensitive (ts) allele, *rsp5-3*, and assessed the effect of Rsp5 inactivation on SG clearance rates initially following HS (42°C, 30mins). Notably, we observed almost no recovery from HS-induced SGs following Rsp5 inactivation relative to WT (**Figure 5A-C**). Specifically, while SG foci/cell, and percent of cells with SGs decreased approximately 60-70% over a 2hr recovery period in WT cells, *rsp5-3* inactivated strains showed little or no change. Next, we tested whether Rsp5 is required for SG clearance after oxidative stress (0.5% w/v NaN₃, 2hr). However, under NaN₃ stress, SG clearance was not affected by Rsp5 inactivation (**Figure 5D-F**).

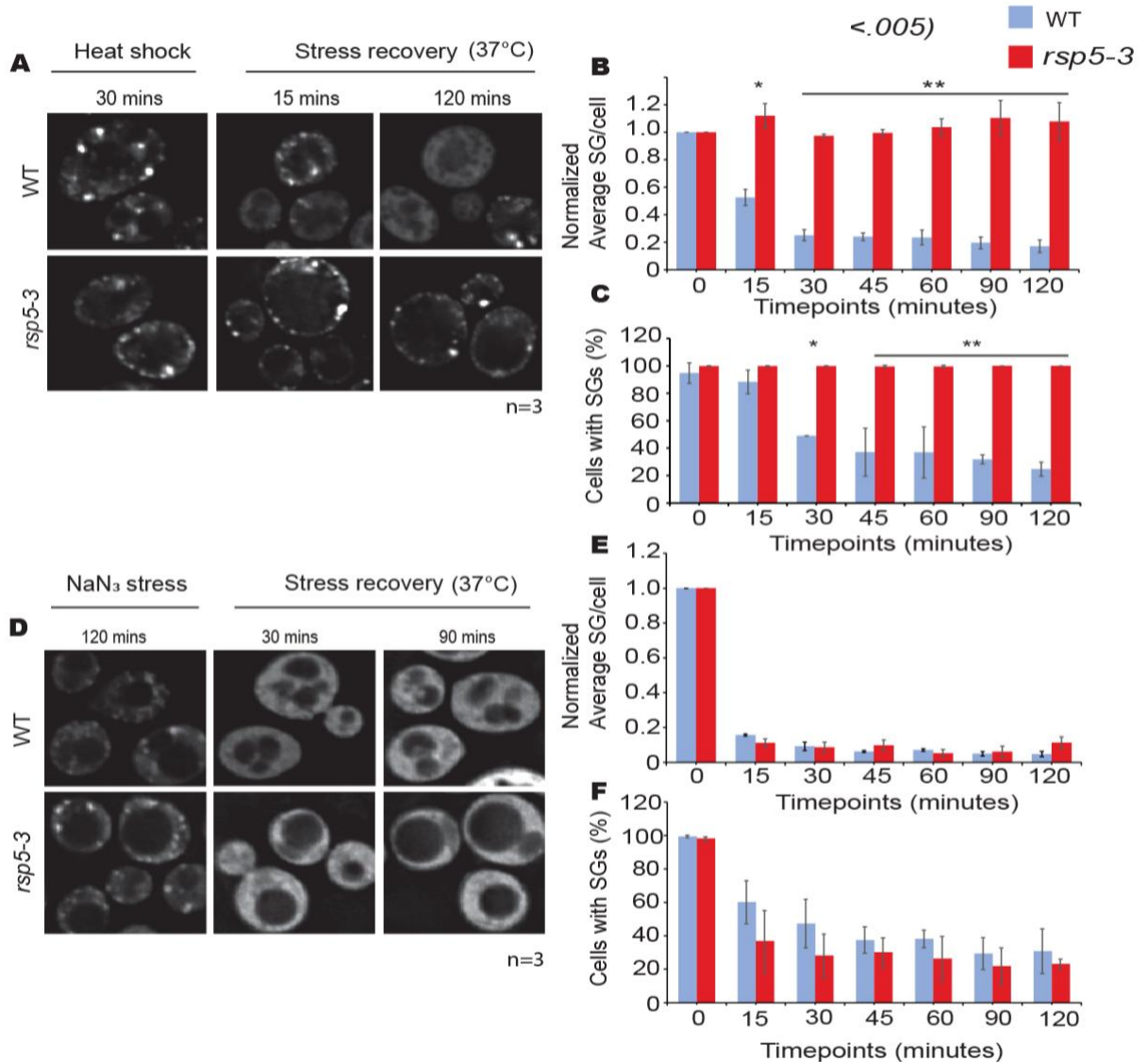


Figure 5: Rsp5 is required for SG clearance after heat shock. (A-F) both temperature sensitive *rsp5-3* and wildtype BY4741 yeast were transformed with pRB1, containing *PAB1*-GFP as a SG marker. **A)** *rsp5-3* and WT were subject to heat shock stress at 42°C for 30 minutes. Cells were allowed to recover from stress at 37°C for 120 minutes. **B)** Average SG foci/cell was quantified using HARLEY [55]. SG foci are identified based on fluorescence intensity of Pab1-GFP in foci compared to cytoplasmic Pab1-GFP fluorescence. **C)** Percentage cells with SGs. **D)** *rsp5-3* and WT were subject to 0.5 mM NaN₃ of stress, placed at 25°C for 120 minutes, followed by recovery in fresh media at 37°C for 120 minutes. **E)** NaN₃ SG foci/cell. **F)** NaN₃ cells with SG. **Panels B, C, E and F:** Mean values ± SD (error bars), n=3 biological replicate data shown. P-value significance (* = <0.05, ** = <0.005).

Next, we tested stationary phase stress, which involves prolonged nutrient starvation (primarily glucose absence). Due to viability issues with our *ts* allele, particularly under this condition, we decided to switch to an auxin inducible degron (AID) system. As done previously [56], we created a C-terminal RSP5-AID tagged strain with expression of *TIR1* under a β -estradiol inducible promoter (**Figure 6A,B**). Western blotting showed ~80% knockdown of Rsp5 in mid log cells within 30 minutes of auxin induction in mid log cells (**Figure 6C**). Additional experiments were conducted to ensure that the addition of β -estradiol, auxin as well as the absence of Rsp5 did not induce SGs (**Figure 6D**).

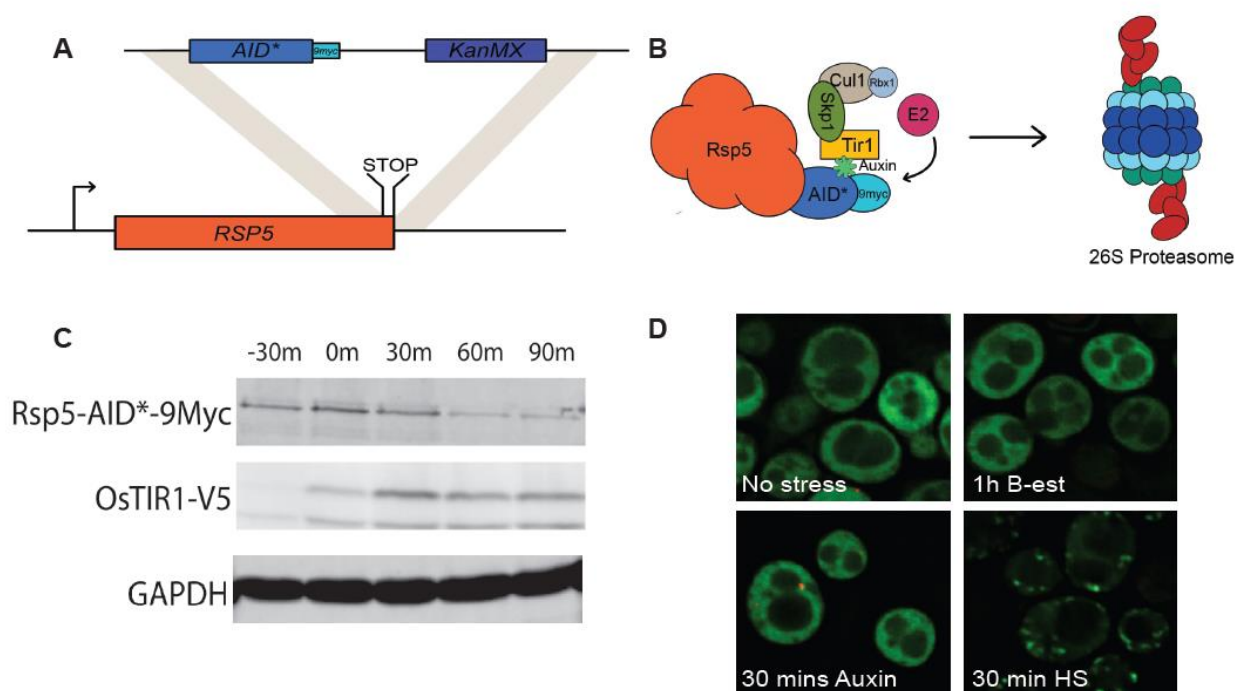


Figure 6: Construction of an Auxin inducible degron (AID) strain allows for controllable and timely degradation of Rsp5. **A)** Schematic representation of the insertion of the degron tag on Rsp5. **B)** Rsp5 degradation is induced by the binding of Tir1 to auxin, then allowing for Tir1 to bind the degron, recruiting factors that mediate the ubiquitination and eventually the degradation via the proteasome. **C)** Rsp5 degradation in mid log cells is optimal following 1 hour of β -estradiol treatment (inducing Tir1) and 30 mins of Auxin addition. **D)** Representative images verifying the addition of β -estradiol, auxin and the absence of Rsp5 does not induce stress granules.

Cells were subjected to 24 hours of nutrient deprivation, followed by 90 mins of recovery in fresh glucose-containing media (**Figure 7A**). Our AID system was also optimized to obtain better Rsp5 KD. In comparison to mid log culture, during SP conditions Rsp5 degradation was less efficient. Therefore, the time for Tir1 and auxin induction were extended to an hour each to stimulate Rsp5 degradation. In contrast to mid-log cells, cells in SP conditions showed via western blot analysis that Rsp5 knock-down was only ~ 40% effective (**Figure 7B**). Despite this, after removing SP, depletion of Rsp5-AID slowed the rate of SG clearance in cells by approximately 3-fold in comparison to WT after 30 minutes (**Figure 7C, D**). In summary, Rsp5 facilitates SG clearance following acute HS and SP stress, but not after oxidative stress, indicating a stress-specific function in SG clearance.

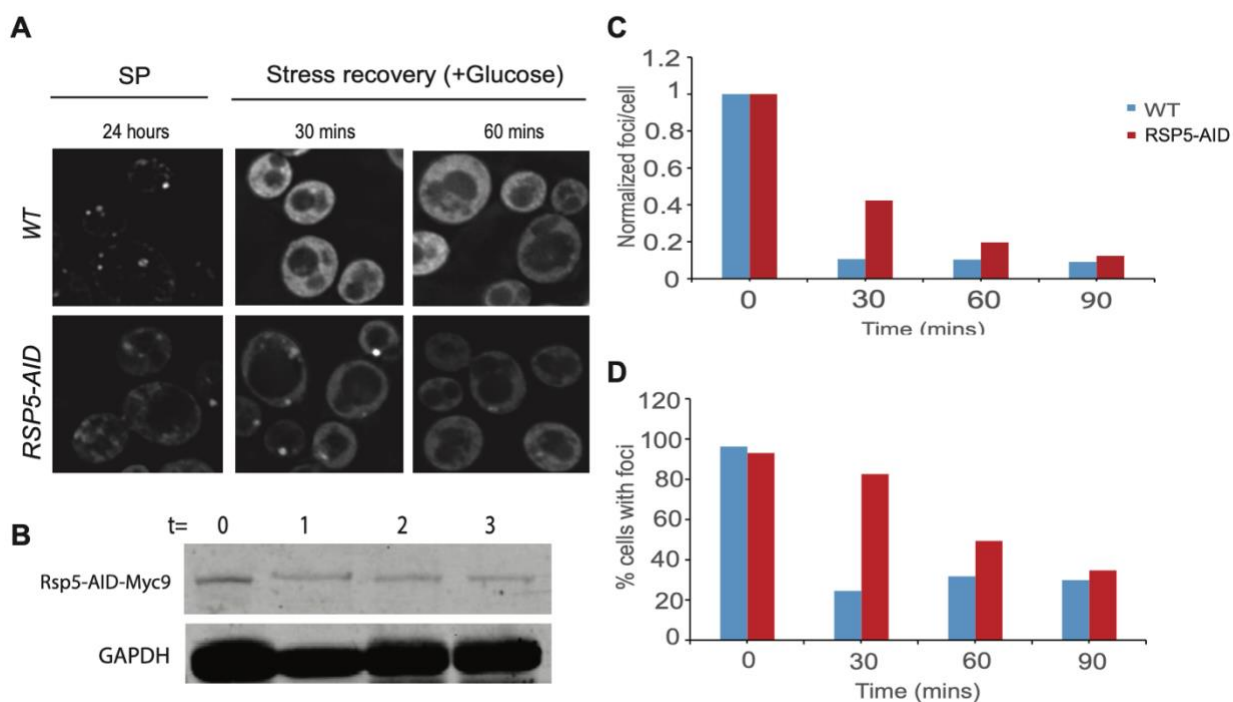


Figure 7: Rsp5 is required for SG clearance after stationary phase stress. (A-D) using baker's field yeast, RSP5-AID was transformed using an EPU plasmid, containing a *PAB1*-GFP as a SG marker. **A)** RSP5-AID with removed and intact Rsp5 were subject to stationary phase stress at 30°C for 24 hours. Cells were allowed to recover from stress at 30°C for 90 mins. **B)** Western blot of Rsp5 degradation, t=0, initial Rsp5 levels, t=1 1h β -estradiol induction, t=2 1h auxin induction, t=3 24h stationary phase. Average SG per cell is quantified using HARLEY [55]. SG foci are identified based on fluorescence intensity of Pab1-GFP in foci compared to cytoplasmic Pab1-GFP fluorescence. n=1 independent experiment shown. **C)** Normalized SG Foci/cell. n=1 independent experiments shown. **D)** Percent of cells with SG foci.

Rsp5 colocalizes more with SGs during stress and the initial phases of stress recovery.

We next wanted to determine if and when Rsp5 colocalizes with SGs during Rsp5-regulated SG clearance. We generated a *GFP-RSP5* strain (see materials and methods) and transformed this with a *DED1-mCherry* plasmid to label SGs. Preliminary data shows that Rsp5 puncta are more colocalized with SGs following 30mins of HS than after the first 5 mins of HS recovery. Qualitative analysis suggests that more than 60% of cells have some Rsp5 foci directly overlapping or juxtaposed to SGs following stress. This reduces to about 40% during the first 5 mins of stress recovery, where partial overlap/juxtaposition of Rsp5 and Ded1 foci also becomes more common. After 30 mins of recovery, there is little to no colocalization of Rsp5 with SGs. After 30 mins, most cells had also largely recovered from stress, meaning few SGs are visible for co-localization analysis (**Figure 8**).

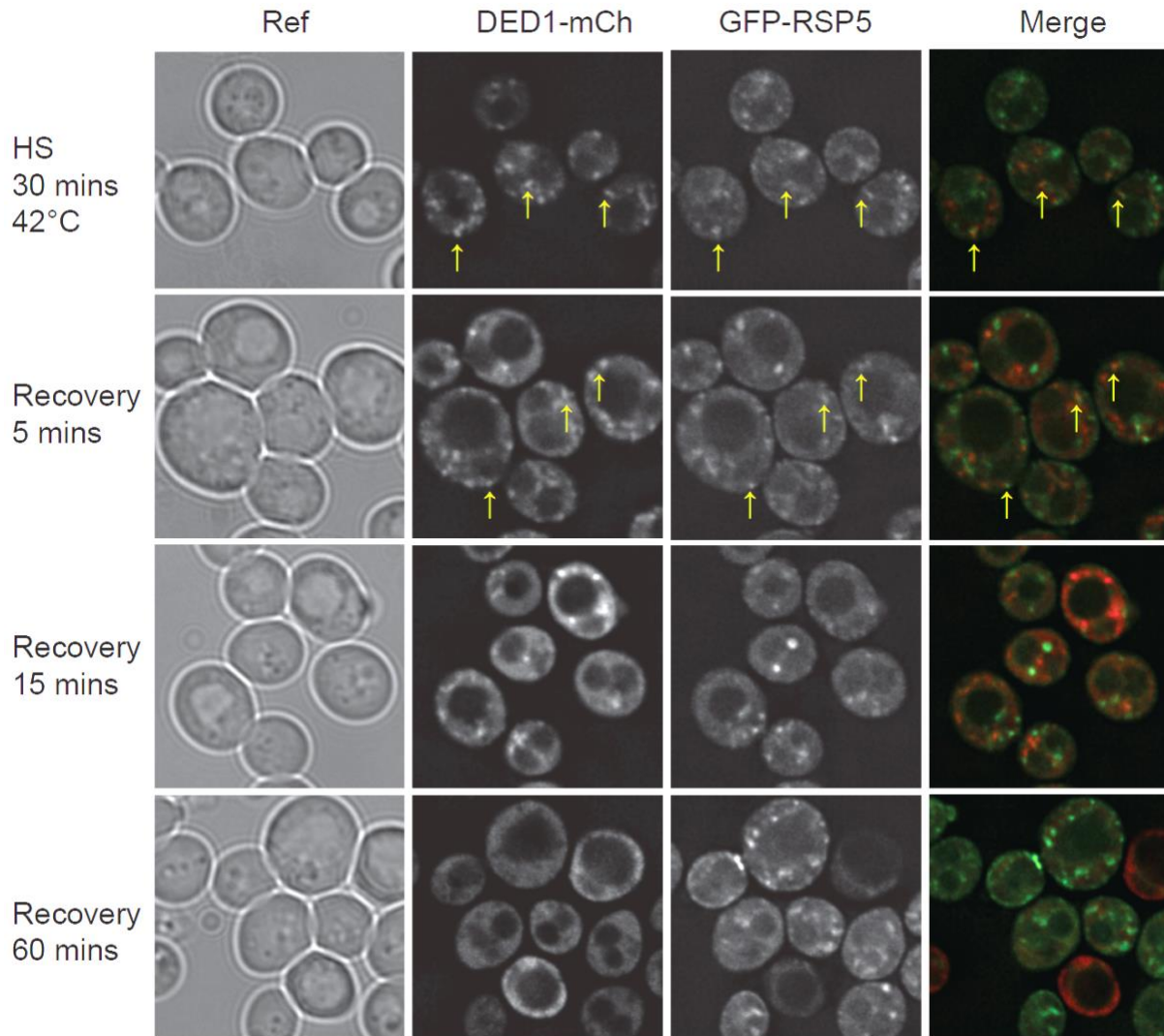


Figure 8: Rsp5 colocalizes with SG after heat shock and during initial recovery. Using a GFP-RSP5 strain, transformed with pRB108 (DED1-mCh), cells were subjected to HS as done before. Images were taken during stress and in various stages of stress recovery. Yellow arrows indicate points of colocalization and adjacent foci.

Discussion:

I observed that Rsp5 is necessary for SG clearance after HS and SP stress, but not oxidative stress. As previously noted, Rsp5 has been observed as the major E3 ligase responsible in yeast for clearing misfolded proteins after HS stress [53]. During HS stress, proteins misfold and as a result have a propensity to aggregate. It is speculated that depending on the type of stress, the proteomic landscape of SGs may change, however studies on this topic have been limited. It is possible that, in comparison to NaN₃ and stationary phase induced SGs, HS induced SGs may be more enriched in misfolded protein species, hence the greatest dependency on Rsp5 for clearance.

My preliminary data shows that Rsp5 colocalization in SGs happens to the greatest extent during HS stress but is also visible in the initial phases of stress recovery (first 5 mins). Notably, Rsp5 and Ded1 foci were often only partially overlapping, particularly during the HS stress recovery at 5 mins. Identifying SG proteins ubiquitinated by Rsp5 during SG clearance is an important future goal which could be addressed by SG purification and Di-gly mass spec [57], in the presence or absence of Rsp5 activity.

The method by which Rsp5 facilitates SG clearance is still unknown. A future direction includes the repetition of these experiments in an *atg15Δ* strain to determine if Rsp5 is mediating SG clearance via vacuolar-mediated targeting (i.e., granulophagy). Consistent with this, Rsp5 is known to preferentially ubiquitinate substrate proteins with K63 Ub linkages [48, 49]. However, it remains formerly possible that other SG clearance mechanisms could be at play (e.g., proteasome, chaperones). Epistasis testing with Rsp5 inhibition and impairment (genetic, chemical blocks) of each putative clearance mechanism is one way to establish whether Rsp5 functions in 1 or more distinct SG clearance pathway.

G3BP1 (in human cells) is ubiquitinated and this facilitates SG clearance [45]. One caveat of the study is that it only examined G3BP1, which is the most well studied SG assembly protein. In a way, this neglects the large array of Ubiquitinated proteins that localize to SGs during HS stress. Moreover, the study did not identify the E3 ligase that mediates the ubiquitination, so I propose Rsp5/NEDD4 as the E3 ligase that facilitates the ubiquitination. Indeed, it is quite remarkable that ubiquitination of a single SG protein out of several hundred had the SG clearance effect reported. In my opinion, even

assuming Rsp5/NEDD4 is ubiquitinating G3BP1, Rsp5 could have multiple targets within SGs that promote clearance. It is also worth noting that earlier this year, another E3 ligase, Trim21, was identified as a mediator of SG clearance specifically after oxidative stress [58], though no clear homolog of this E3 exists in yeast. More broadly though, it is likely in my view that different E3 ligases may play stress-specific roles in SG clearance (e.g., TRIM21 in oxidative stress, perhaps NEDD4 in heat shock), and that multiple E3s could aid SG clearance in each context, given the compositional complexity of SGs. There is a possibility that the cell is using different mechanisms for clearing stress specific SGs could be because if a cell relies solely on a single mechanism to resolve stress, it has a greater chance of the clearance pathway being compromised and resolution not occurring, resulting in a reduction in survival and adaptation of the cell. It would be beneficial for cells to harbor redundant mechanisms of stress mediation, rather than to rely on one protein for cellular recovery. A survival advantage to SG clearance may be another reason why Rsp5 is working during heat shock but not oxidative stress.

As previously discussed, aberrant SGs (less dynamic, constitutive) have been implicated both in diseases (cancer, ALS) as well as the process of aging [59] with speculation that maintaining SGs in a dynamic state, or aiding their clearance, could have therapeutic value. Thus, a better understanding of SG clearance, including in specific disease models, may lead to novel therapeutic approaches.

Materials & Methods

Yeast strains and growth conditions

Yeast strains (**Table 2**) were cultured in Yeast extract-peptone-dextrose (YPD) medium at 30°C while temperature sensitive alleles (i.e., *rsp5-3*) were cultured at the permissive temperature of 25°C. Synthetic defined medium (SD) with glucose and appropriate amino acids was used to grow strains transformed with plasmids (**Table 2**). A standard LiAc method was used for transformations. Homologous recombination was used to construct the strains in this study. To delete *ATG15*, a EUROSCARF HygromycinB resistance cassette was PCR amplified, flanked by regions of homology to the *ATG15 locus*. The PCR product was transformed into BY4742 yeast where gene replacement occurred via homologous recombination. PCR confirmation was used to confirm *ATG15* deletion. This strain was mated with the yeast gene deletion library (BY4741 background) and selected for double mutant haploids according to previously described synthetic genetic array (SGA) methods [60].

Fluorescence microscopy

The methods have been described previously [61]. Briefly, mid-log live yeast cells (OD_{600} 0.4–0.7) and stationary phase (SP) yeast cells (OD_{600} >3.0) were rapidly examined at room temperature on glass slides (Globe Scientific, cat:1301) with 1.5 coverslips (VWR, cat: 48366-227) using a DeltaVision Elite microscope with a $\times 100$ oil-immersion (1.515; Cargille, cat: 16245) objective (NA 1.40), and 15-bit PCO Edge sCMOS camera. Z-stack data (10 slices, 0.4 μ m each) was collected. Images were subject to deconvolution using standard parameters (Enhanced ratio, 10 cycles, medium filtering) using Softworx 7.0.0 (Deltavision software), followed by maximum intensity Z-stack projection. All image analysis was done using HARLEY software [55]. A minimum of 100 cells (in which each cell may contain multiple foci) was quantified for each biological replicate.

Heat Shock and NaN₃ stress addition

Mid log cells were cultured and grown overnight (OD_{600} 0.4–0.7). Cells of both *Rsp5* null and WT were subjected to heat shock (HS) stress at 42°C for 30 mins, followed by 120 min recovery at 37°C (for experiments involving *rsp5-3*) or 30°C (for *RSP5-AID* allele).

For NaN_3 stress, WT and *rsp5-3* cells were treated with 0.5% w/v NaN_3 at 25°C (TS) for 2 hours, followed by 120 min recovery at 37°C. Samples were collected for imaging immediately after stress, and at specific recovery time points including 15, 30, 60, 90 and 120 mins. Samples were spun down at 13000 xg for 2 mins and had additional media removed. Standard microscopy slides were prepared with 6 μ L of cells which were covered with a 1.5 coverslip for imaging.

Yeast strain construction

GFP-RSP5

Strain (YER125W) harboring *GAL1-SceI*, and endogenous *RSP5*, N-terminally tagged with GFP was generated using an *RSP5* acceptor strain from the N-terminal SWAT library collection via previously reported methods [62, 63]. Briefly, the *RSP5* acceptor strain was mated with a donor strain (yMS2085) containing a “seamless GFP” tagging plasmid (pRB448). Diploids were selected on SD -Ura, 200mg/ mL G418, sporulated on -N media, and haploid cells were then selected for A/alpha mating type and retention of the donor plasmid. Next, *SceI* was induced on 2% galactose media, which liberated the seamless GFP cassette from the donor plasmid and induced a double stranded break in the *RSP5* N-terminal SWAT cassette. Following homologous recombination repair between complementary sequences in the GFP plasmid fragment and the SWAT cassette, N-terminally tagged *RSP5*, under control of its native promoter and 5'UTR was generated. Successful recombination was verified by 5-FOA counter selection to verify removal of the *URA3* selection marker in the SWAT cassette, and PCR amplification of the *RSP5* locus.

RSP5-Auxin inducible degron

Inducible and rapid degradation of Rsp5 via an auxin-inducible degron (AID) system was pursued to circumvent the essential nature of the gene and examine Rsp5 function in the absence of temperature shifts necessary to study Rsp5 ts allele function. The wild-type strain BY4741 was modified to express an *RSP5*-auxin inducible degradation tag as described previously [56]. We designed forward and reverse primers for plasmid pRB461,

which harbors the AID tag, a 9myc tag, and a KanMX selective cassette. The primers were also designed with flanking sequences at the C-terminal portion of the *RSP5* open reading frame before the stop codon. The PCR amplification was performed on the AID cassettes and the amplification was verified on an agarose gel. The AID tag was integrated at the C-terminus of *RSP5* in strain BY4741. Plasmid pRB471 was then transformed which harbors *TIR1* (Auxin-binding adaptor protein that links degron tagged *RSP5* to proteasomal turnover) under a β -estradiol inducible promoter. For KanMX selection, transformed cultures were plated on selective YDP KanMX plates. PCR was used to verify successful genome editing using primers flanking the entire *RSP5* gene.

Western blotting confirmed the speed and efficiency of Rsp5-AID turnover by probing with an anti-Myc antibody.

Mid-log cells Before stress, mid-log cultures were treated with 10 nM of β -estradiol for 1 hour, followed by 30 minutes of auxin at 100mM final concentration.

SP cells For SP cells, after inoculation of a fresh overnight culture at OD₆₀₀ 0.2, followed by 20.5 hours of growth, culture was treated with 10nM of β -estradiol for 1 hour, followed by 100 mM of Auxin for 1 hour. Cultures were then left to reach SP of 24h

Colocalization Using our created N-terminal GFP-RSP5 strain, we subjected cells to HS stress and recovery protocol as mentioned above. Fluorescence microscopy also followed the same methods as above. Collected time points; After stress and 5, 15, 30, 60 minutes into recovery.

Table 2. Strains and Plasmids Used in this Study		
Yeast Strains		
Name	Properties	Reference
yRB001	<i>MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0</i>	"BY4741" - standard WT strain
yRB241	<i>his3Δ1, leu2Δ, ura3Δ0, met15Δ0, RSP5::rsp5-3-Kan</i>	Zhijian et al 2011
yRB358	BY4741 background. Rsp5 endogenously tagged with AID*-9myc. PCR and western checked	Mendoza-Ochoa, G. I., 2019
YER125W	RSP5 N-terminal GFP:SWAP Library	Yofe et al 2016
Plasmids		
Name	Properties	Reference
pRB1	<i>URA3, PAB1-GFP, EDC3-mCherry</i>	Ross Buchan
pRB108	<i>URA3: DED1-mCherry</i>	Ross Buchan
pRB471	<i>KanMx: TIR1</i>	Mendoza-Ochoa, G. I., 2019
pRB448	pSD-N9 X-Ntag Donor Seamless sfGFP #1 G418	Yofe et al 2016

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