The Orb6-Sts5 Axis Regulates Stress Granule Formation and Heat Stress Response in Fission Yeast

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Running Head: Orb6-Sts5 Regulates Stress Granules

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Abbreviations: Processing Bodies, P-Bodies; RNA Binding Protein, RBP; Ribonucleoprotein RNP; Nuclear Dbf2 Related, NDR; Guanine Nucleotide Exchange Factor, GEF; Intrinsically Disordered Domain, IDD.

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Abstract:

The NDR/LATS family kinases are a subclass of the AGC serine/threonine kinases which are important for morphogenesis and cell growth control. Using the model organism *Schizosaccharomyces pombe*, we previously reported that the NDR/LATS kinase Orb6 phosphorylates the RNA-binding protein (RBP) Sts5 serine 86 residue on its Intrinsically Disordered Domain (IDD). When dephosphorylated, Sts5 forms ribonucleoprotein (RNP) granules that colocalize with processing bodies (P-Bodies) and translationally repress mRNAs important for polarized cell growth. Here we report that Sts5 puncta colocalize with both P-Bodies and stress granules (SG) in response to glucose starvation, as well as heat, oxidative, and hyperosmotic stress. We find that loss of Sts5 decreases the number of stress granules, indicating that Sts5 has a role in promoting stress granule formation. Conversely, inhibition of Orb6 kinase promotes Sts5 aggregation and stress granule formation. In addition, loss of Sts5 decreases cell survival after heat stress, whereas decreasing Orb6 protein levels or including the *sts5S86A* mutation, which promotes Sts5 aggregation, leads to increased survival. These data indicate that the Orb6-Sts5 axis is not only important for regulation of polarized growth but also for response to environmental stress, as dysregulation of the Orb6-Sts5 axis affects stress granule formation and cell survival.

Introduction:

Nuclear Dbf2-related (NDR) kinases are highly conserved members of the AGC protein kinase family which regulate essential cellular processes including morphogenesis, growth and proliferation, mitosis, and apoptosis (Verde *et al.*, 1995; Verde *et al.*, 1998; Hergovich *et al.*, 2006; Hergovich *et al.*, 2007; Vichalkovski *et al.*, 2008; Chiba *et al.*, 2009; Hergovich *et al.*, 2009; Cornils *et al.*, 2011; Yang *et al.*, 2014). Several studies have implicated dysregulation of NDR kinases in the development of cancer, and NDR also plays a role in neuronal differentiation (Millward *et al.*, 1998; Hauschild *et al.*, 1999; Ross *et al.*, 2000; Adeyinka *et al.*, 2002; Cornils *et al.*, 2010; Napoletano *et al.*, 2011; Zhang *et al.*, 2015). Given the role of these proteins in the development of disease, it is crucial to identify the substrates of NDR kinases, and to elucidate their underlying mechanisms of action. The fission yeast *Schizosaccharomyces pombe* is a robust model for the study of cell morphogenesis and growth due to its well-defined cylindrical shape and growth pattern, which can be easily quantified (Mitchison and Nurse, 1985). Since NDR kinases are highly

conserved amongst yeast and humans, *S. pombe* is an ideal system to elucidate the targets and mechanism of action of NDR kinases (Verde *et al.*, 1995; Verde *et al.*, 1998; Hou *et al.*, 2003; Hergovich *et al.*, 2006; Das *et al.*, 2009; Das *et al.*, 2015; Nunez *et al.*, 2016; Chen *et al.*, 2019; Tay *et al.*, 2019).

S. pombe has a NDR/LATS kinase known as Orb6, which is responsible for regulation of polarized cell growth (Verde *et al.*, 1995). We previously demonstrated that Orb6 governs cell polarity by spatially regulating the conserved GTPase Cdc42 via phosphorylation of the guanine nucleotide exchange factor Gef1 (Das *et al.*, 2009; Das *et al.*, 2012; Das *et al.*, 2015). More recently, we found a novel and genetically separable function of Orb6 in promoting polarized cell growth by spatiotemporal regulation of RNA-binding protein Sts5 (Nunez *et al.*, 2016; Chen *et al.*, 2019). Sts5 is an RNA binding protein (RBP) homologous to human Dis3L2, which is associated with Perlman's syndrome and Wilm's tumor in humans (Toda *et al.*, 1996; Jansen *et al.*, 2009; Kurischko *et al.*, 2011; Astuti *et al.*, 2012; Vaggi *et al.*, 2012; Malecki *et al.*, 2013; Lv *et al.*, 2015; Robinson *et al.*, 2015). Sts5 functions to repress translation of specific mRNAs, many of which encode proteins involved in polarized cell growth, via binding and sequestration into ribonucleoprotein (RNP) granules (Nunez *et al.*, 2016). This process is mediated by the Sts5 intrinsically disordered domain (IDD), which is responsible for aggregation of the Sts5 protein and coalescence into RNP granules, under conditions of nutritional stress (Nunez *et al.*, 2016; Chen *et al.*, 2019). However, under conditions favorable to growth, Orb6 kinase inhibits Sts5 puncta formation via phosphorylation of Sts5 at serine 86, promoting Sts5 physical association with the 14-3-3 protein Rad24 (Chen *et al.*, 2019). Rad24 binding represses formation of Sts5 puncta, thus promoting translation of Sts5 bound mRNAs (Nunez *et al.*, 2016). Environmental stress such as nitrogen starvation, or crowded growth conditions leading to stationary phase, inhibit Orb6 kinase activity, which results in the translational repression of mRNAs involved in polarized growth via formation of Sts5 puncta (Nunez *et al.*, 2016; Chen *et al.*, 2019).

Consistent with the function of Sts5 in translational regulation, Orb6 inhibition causes Sts5 puncta to partially co-localize with processing bodies (P-Bodies) (Nunez *et al.*, 2016). P-Bodies are evolutionarily conserved cytoplasmic ribonucleoprotein (RNP) granules enriched in RNA decay machinery that control mRNA localization and stability (Ingelfinger *et al.*, 2002; van Dijk *et al.*, 2002; Wang *et al.*, 2002; Sakuno *et al.*, 2004). During nitrogen or glucose deprivation, Sts5 proteins assemble into puncta that partially co-localize with the P-Body marker *Dcp1-mCherry* (Nilsson and Sunnerhagen, 2011; Nunez *et al.*, 2016; Chen

et al., 2019). In this paper, we report that other environmental stressors such as osmotic stress, oxidative stress, and heat stress, also promote Sts5 coalescence into RNP granules. We find that under these stress conditions Sts5 granules colocalize with stress granules, another type of mRNA containing cytoplasmic aggregate which forms in response to translational repression and contain the stress granule-specific marker Pabp1/Pab1 (Nilsson and Sunnerhagen, 2011). Consistent with these findings, loss of Sts5 decreases, whereas Orb6 kinase inhibition increases, stress granule formation and resilience to heat stress. Thus, the Orb6-Sts5 regulatory axis modulates the formation of stress granules, and also cell survival in response to heat stress.

Results and Discussion:

Sts5 Puncta Co-Localize with Both P-Bodies and Stress Granules in Response to Oxidative, Osmotic, and Heat Stress

We previously demonstrated that Sts5 partially co-localizes with P-Bodies following Orb6 inhibition, or in media lacking glucose or nitrogen (Nunez *et al.*, 2016). Other stresses, such as osmotic stress, oxidative stress, and heat stress, are also known to induce the formation of P-Bodies (Nilsson and Sunnerhagen, 2011). To determine if Sts5 would co-localize with P-Bodies when cells are exposed to these stressors, log phase s*ts5-3xGFP dcp1-mCherry* cells were exposed to either oxidative, osmotic, or heat stress, and Sts5 puncta and P-Body formation was assessed with microscopy. In untreated control cells, Sts5-3xGFP remains diffuse in the cytoplasm with few puncta observed, and P-Bodies form at low numbers, as visualized by the P-Body marker Dcp1-mCherry (Figure 1, a-c). However, for cells exposed to 10 mM H_20_2, Sts5 puncta form and co-localize with P-Bodies (Figure 1, d-f). This result was also observed for cells exposed to 1 M NaCl for 30 minutes (Figure 1, g-i), or to 42°C heat for 20 minutes (Figure 1, j-l).

Both P-Bodies and RNA binding proteins are thought to promote the assembly of another type of cytoplasmic ribonucleoprotein granules known as stress granules (Buchan *et al.*, 2008; Wheeler *et al.*, 2016), which have been closely associated with cancer for their role in stress adaptation, and also with neurodegenerative diseases due to their ability to undergo phase transitions into biomolecular condensates (Gilks *et al.*, 2004; Ramaswami *et al.*, 2013; Anderson *et al.*, 2015; Aguzzi and Altmeyer, 2016). Stress granules are membrane-less organelles, which are often spatially associated with and contain many of the

same classes of proteins as P-Bodies, such as RNA binding proteins (reviewed in (Buchan and Parker, 2009; Guzikowski *et al.*, 2019)). The proteomes of stress granules and P-Bodies generally have little overlap in *S. cerevisiae* (Guzikowski *et al.*, 2019), and stress granules may include components not found in P-Bodies such as translation initiation factors and translationally stalled mRNAs (Anderson and Kedersha, 2009; Buchan and Parker, 2009; Poblete-Duran *et al.*, 2016). However, there are still a number of proteins that are common to both types of RNP granules, and it has been suggested that transfer of components can occur between P-Bodies and stress granules during docking events (Kedersha *et al.*, 2005; Stoecklin and Kedersha, 2013). Given this relationship, and since stress granules are often associated with mRNA binding proteins (Jain *et al.*, 2016), it is plausible that Sts5 puncta are also associated with stress granules.

To determine if Sts5 co-localizes with stress granules, the poly(A)-binding protein marker Pabp1-DsRed was used to visualize stress granules in the *Sts5-3xGFP* background under conditions of glucose limitation, which is known to induce formation of both stress granules and Sts5 puncta (Nilsson and Sunnerhagen, 2011; Nunez *et al.*, 2016). s*ts5-3xGFP pabp1-DsRed* cells were suspended in EMM medium with or without 2% glucose for 20 minutes. In glucose rich medium, both the Sts5-3xGFP and Pabp1-DsRed markers were observed as mostly diffuse within the cytoplasm with the formation of very few puncta (Figure 2A, a-c). In contrast, Sts5 puncta were clearly observed following glucose starvation, and these puncta partially co-localize with stress granules (Figure 2A, d-f).

Since Sts5 was found to coalesce into cytoplasmic puncta in response to oxidative stress, osmotic stress, or heat stress (Figure 1), and because stress granules are known to form under these conditions (Mahboubi and Stochaj, 2017; Guzikowski *et al.*, 2019), we tested if Sts5 co-localizes with stress granules following these additional stress types. s*ts5-3xGFP pabp1-DsRed* cells were exposed to oxidative stress (Figure 2B, d-f), hyperosmotic stress (Figure 2B, g-i), or heat stress (Figure 2B, j-l), and found that Sts5 puncta readily associate with stress granules, whereas Sts5 puncta and stress granules do not assemble in untreated controls (Figure 2B, a-c).

To further support these findings, we investigated the localization of *ssp1* mRNA, which encodes a CamK kinase (Matsusaka *et al.*, 1995; Hanyu *et al.*, 2009). *ssp1* is a Sts5-bound mRNA which co-localizes with P-Bodies and is translationally repressed upon formation of Sts5 puncta (Nunez *et al.*, 2016). Thus, we performed RNA-FISH analysis to localize *ssp1* RNA in *sts5-3xGFP pabp1-DsRed* cells cultured in EMM

medium with or without glucose. We observed co-localization of Sts5 puncta, *ssp1* mRNA, and stress granules in cells cultured in medium lacking glucose (Figure 2C, a-d). Conversely, for cells in glucose-rich medium, these factors displayed a diffuse localization (data not shown)(Nunez *et al.*, 2016).

These findings indicate that, in addition to responding to nutritional cues such as glucose or nitrogen starvation (Nunez *et al.*, 2016; Chen *et al.*, 2019), Sts5 also assembles into cytoplasmic RNP granules in response to more diverse environmental stressors such as oxidative stress, osmotic stress, and heat stress (Figures 1, 2A, 2B). In addition, Sts5 puncta associate with both P-Bodies and stress granules under these diverse stress conditions (Figures 1, 2A, 2B). One reversible function of stress granules is to alter the translational landscape of the cell by decreasing specific mRNA availability for translation, such as *ssp1* mRNA (Figure 2C) (Buchan and Parker, 2009). Consistent with this idea, we previously found that the Ssp1 protein levels drastically increase upon heat stress, when *sts5* is deleted (Nuñez et al, 2016).

Sts5 Promotes Stress Granule Assembly

While it is known that stress granules initially assemble through a process known as liquid-liquid phase separation (Aguzzi and Altmeyer, 2016), much remains to be elucidated regarding the mechanisms and factors involved in their formation (Nilsson and Sunnerhagen, 2011; Nunez *et al.*, 2016). Coalescence of RBPs into puncta is thought to promote nucleation of both P-Bodies and stress granules (Brangwynne *et al.*, 2009; Kato *et al.*, 2012; Brangwynne, 2013; Lee *et al.*, 2013; Hyman *et al.*, 2014; Becker and Gitler, 2015; Elbaum-Garfinkle *et al.*, 2015; Kroschwald *et al.*, 2015; Lin *et al.*, 2015; Patel *et al.*, 2015). Formation of these RBP “seeds” is often regulated by post-translational modification (such as citrullination, methylation, and notably, phosphorylation) of an intrinsically disordered domain which controls RBP self-aggregation (Hofweber and Dormann, 2019; Owen and Shewmaker, 2019). Sts5 contains an intrinsically disordered domain, which is phosphorylated by Orb6 kinase at serine 86 (Chen *et al.*, 2019). We have previously demonstrated that Orb6 inhibition promotes the formation of P-Bodies upon glucose deprivation in a Sts5 dependent manner (Nunez *et al.*, 2016). This suggests that Sts5 aggregates may serve as a nucleation site for P-Body formation. Since Sts5 also co-localizes with stress granules following stress (Figures 2A, 2B), we wanted to determine whether Sts5 plays a role in seeding stress granules.

To evaluate this possibility, we followed the formation of stress granules in the *sts5*Δ mutant using the stress granule marker Pabp1-DsRed. In media containing glucose, Pabp1-DsRed was largely diffuse within the cytoplasm in both the *sts5*Δ and control strains (Figure 3A; a, c). However, after culturing cells for 20 minutes in media lacking glucose, the number of stress granules was decreased by 44% for the *sts5*Δ mutant as compared to the control (Figure 3A, b, d; Figure 3B, ***$p<0.001$, unpaired t-test).

Since Orb6 regulates Sts5 coalescence, we also created an *orb6-as2 pabp1-DsRed* strain (which expresses a chemically inhibitable allele of *orb6*) to determine if inhibition of Orb6 kinase could drive stress granule formation. *orb6-as2 pabp1-DsRed* cells were treated with 1-NA-PP1 inhibitor and monitored for the formation of stress granules over a three-hour time course. We found that inhibition of Orb6-as2 at 25°C does not induce the formation of stress granules (Figure 3C; a, c), These findings indicate that Orb6 kinase inhibition alone is not sufficient to induce stress granule formation, while it is sufficient to promote P-Body formation, as we previously reported (Nunez *et al.*, 2016). However, when 1-NA-PP1 treated cells were subsequently heat shocked for 20 minutes at 42ºC, we observed a remarkable increase in the number of stress granules, as compared to the heat-shocked DMSO control (Figure 3C; b, d). The number of stress granules formed was quantified for three independent experiments (N=90 cells/condition), and the number of stress granules increased, on average, by 54% for the 1-NA-PP1 treated cells as compared to the heat shocked DMSO control (Figure 3D; ***$p<0.001$, unpaired t-test).

Overall, these data indicate that the presence of Sts5 is important for the formation of stress granules, and that Orb6 kinase plays a role in this process by modulating the extent of Sts5 granule formation. Since Orb6 kinase inhibition alone is not sufficient to drive stress granule formation, it is likely that additional factor(s) play a role in driving Sts5-mediated stress granule formation. For example, other factors such as the vigilin homologue Vgl1 (Wen *et al.*, 2010), signaling factors such as calcineurin (Higa *et al.*, 2015), and protein kinase C Pck2 (Kanda *et al.*, 2021) also associate with stress granules in response to thermal stress, and stress granule formation is also stimulated in response to heat stress by the RNA-binding protein Nrd1 (Satoh *et al.*, 2012). Additionally, the intrinsically disordered N-terminus of Sts5 is phosphorylated on multiple residues, suggesting it is the target of additional protein kinases (Magliozzi *et al.*, 2020).

Sts5 Activity Modulates Heat Resilience in S. pombe

Stress granules provide a mechanism whereby cells can rapidly adapt to environmental stressors by immediately and reversibly halting translation of specific mRNAs (Mahboubi and Stochaj, 2017). These condensates can aid in protection from specific stress, such as viral infection (Miller, 2011; Onomoto *et al.*, 2014) or modulate susceptibility to heat stress (Kroschwald and Alberti, 2017; Riback *et al.*, 2017). Since Sts5 responds to heat, oxidative, and osmotic stress by co-localizing with both stress granules and P-Bodies (Figures 1, 2) and is important for stress granule formation (Figure 3, A-B), we hypothesized that modulating the assembly of Sts5 into RNP granules plays a role in cell protection from stress. Thus, we tested how loss of Sts5 (*sts5*Δ), or the *sts5S86A* mutation that causes increased Sts5 puncta formation (Chen *et al.*, 2019), affect cell survival following exposure to stress. To test this, wild-type, *sts5*Δ, *sts5-HA*, and *sts5S86A-HA* strains were diluted to an equivalent optical density, exposed to a 48ºC heat shock for 20 minutes, and survival was determined by plating cells to calculate CFUs/mL as percent survival compared to untreated control strains. Following heat shock, the *sts5*Δ strain exhibited a decrease in survival (-0.92-fold) as compared to the wild-type control (Figure 4A; *p<0.001, One-Way ANOVA with Tukey's test). Conversely, the *sts5S86A-HA* mutant exhibited an increase in survival as compared either the wild-type (2.5-fold) or *sts5-HA* controls (2.88-fold) (Figure 4A; ***p< 0.001, One-Way ANOVA with Tukey's test). The *sts5-HA* strain exhibited similar survival as compared to the wild type, as expected.

To further investigate the impact of Sts5 modulation on heat tolerance (exposure to heat for extended times), wild-type, *sts5*Δ, *sts5-HA*, and *sts5S86A-HA* log-phase cells were diluted to an equivalent optical density and split into sample sets that were incubated for 6 hours at either 25°C or restrictive temperature (36.5°C) in a shaking incubator. Following incubation, cells were serially diluted and plated, and plates were incubated at 25°C. CFU's/mL were calculated as a relative percent survival for samples exposed to 36.5°C compared to untreated controls for each strain (Figure 4B). Again, it was found that the *sts5*Δ strain exhibited decreased survival compared to wild type, whereas the hypermorphic *sts5S86A-HA* strain displayed increased survival, as compared to the *sts5-HA* control (Figure 4B; ***p<0.001, One-Way ANOVA with Tukey's post hoc test).

Thus, our data indicate that Sts5 coalescence is important for survival and heat stress resilience following various durations and intensities of exposure to elevated temperatures. Therefore, we determined

whether Sts5 granule assembly is modulated by increased temperature and whether the *sts5S86A* mutation increases formation of Sts5 puncta following exposure to heat. To do this, *sts5-3xYFP* and *sts5S86A-3xYFP* strains were cultured at 29°C, 32°C, 35.5°C, or 42°C for 30 minutes, while control *sts5-3xYFP* and *sts5S86A-3xYFP* cells were maintained at 25°C throughout the experiment. Following heat stress, microscopy was performed and the number of Sts5 puncta were counted at each temperature. The *sts5-3xYFP* control began to form visible puncta only at 35.5°C (Figure 4C; a-e), whereas the *sts5S86A-3xYFP* mutant began to form puncta already at 25°C (Figure 4C, f-j). These differences were statistically significant (Figure 4D; One way ANOVA with Tukey's post hoc test, ***$p<0.001$, **$p<0.01$), and *Sts5S86A* predisposition to the formation of puncta was readily apparent until the temperature reached 42°C at which point the control formed approximately the same number of puncta.

Our findings indicate an important role for serine 86 phosphorylation in modulating the extent of Sts5 puncta assembly in response to increased temperatures (Figure 4, C-D). Since Orb6 kinase phosphorylates serine 86 (Chen *et al.*, 2019), we tested if downregulation of Orb6 kinase also increases cell resilience to heat stress. To transcriptionally repress Orb6, a thiamine-repressible o*rb6-as2* strain was used. Log phase wild-type and *orb6-as2* cells were pre-cultured with or without 15 μM thiamine for 16 hours at 32°C. Samples were diluted to an equivalent optical density and were heat shocked at 48°C for 15 minutes while control samples were maintained at 32°C. Following heat shock, cells were plated on media lacking thiamine, and CFU's/mL were calculated as described previously. The *orb6-as2* strain, when pre-cultured in the presence of thiamine, exhibited a striking 17.09-fold increase in survival following heat shock compared to the untreated control (Figure 4E; One-Way ANOVA and Tukey's test, ***$p<0.001$). This striking increase in resilience was readily observed on Petri plates (Supplemental Figure 1) and was notably higher than the relative increase in heat resilience observed for the *sts5S86A* mutant (Figure 4, A-B). Overall, these data indicate that the Orb6 kinase modulates stress granule assembly and resilience to heat stress, in part, by phosphorylating mRNA binding protein Sts5 on serine 87 (see model, Figure 5).

Since stress granules are important for responding to stress (Mahboubi and Stochaj, 2017), our findings suggest that the loss of Sts5 decreases cell survival by decreasing stress granule formation. Additionally, since Sts5 is important for translational regulation of Sts5-bound mRNA (Nunez *et al.*, 2016), it is plausible that decreased survival for *sts5*Δ is due to changes in the translational landscape of the cell.

Consistent with this idea, the *sts5S86A* mutant, which is predisposed to forming Sts5 puncta (Figure 4, C-D), exhibits increased resilience to heat stress (Figure 4, A-B). Inhibition of Orb6, which also induces Sts5 coalescence (Nunez *et al.*, 2016; Chen *et al.*, 2019), drastically increases resilience to heat stress (Figure 4E). Since this increase is much greater for Orb6 inhibition than for the *Sts5S86A* mutation, it is likely that Orb6 has additional roles in resilience which are independent of Sts5.

We previously showed that Orb6 kinase activity is decreased during nutritional deprivation, a stressful condition leading to cellular quiescence and dormancy (Chen *et al.*, 2019). Additionally, we found that either Orb6 kinase repression or *sts5S86A* mutation promotes extended chronological lifespan (Chen *et al.*, 2019). In fungi, dormancy is associated with a solidification of the cytoplasm (Munder *et al.*, 2016), and increased resilience to stress. For example, in the budding yeast *Saccharomyces cerevisiae*, cellular quiescence, when induced by limiting nitrogen, glucose, or phosphate, promotes resilience to heat stress (Klosinska *et al.*, 2011). Therefore, it is possible that the Orb6-Sts5 axis modulates stress response by regulating the assembly of different types of RNP granules, and thus fostering cell resilience during dormancy. These findings provide insight into the formation and differential regulation of RNP granules, which play an important role in human disease, as aberrant cytoplasmic condensates are associated with cancer, neurodegeneration, and infectious diseases (Alberti and Dormann, 2019). Furthermore, our findings highlight a novel role for NDR kinase in the control of RNP phase separation and suggest that Orb6 kinase may have a fundamental role in promoting biophysical changes in the cell cytoplasm.

Materials and Methods:

Strains and Growth Medium

Schizosaccharomyces pombe strains used in this study were derived from wild-type strains 972 or 975 and are displayed in Supplemental Table 1. *S. pombe* was maintained in yeast extract plus supplements (YES) or Edinburgh minimal medium supplemented with 0.5% ammonium chloride (EMM) and additional supplements as needed (Histidine, Leucine, Adenine, or Uracil) at a concentration of 225 mg/L. All strains were cultured at 25°C unless otherwise noted. Liquid cultures were incubated at 180 RPM in a shaking incubator, and all cultures were diluted daily such that they were maintained in logarithmic growth for a minimum of 8 generations prior to performing experiments.

Mating of yeast was performed on sporulation agar (SPA) medium at 25°C. Following digestion with glusulase (PerkenElmer, NEE-154001EA), colonies were selected using pombe minimal glutamate (PMG) medium containing necessary supplements and 100 μg/mL Natamycin or 150 μg/mL G418 as needed.

Microscopy

For experiments utilizing microscopy, samples were imaged with an Olympus BX61 fluorescent microscope using appropriate filters (GFP, YFP, or Cy3). Exposure times range from 2000-2500ms for each experiment and remain consistent among all samples for that experiment. All images are deconvolved projections from 12 Z-stacks separated by a step size of 0.3 μM (Bar = 5 μM) that were produced with SlideBook software (Intelligent Imaging Innovations; Denver, CO) and subsequently exported to ImageJ v1.53a for analysis (Schneider *et al.*, 2012).

RNP Granule Induction

To determine localization of Sts5 puncta relative to P-Bodies, we previously constructed a *sts5-3xGFP dcp1-mCherry* (FV2267) strain (Nunez *et al.*, 2016). *sts5-3xGFP dcp1-mCherry* was cultured in YES and diluted to $O.D._{595nm}$= 0.1 and cells were allowed to grow at this $O.D._{595nm}$ for a minimum of 1 hour. Samples were pelleted and resuspended in YES containing either 10 mM H_2O_2 for 1 hour, 1M NaCl for 30 minutes, or heat shocked at 42°C for 20 minutes. Control samples were resuspended in YES only. To determine localization of Sts5 puncta relative to stress granules, we introduced the marker Pabp1-DsRed, constructing a *sts5-3xGFP pabp1-DsRed* strain (FV2361). We tested the localization of Sts5 relative to stress granules under the same conditions described above (heat, hyperosmotic, and oxidative stress). To test the effects of glucose limitation, cells were grown in minimal medium (EMM) containing 2% glucose for at least 8 generations, and then were centrifuged, washed with the appropriate medium, and resuspended either in EMM lacking glucose or in EMM with 2% glucose. Images are representative of samples from three independent experiments.

RNA Fluorescent In Situ Hybridization (RNA FISH)

Localization of *ssp1* mRNA was determined using RNA-FISH as previously described, with modifications according to Nunez *et al.* (Brengues and Parker, 2007; Nilsson and Sunnerhagen, 2011; Heinrich *et al.*, 2013; Nunez *et al.*, 2016). *sts5-3xGFP pabp1-DsRed* cells (FV2361) were cultured in EMM lacking glucose for 20 minutes prior to fixation and hybridization of RNA was performed with 20-mer DNA oligonucleotides (Stellaris) labeled with Quasar 705 fluorochromes (Bar = 5 µm).

Glucose Starvation and Orb6 Inhibition Assays

To determine if Sts5 was required for the formation of stress granules, a glucose starvation assay was performed with sts5+ *pabp1-DsRed* (FV1684) and *sts5*Δ *pabp1-DsRed* (FV3192) mutants. Samples were cultured to log phase in minimal medium (EMM) and were washed once with 5 mL minimal medium lacking glucose. Cells were then resuspended in 5 mL minimal medium lacking glucose and were incubated at 25°C and 180 RPM in a shaking incubator for 20 minutes. Following incubation, 1 mL aliquots were centrifuged at 4000 RPM for 1 minute and resuspended in a small amount of residual medium. Microscopy was performed as previously described and the number of stress granules was manually counted. This experiment was performed in triplicate, and N=90 cells per strain per strain per condition in total.

For Orb6 inhibition assays, *orb6-as2 pabp1-DsRed* (FV3226) and *pabp1-DsRed* control (FV1684) strains were cultured to log phase in minimal medium containing adenine. 1-NA-PP1 was added to samples at a final concentration of 50 µM, and microscopy was performed as previously described for time-points of 30 m, 1 hour, 2 hours, and 3 hours post addition of inhibitor. For assays in which Orb6 was inhibited and then cells were heat shocked, 1-NA-PP1 was added to cells 15 minutes prior to heat shock, whereas DMSO was added for control cells. Samples were then shifted to a 42ºC shaking water bath incubator for 20 minutes. Additional controls were maintained at 25 ºC. Microscopy was performed as previously described and stress granules were manually counted. This experiment was performed in triplicate, and N = 90 cells per strain per condition in total.

Heat Shock Survival Assay

Wild-type (FV2644), *sts5*Δ (FV2674), *sts5-HA* (FV2645), and *sts5S86A-HA* (FV2649) strains were cultured

in EMM and maintained in log-phase for a minimum of eight generations. Each strain was diluted to $O.D._{595nm}$ = 0.2 (approximately $4x10^6$ cells/mL) in 5 mL EMM and allowed to grow at this density for one hour at 25°C shaking at 180 RPM. Samples were either maintained at 25°C or heat shocked at 48°C for 20 minutes. Following heat shock, experimental and control samples were each serially diluted, plated in triplicate on EMM plates, and incubated at 25°C for approximately 72 hours. Colony forming units per mL (CFU's/mL) were calculated for each condition, and percent survival was calculated by dividing CFU's/mL for heat shocked replicates by their respective average untreated control CFU's/mL. This experiment was performed in biological triplicate, and data was normalized to the average wild-type percent survival for each independent experiment. Following normalization, data from independent experiments was combined, and a one-way ANOVA with Tukey's post hoc test was performed to determine statistical significance (α = 0.05). To determine the impact of intermediate to long-term restrictive temperature on these strains, this experiment was repeated with modifications. In this case, samples were incubated at 25°C or 36.5°C for 6 hours. Following incubation, cells were serially diluted and plated in triplicate, and incubated at 25°C for approximately 72 hours. Calculation of CFU's/mL and normalization of data was performed as previously described, and this experiment was performed in biological triplicate.

For heat shock survival analysis of the *orb6-as2* strain (FV2527) and its respective wild-type control (FV2530), cells were initially pre-cultured in minimal medium supplemented with adenine at 180 RPM and 32ºC. In order to repress the expression of *orb6-as2* the culture was split into two sets, either with or without thiamine at a final concentration of 15 µM and was allowed to grow for ~16 hours. Cells were diluted to an equivalent $O.D._{595nm}$ of 0.2 ($4x10^6$ cells/mL) in appropriate media, and cells were allowed to grow for an additional 2 hours. Samples were then heat shocked in a 48ºC shaking water bath for 15 minutes while controls were maintained at 32ºC. All samples were then serially diluted and plated on minimal medium supplemented with adenine, but lacking thiamine, for subsequent quantification of CFU's/mL as previously described.

Temperature Dependent Analysis of Sts5 Puncta

sts5-3xYFP (FV2518) and *sts5S86A-3xYFP* (FV2522) strains were used to determine whether Sts5 puncta are induced at lower temperatures when Sts5 is hyperactive as compared to a wild-type allele. Each strain

was cultured to log-phase in EMM, diluted to $O.D._{595nm}$= 0.1, and cells were allowed to grow for a minimum of 1 hour at 25°C. Aliquots of each strain were created, and samples were heat shocked at 29°C, 32°C, 35.5°C, or 42°C for 30 minutes. Control cells were maintained at 25°C throughout the experiment. Following exposure to heat shock, samples were imaged using an Olympus BX61 microscope. To determine differences in number of puncta, 20 cells per strain were manually scored for Sts5 (YFP) puncta at each temperature. This experiment was replicated independently in triplicate and statistical analysis was performed using One-Way ANOVA and Tukey's post hoc test for statistical significance (α = 0.05) within GraphPad Prism v8.4.1.

Author Contributions:

RT wrote the manuscript, designed figures, generated strains, designed and executed stress granule assembly experiments, and performed heat shock survival assays. CC designed and executed co-localization experiments, generated strains, and performed quantification of Sts5 puncta following heat stress. IN performed RNA-FISH analysis of *Ssp1*. PH provided technical support to RT and CC. FV designed experiments, edited the manuscript, and provided funding.

Conflict of Interest Statement:

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1. Sts5-3xGFP co-localized with P-bodies after different types of stress. sts5-3xGFP dcp1-mCherry cells were cultured in YES medium, centrifuged, and resuspended in YES medium (a-c) as an untreated control. To induce stress, cells were resuspended in YES containing either 10 mM H_2O_2 for 1 hour (d-f), 1M NaCl for 30 minutes (g-i), or heat shocked at 42ºC for 20 minutes (j-l). In all three conditions Sts5-3xGFP forms cytoplasmic puncta which partially co-localized with the P-body marker Dcp1-mCherry, whereas the untreated controls display little to no formation of P-Bodies or puncta. Images are deconvolved projections from Z-stacks separated by a step size of 0.3 µM (Bar = 5 µM).

Figure 2. Sts5-3xGFP co-localized with stress granules and Sts5-*ssp1* associated mRNA following stress. (A) *sts5-3xGFP pabp1-DsRed* cells were cultured in medium with glucose (A; a-c) or without glucose (A; d-f) for 30 minutes. Upon glucose deprivation, Sts5-3xGFP coalesced into cytoplasmic puncta which partially co-localized with the stress granule marker Pabp1-DsRed. (B) *sts5-3xGFP pabp1-DsRed* cells were cultured in YES medium as the untreated control condition (B; a-c). To induce stress, cells were resuspended in YES containing either 10 mM H_2O_2 for 1 hour (B; d-f), 1 M NaCl for 30 minutes (B; g-i), or heat shocked at 42ºC for 20 minutes (B; j-l). In all three conditions, Sts5-3xGFP formed cytoplasmic puncta which partially co-localized with the stress granule marker Pabp1-DsRed. (C) RNA FISH visualization of *ssp1* mRNA in fixed cells cultured for 20 minutes in minimal medium containing 0% glucose. Hybridization of RNA was performed with 20-mer DNA oligonucleotides (Stellaris) labeled with Quasar 705 fluorochromes. *ssp1* mRNA co-localized with Sts5-3xGFP and the stress granule marker Pabp1-DsRed (arrows). Images are deconvolved projections from Z-stacks separated by a step size of 0.3 µM (Bar = 5 µM).

Figure 3. Orb6 and Sts5 regulate stress granule formation. (A) *pabp1-DsRed* and *sts5*Δ *pabp1-DsRed* cells were cultured in EMM medium with (a, c) or without (b, d) glucose for 20 minutes. In untreated controls, the stress granule marker Pabp1-DsRed remained diffuse throughout the cytoplasm. Following glucose deprivation, *sts5*Δ cells formed less stress granules than the control (b, d). (B) The number of stress granules were quantified for three separate experiments (N=90 cells/strain/condition total), and there was a statistically significant decrease in stress granule formation for the *sts5*Δ mutant as compared to the control (***$p<0.001$; unpaired t-test). (C) Inhibition of Orb6 primes cells for the formation of stress granules.

orb6-as2 pabp1-DsRed cells were cultured in minimal medium containing adenine, and 15 minutes prior to heat shock 1-NA-PP1 inhibitor was added, or DMSO for controls. In cells maintained at 25ºC, Pabp1-DsRed remained diffuse within the cytoplasm (a, c). However, in cells that were heat shocked for 20 minutes at 42ºC (15 minutes post addition of 1-NA-PP1), there was an increase in stress granule formation compared to the DMSO control (b, d). (D) This experiment was performed three times and the increase in stress granule formation was statistically significant (N=90 cells/strain/condition total; ***p<0.001, unpaired t-test). Representative images are deconvolved projections from Z-stacks separated by a step size of 0.3 μM (Bar = 5 μM).

Figure 4. Orb6 and Sts5 modulate survival following heat stress. (A) Wild-type, *sts5*Δ, *sts5-HA*, and *sts5S86A-HA* cells were exposed to a 48ºC temperature shift for 20 minutes, while untreated controls were incubated at 25ºC. Following heat stress, *sts5*Δ cells exhibit decreased survival, whereas the *sts5S86A-HA* mutant exhibited increased survival (***p<0.001; One-Way ANOVA with Tukey's test). (B) The experiment in A was repeated, but cells were instead incubated for 6 hours at either 25ºC or 36.5ºC. Again, *sts5*Δ cells displayed decreased survival compared to wild type, whereas *sts5S86A-HA* had increased survival compared to the *sts5-HA* control (***p<0.001; One-Way ANOVA with Tukey's test). (C) In the *sts5S86A-3xYFP* mutant, the number of Sts5 granules were increased at temperatures in which the *sts5-3xYFP* control did not form granules. (D) Quantification from results obtained in C. There is a statistically significant increase in average puncta per cell for the *sts5S86A-3xYFP* strain as compared to the control for cells incubated between 25ºC and 35.5ºC (N=60 cells/strain, ***p<0.001, **p<0.01, *p<0.05; One-Way ANOVA with Tukey's test). (E) Wild-type and *orb6-as2* strains were cultured in the presence (+T) or absence of thiamine, heat shocked, and plated for CFUs/mL. Inhibition of Orb6 results in a 17-fold increase in survival as compared to the untreated control (***p<0.001; One-Way ANOVA with Tukey's test).

Figure 5. Model for Sts5 activation and response to stress. (A) Under optimal growth conditions, the RNA binding protein Sts5 is phosphorylated by the NDR kinase Orb6. This results in binding of Sts5 to the 14-3-3 protein Rad24 and promotes translation of Sts5 bound mRNA. Under these conditions, there is little to no formation of RNP granules. (B) Following exposure to heat stress, oxidative stress, osmotic stress, or

nutrient starvation, Sts5 coalesces into cytoplasmic puncta which partially co-localize with P-Bodies and stress granules. (C) Loss of Sts5 decreases the formation of stress granules, and cells are less tolerant to heat stress. (D) Inhibition of Orb6 drastically increases resilience to heat stress and promotes the formation of stress granules after heat stress. The *sts5S86A* mutant exhibits increased Sts5 coalescence and is more resilient to heat stress.

Supplemental Figure 1. Representative image of the *orb6-as2* heat shock assay (10-2 dilution) from the heat shocked sample set. Inhibition of Orb6 drastically increases survival (bottom right) after exposure to a 48ºC heat shock for 15 minutes, as compared to untreated or wild-type controls.

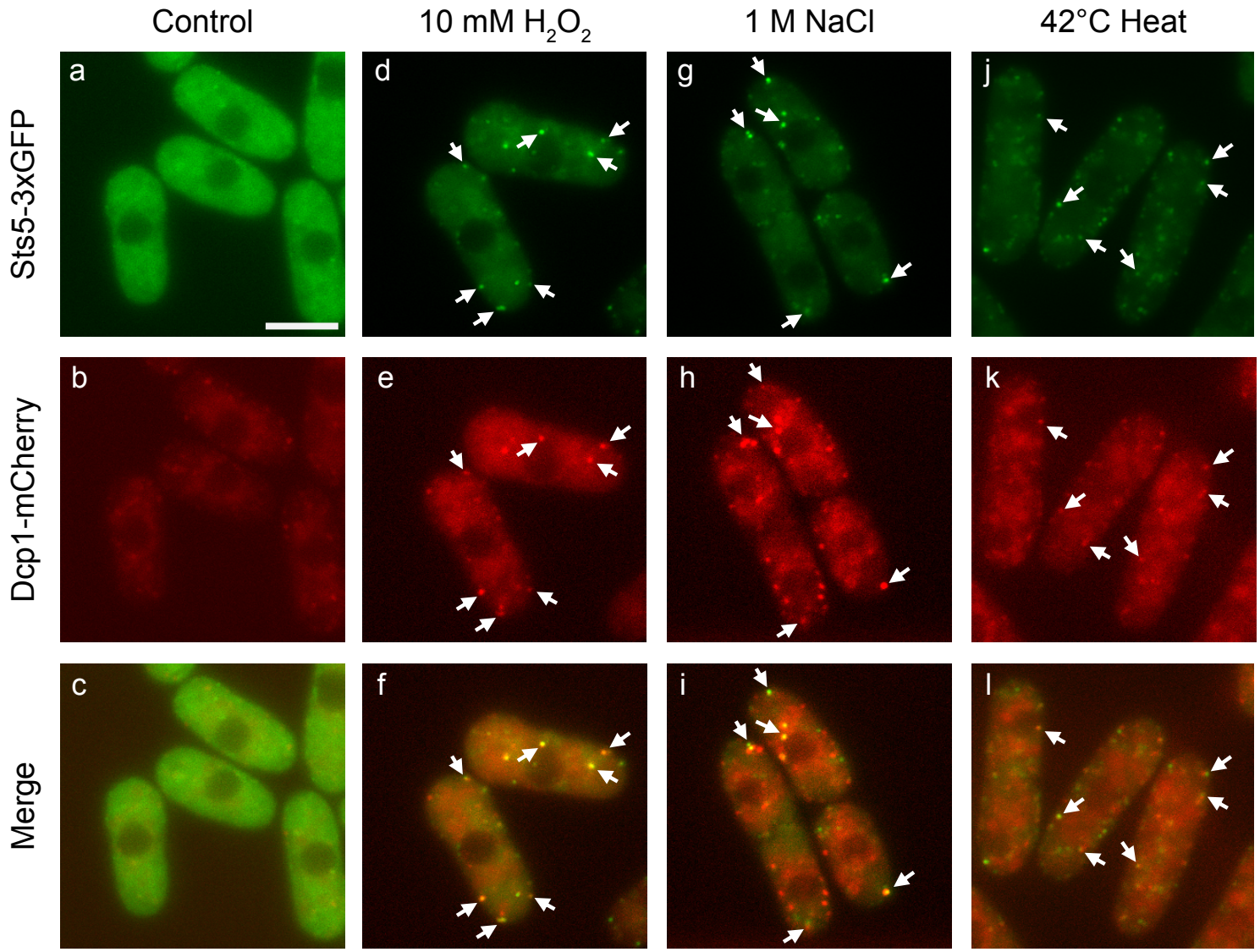


Figure 1. Sts5-3xGFP co-localized with P-bodies after different types of stress. *sts5-3xGFP dcp1-mCherry* cells were cultured in YES medium, centrifuged, and resuspended in YES medium (a-c) as an untreated control. To induce stress, cells were resuspended in YES containing either 10 mM H_2O_2 for 1 hour (d-f), 1M NaCl for 30 minutes (g-i), or heat shocked at 42ºC for 20 minutes (j-l). In all three conditions Sts5-3xGFP formed cytoplasmic puncta which partially co-localized with the P-body marker Dcp1-mCherry, whereas the untreated controls displayed little to no formation of P-Bodies or puncta. Images are deconvolved projections from Z-stacks separated by a step size of 0.3 μM (Bar = 5 μM).

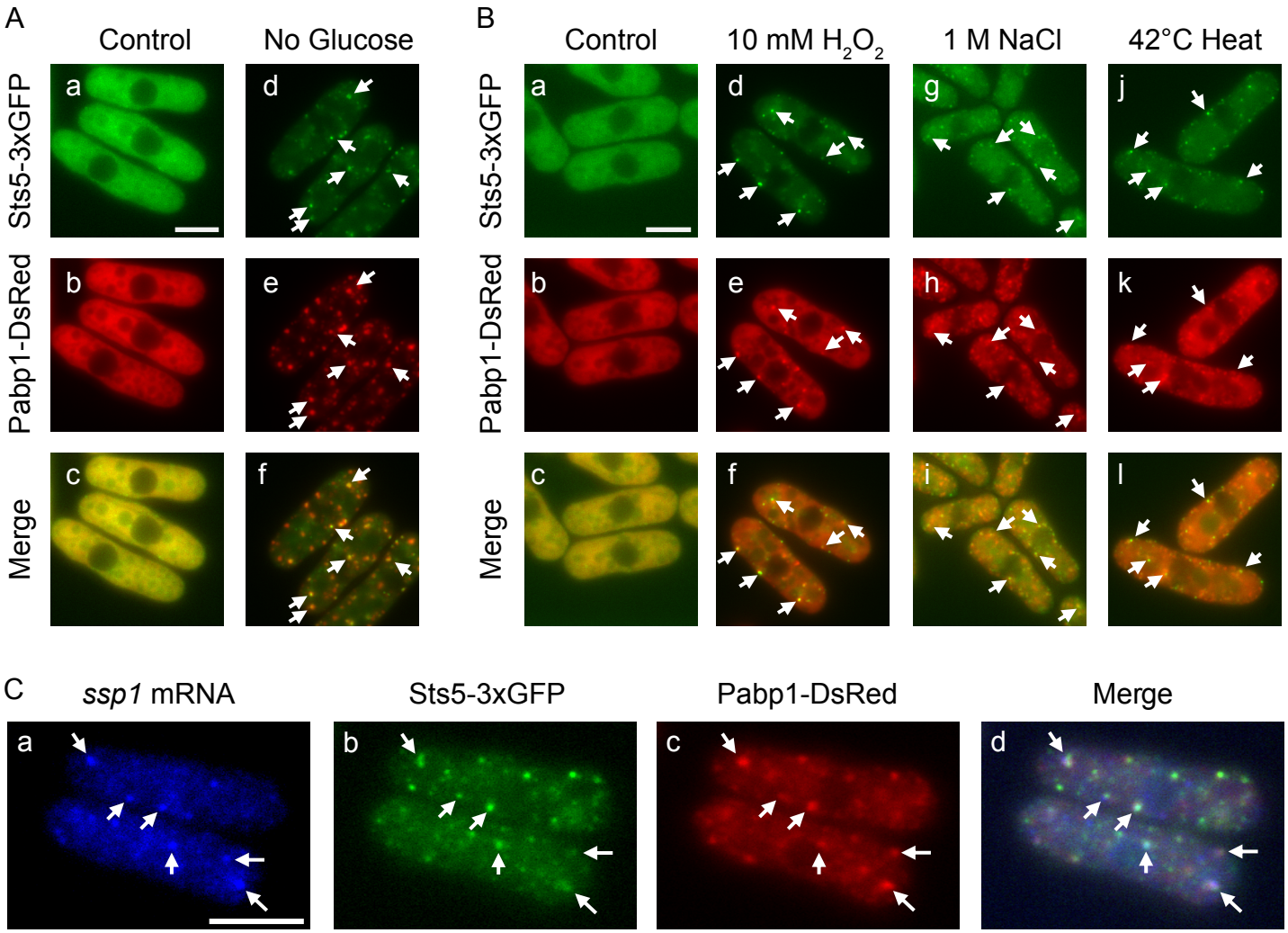


Figure 2. Sts5-3xGFP co-localized with stress granules and Sts5-*ssp1* associated mRNA following stress. (A) *sts5-3xGFP pabp1-DsRed* cells were cultured in EMM medium with glucose (A; a-c) or without glucose (A; d-f) for 30 minutes. Upon glucose deprivation, Sts5-3xGFP coalesced into cytoplasmic puncta which partially co-localized with the stress granule marker Pabp1-DsRed. (B) *sts5-3xGFP pabp1-DsRed* cells were cultured in YES medium as the untreated control condition (B; a-c). To induce stress, cells were resuspended in YES containing either 10 mM H_2O_2 for 1 hour (B; d-f), 1 M NaCl for 30 minutes (B; g-i), or heat shocked at 42ºC for 20 minutes (B; j-l). In all three conditions, Sts5-3xGFP formed cytoplasmic puncta which partially co-localized with the stress granule marker Pabp1-DsRed. (C) RNA FISH visualization of *ssp1* mRNA in fixed cells cultured for 20 minutes in minimal medium containing 0% glucose. Hybridization of RNA was performed with 20-mer DNA oligonucleotides (Stellaris) labeled with Quasar 705 fluorochromes. *ssp1* mRNA co-localized with Sts5-3xGFP and the stress granule marker Pabp1-DsRed (arrows). Images are deconvolved projections from Z-stacks separated by a step size of 0.3 μM (Bar = 5 μM).

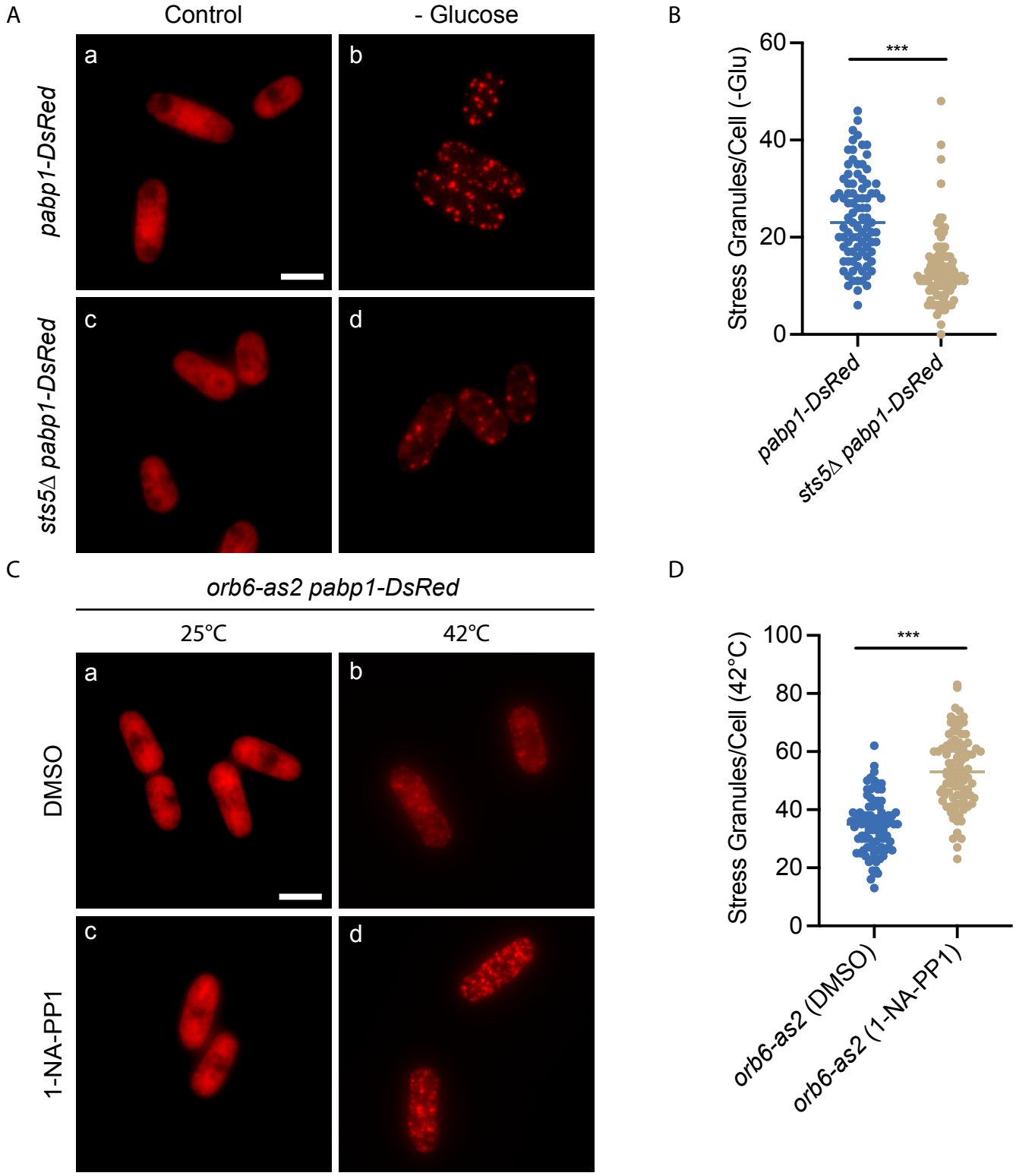


Figure 3. Orb6 and Sts5 regulate stress granule formation. (A) *pabp1-DsRed* and *sts5Δ pabp1-DsRed* cells were cultured in EMM medium with (a, c) or without (b, d) glucose for 20 minutes. In untreated controls, the stress granule marker Pabp1-DsRed remained diffuse throughout the cytoplasm. Following glucose deprivation, *sts5Δ* cells formed less stress granules than the control (b, d). (B) The number of stress granules were quantified for three separate experiments (N=90 cells/strain/condition total), and there was a statistically significant decrease in stress granule formation for the *sts5Δ* mutant as compared to the control (***p<0.001; unpaired t-test). (C) Inhibition of Orb6 primes cells for the formation of stress granules. *orb6-as2 pabp1-DsRed* cells were cultured in minimal medium containing adenine, and 15 minutes prior to heat shock 1-NA-PP1 inhibitor was added, or DMSO for controls. In cells maintained at 25ºC, Pabp1-DsRed remained diffuse within the cytoplasm (a, c). However, in cells that were heat shocked for 20 minutes at 42ºC (15 minutes post addition of 1-NA-PP1), there was an increase in stress granule formation compared to the DMSO control (b, d). (D) This experiment was performed three times and the increase in stress granule formation was statistically significant (N=90 cells/strain/condition total; ***p<0.001, unpaired t-test). Representative images are deconvolved projections from Z-stacks separated by a step size of 0.3 μM (Bar = 5 μM).

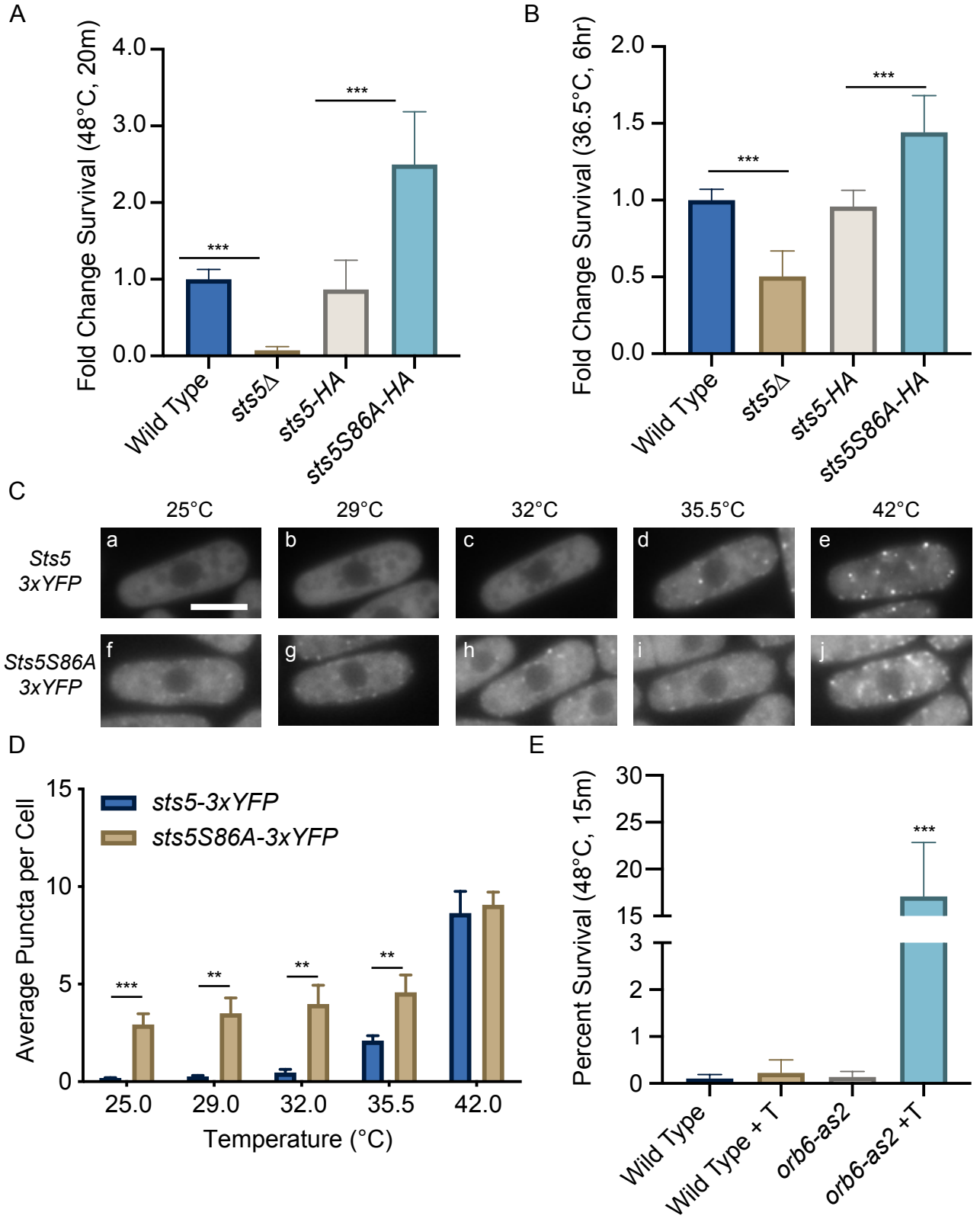


Figure 4. Orb6 and Sts5 modulate survival following heat stress. (A) Wild-type, *sts5*Δ, *sts5-HA*, and *sts5S86A-HA* cells were exposed to a 48ºC temperature shift for 20 minutes, while untreated controls were incubated at 25ºC. Following heat stress, *sts5*Δ cells exhibit decreased survival, whereas the *sts5S86A-HA* mutant exhibited increased survival (***p<0.001; One-Way ANOVA with Tukey's test). (B) The experiment in A was repeated, but cells were instead incubated for 6 hours at either 25ºC or 36.5ºC. Again, *sts5*Δ cells displayed decreased survival compared to wild type, whereas *sts5S86A-HA* had increased survival compared to the *sts5-HA* control (***p<0.001; One-Way ANOVA with Tukey's test). (C) In the *sts5S86A-3xYFP* mutant, the number of Sts5 granules were increased at temperatures in which the *sts5-3xYFP* control did not form granules. (D) Quantification from results obtained in C. There is a statistically significant increase in average puncta per cell for the *sts5S86A-3xYFP* strain as compared to the control for cells incubated between 25ºC and 35.5ºC (N=60 cells/strain, ***p<0.001, **p<0.01, *p<0.05; One-Way ANOVA with Tukey's test). (E) Wild-type and *orb6-as2* strains were cultured in the presence (+T) or absence of thiamine, heat shocked, and plated for CFUs/mL. Inhibition of Orb6 results in a 17-fold increase in survival as compared to the untreated control (***p<0.001; One-Way ANOVA with Tukey's test).

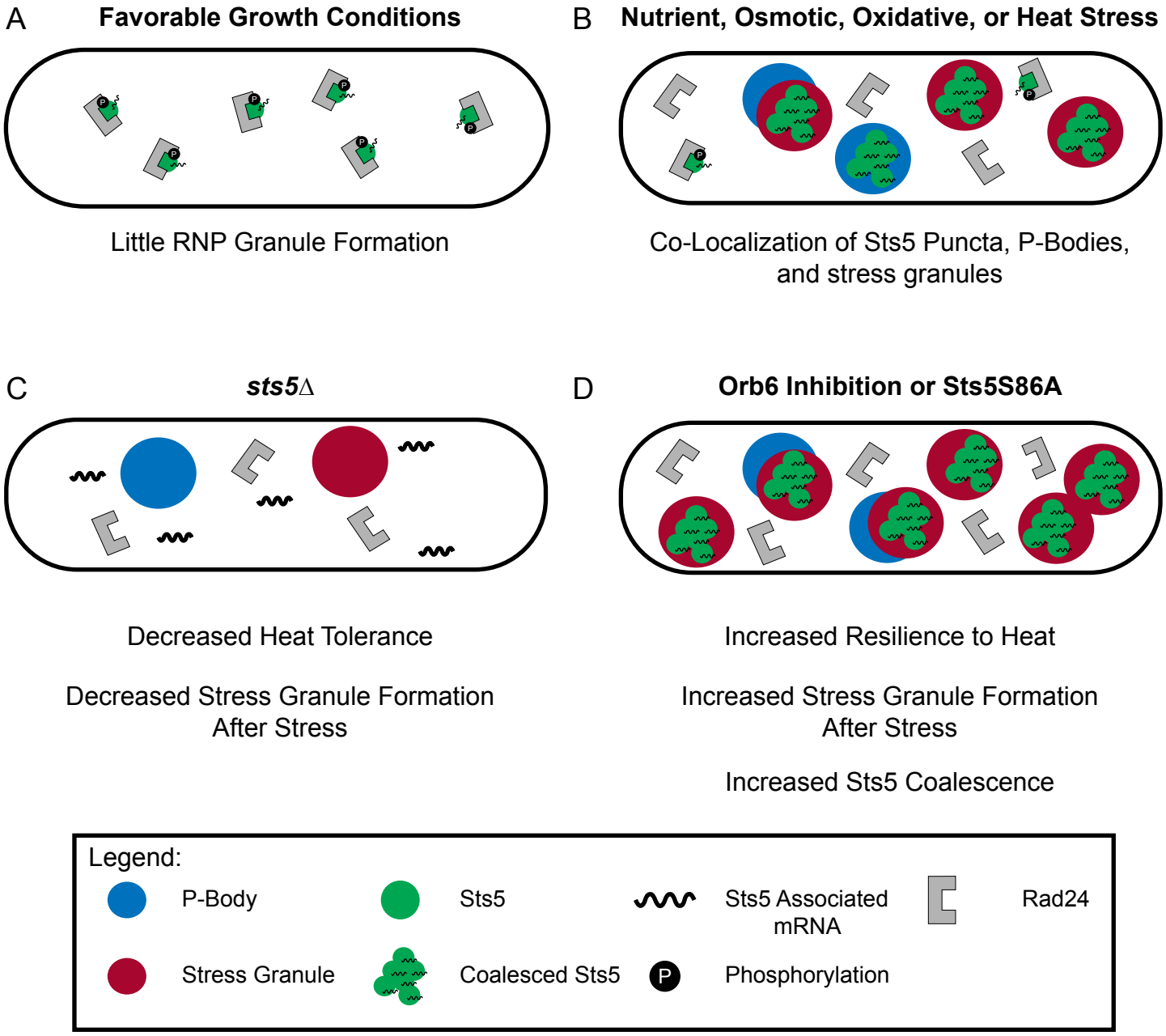
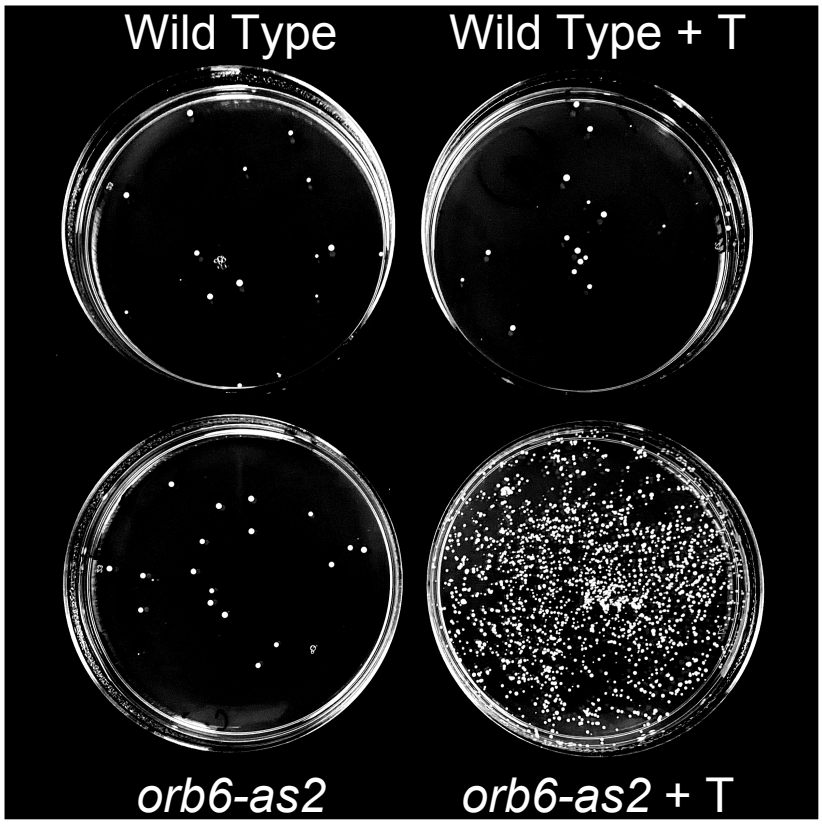


Figure 5. Model for Sts5 activation and response to stress. (A) Under optimal growth conditions, the RNA binding protein Sts5 is phosphorylated by the NDR kinase Orb6. This results in binding of Sts5 to the 14-3-3 protein Rad24 and promotes translation of Sts5 bound mRNA. Under these conditions, there is little to no formation of RNP granules. (B) Following exposure to heat stress, oxidative stress, osmotic stress, or nutrient starvation, Sts5 coalesces into cytoplasmic puncta which partially co-localize with P-Bodies and stress granules. (C) Loss of Sts5 decreases the formation of stress granules, and cells are less tolerant to heat stress. (D) Inhibition of Orb6 drastically increases resilience to heat stress and promotes the formation of stress granules after heat stress. The *sts5S86A* mutant exhibits increased Sts5 coalescence and is more resilient to heat stress.



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Supplemental Table 1: *S. pombe* strains used in this study.

Strain	Genotype	Source
FV1684	*pabp1-DsRed::KanMX6*	(Nilsson and Sunnerhagen, 2011)
FV2267	*sts5-3xGFP::NatMX6 dcp1-mCherry::hph*	(Nuñez *et al.,* 2016)
FV2361	*sts5-3xGFP::NatMX6 pabp1-DsRed::KanMX6*	This Study.
FV2518	*sts5Δ::NatMX6 sts5-HA::KanMX6*	(Chen *et al.,* 2019)
FV2522	*sts5Δ::NatMX6 sts586A-HA::KanMX6*	(Chen *et al.,* 2019)
FV2527	*orb6Δ::ura4+ pJK148orb6-as2::leu1+*	(Chen *et al.,* 2019)
FV2530	Wild Type, PN972	(Leupold, 1949)
FV2644	Wild Type, PN975	(Leupold, 1949)
FV2645	*sts5Δ::NatMX6 sts5-HA::KanMX6*	(Chen *et al.,* 2019)
FV2649	*sts5Δ::NatMX6 sts5S86A-HA::KanMX6*	(Chen *et al.,* 2019)
FV2674	*sts5Δ::KanMX6*	(Chen *et al.,* 2019)
FV3192	*sts5Δ::NatMX6 pabp1-DsRed::KanMX6*	This Study.
FV3226	*orb6Δ::ura4+ pjk148orb6-as2::leu1+ pabp1-DsRed::KanMX6*	This Study.