Article

Coordination of Translational Control and Protein Homeostasis during Severe Heat Stress

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Summary

Background: Exposure of cells to severe heat stress causes not only misfolding and aggregation of proteins but also inhibition of translation and storage of mRNA in cytosolic heat stress granules (heat-SGs), limiting newly synthesized protein influx into overloaded proteome repair systems. How these two heat stress responses connect is unclear.
Results: Here, we show that both *S. cerevisiae* and *D. melanogaster* heat-SGs contain mRNA, translation machinery components (excluding ribosomes), and molecular chaperones and that heat-SGs coassemble with aggregates of misfolded, heat-labile proteins. Components in these mixed assemblies exhibit distinct molecular motilities reflecting differential trapping. We demonstrate that heat-SG disassembly and restoration of translation activity during heat stress recovery is intimately linked to disaggregation of damaged proteins present in the mixed assemblies and requires Hsp104 and Hsp70 activity.
Conclusions: Chaperone-driven protein disaggregation directly coordinates timing of translation reinitiation with protein folding capacity during cellular protein quality surveillance, enabling efficient protein homeostasis.

Introduction

The structural fragility of proteins makes cells vulnerable to damage from various environmental stressors. In organisms from bacteria to humans, exposure to heat is a particularly important form of stress. Excess heat only a few degrees above optimal physiological temperature causes misfolding and aggregation of a broad spectrum of proteins, rapidly endangering cell viability [1]. Cells have therefore evolved powerful, compartment-specific stress responses that adjust to the degree of protein misfolding in order to maintain protein homeostasis [2].

The accumulation of misfolded proteins in the cytosol and nucleus of eukaryotic cells activates the heat-shock transcription factor enabling expression of protein quality control machinery components [3]. These are the ubiquitin-proteasome system and the molecular chaperones, including the Hsp70-Hsp40-Hsp110 network. Together, these systems secure regular folding pathways and eliminate misfolded proteins by refolding or degradation [4].

When heat-induced damage exceeds the capacity of these systems, misfolded proteins aggregate and sequester into intracellular deposits, considered to be cytoprotective [4, 5]. Multiple factors control protein aggregation. These include nature and concentration of the aggregating protein species, activity of the aggregate organizing cellular machinery, availability of chaperones, and disaggregation activity of the Hsp70-Hsp40-Hsp110 network (Ssa1-Ydj1/Sis1-Sse1 in *S. cerevisiae*) and the cooperating AAA+ ATPase Hsp100 (Hsp104 in *S. cerevisiae*) [1, 4, 6]. Severe heat stress well above the physiological growth temperature (>37°C for *S. cerevisiae*, >35°C for *D. melanogaster*, and >42°C for mammalian cells) causes an additional global inhibition of bulk protein synthesis, limiting protein influx to severely damaged proteomes. Selective synthesis of heat shock proteins (HSPs), which are exempt from the global inhibition of translation, boosts the capacity of the protein quality control machinery [7].

Global inhibition of protein synthesis also occurs in response to a number of other stress conditions, including nutrient starvation, oxidative stress, unfolded protein response, and cold shock [8–11]. Under most of these conditions, protein synthesis is blocked at the level of translation initiation through stress-specific activation of GCN2, HRI, PERK, or PKR [12, 13]. These kinases phosphorylate the translation initiation factor eIF2α, depleting the cellular pool of the ternary eIF2-guanosine triphosphate (GTP)-tRNA$_i$Met complex, which impedes the delivery of initiator tRNA^{Met} to the 40S ribosomal subunit. Following arrest of translation initiation, polysomes disassemble and stalled preinitiation complexes aggregate into large cytoplasmic structures termed stress granules (SGs) [14, 15]. Besides arrested mRNAs, SGs contain components of the translation machinery, including eIF4E (Cdc33 in yeast) and eIF4G (Tif4631/Tif4632 in yeast), the poly(A)-binding protein PABP (Pab1 in yeast), the 40S ribosomal subunits, and RNA binding proteins, such as TIA-1 (Pub1 in yeast) which contain aggregation-prone domains [16, 17]. SGs are thought to store partially assembled translation initiation complexes that rapidly resume translation upon stress relief. SGs may also exchange mRNA and protein components with P-bodies, where mRNAs are committed to degradation [18]. Sequestering of signaling molecules such as RACK1 and TRAF2 in SGs may also affect cell-death decisions [19, 20]. SGs further show stress- and organism-specific differences with respect to their composition, size, number, and exchange rates of SG components [14, 21].

Although heat-SGs were identified early in plant and mammalian cells, their composition and mechanism of formation, in particular the role of eIF2α phosphorylation, remain poorly understood and are controversial [22–24]. In mammalian cells, formation of heat-SGs requires eIF2α phosphorylation by the HRI kinase, whereas heat-SGs in *S. cerevisiae*, *T. brucei*, and *D. melanogaster* assemble independently of eIF2α phosphorylation through an unknown pathway [25–27]. Comprehensive

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understanding of the cellular response to heat stress requires clarification of how the multiple events integrate. These events include protein misfolding and aggregation, translation repression, and SG assembly, together with a sequence of reversal events during the recovery phase. Here, we investigate the connection between protein aggregation, translation control, and SG formation during robust heat shock. We show that thermolabile proteins and stalled translation initiation complexes form mixed mRNA-protein assemblies in *S. cerevisiae* and *D. melanogaster* cells at increased (but still viable) temperature. Upon stress relief, the presence of misfolded proteins in heat-SGs serves as a damage-calibrated timer that permits reinitiation of translation only when the protein quality control machinery has successfully eliminated the major part of unfolded proteins in the cytosol.

Results

SGs Colocalize with Misfolded Proteins during Robust Heat Shock in Yeast

Robust heat shock of yeast cells results in both SG formation and aggregation of thermally denatured proteins [5, 26]. This prompted us to investigate the relationship between heat-SGs and protein aggregates. As a reporter for heat-denatured proteins, we used firefly luciferase fused to GFP (GLuc), which aggregates with endogenous yeast proteins when exposed to temperatures above 37°C [28]. Yeast cells coexpressing GLuc and the SG marker poly(A)-binding protein (Pab1) fused to mCherry (Pab1Ch) were exposed to a 10 min heat shock at 37°C, 40°C, 43°C, and 46°C. Importantly, these heat treatments did not compromise cell survival within the 10 min exposure time; only longer exposure times at 46°C progressively decreased viability (Figure S1A available online). SG formation and protein aggregation in these cells were monitored by confocal fluorescence microscopy (Figure 1A), and the rate of global protein synthesis was estimated from polysome profiles (Figure 1B). As reported previously [28], GLuc formed multiple aggregates already with mild heat shock at 37°C. The number of GLuc aggregates increased dramatically at 40°C, whereas SG formation was inefficient and polysomal ribosomes were only modestly reduced from 83% of total ribosomes at 30°C to 65% at 40°C. Evidently, intermediate heat shock conditions (40°C) result in massive aggregation of GLuc but are not sufficient to trigger strong translational repression and SG formation. At 46°C, however, polysomal ribosomes dropped sharply from 83% to 17% of total ribosomes, which coincided with extensive SG formation (Figure 1A). Strikingly, aggregated GLuc or mCherry-luciferase (ChLuc) colocalized almost completely with several different SG markers at this temperature (Figures 1A and S1B).

To determine whether other misfolded proteins coaggregate in heat-SGs similar to GLuc, we analyzed the human von Hippel-Lindau (VHL) tumor suppressor protein. VHL maintains a native, soluble state when associated with its elongin-binding partners, which are absent in yeast. VHL therefore misfolds in yeast cells and either shows temperature-dependent aggregate formation or is degraded by the ubiquitin-proteasome system [29]. In cells grown at 30°C, mCherry-VHL remained diffuse but formed multiple aggregate foci after a 10 min heat shock at 46°C (Figure 1C). VHL foci displayed a high degree of colocalization with Pab1-GFP (Pab1G) foci, similar to GLuc foci.

Given the strong colocalization of SGs and misfolded protein aggregates under severe heat shock conditions, we asked

whether protein aggregates could serve as scaffold for SG formation. Cells expressing both GLuc and Pab1Ch were initially incubated at 42°C for 5 min, which causes strong GLuc aggregation and reduction of luciferase activity to 5% but no SG formation (Figures S1C and 1D). When cells were subsequently shifted to 46°C for 10 min, heat-SGs largely overlapped with preexisting GLuc aggregates (Figure 1D). This indicates that misfolded protein aggregates form independently at lower temperatures, whereas SG markers and protein aggregates colocalize upon further increase in stress severity.

We next investigated whether heat-stress-induced protein aggregates containing SG markers are indeed bona fide SGs, which also contain mRNA. We used fluorescence in situ hybridization (FISH) to characterize the RNA content of heat-SGs in yeast cells. An oligo(dT)$_{50}$ probe showed that poly(A)-mRNA appeared largely diffuse throughout the cytoplasm of cells at 30°C but upon shift to 46°C colocalized with Pab1Ch in heat-SGs (Figure 1E).

SGs have been reported to contain 40S ribosomal subunits, but this feature is controversial for yeast SGs and seems to depend on the type of stressor [26, 30]. Figure S1D shows that fluorescent reporters for two 40S subunit proteins, Rps2-GFP and Rps30A-GFP, remain diffuse and cytosolic at 46°C, similar to the 60S subunit protein reporter Rpl25-GFP. This was confirmed with a FISH probe specific for eukaryotic 18S rRNA, which also showed a diffuse staining in heat-shocked cells (Figure 1F). Unlike the 40S subunit, translation initiation factors, including eIF4E, eIF4G, eIF4B, and eIF3, accumulated markedly in heat-SGs (Figure S1E).

We next asked whether heat-SGs also colocalize with amyloid protein aggregates. To test this, we used the amyloidogenic exon1 fragment of human huntingtin with an expansion of 103 glutamines (103Q), which forms amyloids constitutively in yeast [31]. Before heat shock, 103Q-GFP formed a single focus in most cells. Raising the temperature to 46°C induced multiple smaller and less-intense 103Q-GFP foci per cell (Figures 1G and S1F). A heat-induced transition of 103Q from the single focus to the more fragmented multiple foci state has also been described in mammalian cells and can be explained by increased chaperone expression during the heat-shock response [32]. Importantly, unlike GLuc and mCherry-VHL foci, 103Q-GFP foci remained distinct from SGs (Figure 1G).

Together, these data show that translation arrest in yeast during severe heat stress correlates tightly with pronounced formation of SGs, which contain poly(A)-mRNA, multiple translation initiation factors, and misfolded proteins, but no ribosomal subunits. Coaggregation of heat-SG components and misfolded proteins is a general phenomenon and not substrate specific.

SGs Colocalize with Molecular Chaperones

The formation and fate of misfolded protein aggregates inside cells is strongly influenced by molecular chaperones. Association with small HSPs (e.g., yeast Hsp26) sequesters misfolded proteins into aggregate structures that facilitate subsequent refolding [33]. In yeast, the Hsp70 protein Ssa1 (one out of four Ssa homologs of yeast) with its Hsp40 cochaperones (Sis1 and Ydj1) cooperates with the Hsp104 disaggregase to associate with and solubilize aggregated proteins in an ATP-dependent manner [34]. We asked whether heat-activated Hsp26 and the Ssa-Hsp104 bichaperone system might associate with mixed heat-SG/protein aggregate assemblies in yeast. To monitor chaperone association, we employed functionally active Hsp104-yEmCitrine (Hsp104Ci), GFP-Ssa1
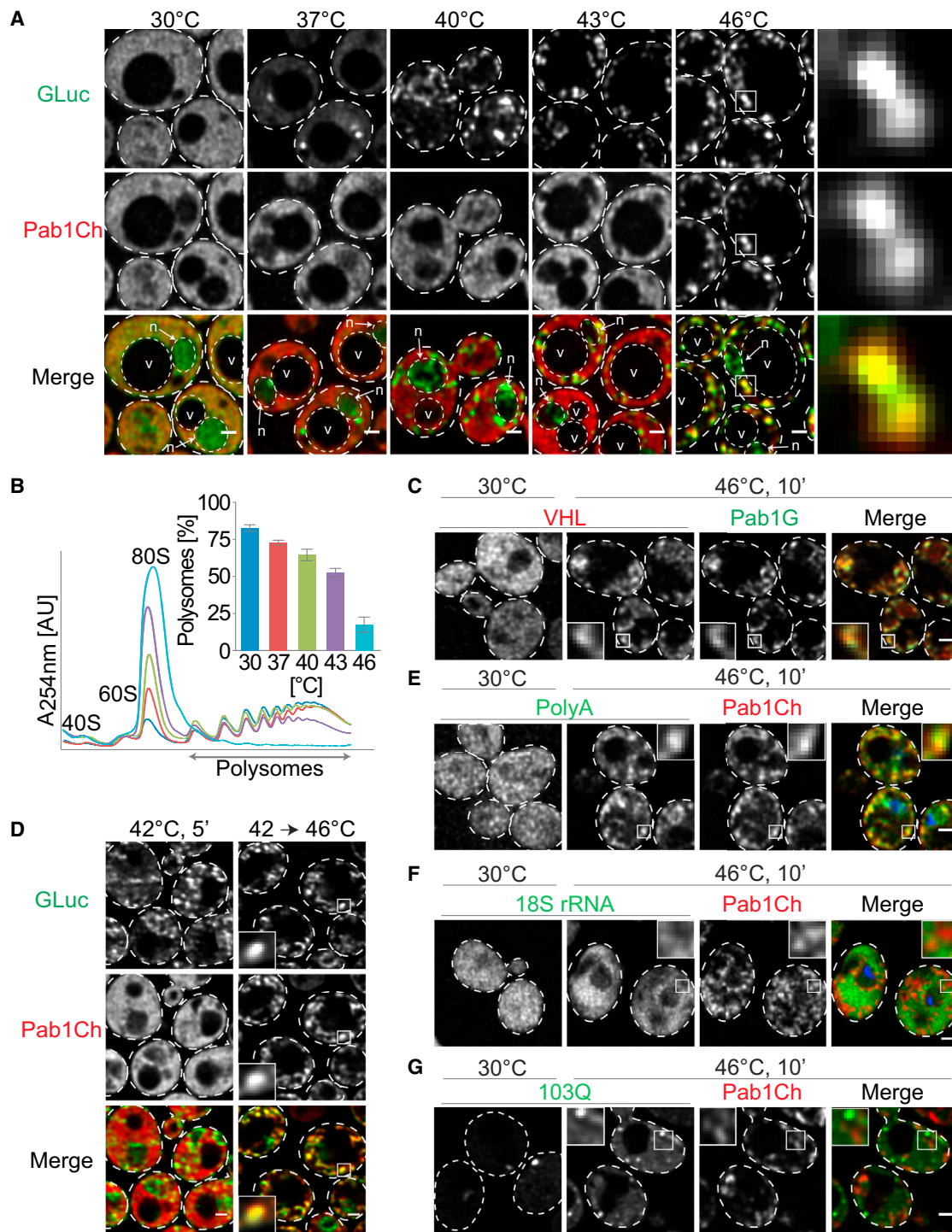
Figure 1. Heat-SGs Contain mRNA and Misfolded Protein Species, but No 40S Ribosomal Subunits

(A) Yeast cells expressing GFP-luciferase (GLuc) and Pab1-mCherry (Pab1Ch) were incubated at 37°C, 40°C, 43°C, or 46°C for 10 min, fixed, and imaged by confocal microscopy.

(B) Polysome profiles of yeast cells treated as in (A); the percentage of polysomal ribosomes ± SD (n = 3) is shown in the graph.

(C) Δpdr5 cells expressing Pab1-GFP (Pab1G) and VHL-mCherry exposed to heat shock.

(D) Yeast cells expressing GLuc and Pab1Ch were incubated at 42°C for 5 min and subsequently shifted to 46°C for 10 min.

(E and F) Yeast cells expressing Pab1Ch exposed to heat shock and stained by FISH for poly(A)-mRNA (E) or 18S rRNA (F).

(G) Wild-type cells expressing Htt103Q-GFP (103Q) and Pab1Ch exposed to heat shock.

Dashed lines indicate borders of the entire cell, vacuoles (v), and nuclei (n). Single confocal sections are shown. The scale bars represent 1 μm. See also Figure S1.
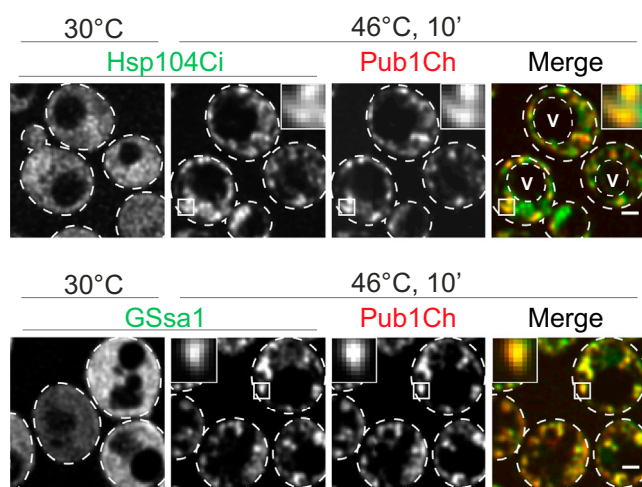
Figure 2. Molecular Chaperones Localize to Heat-SGs

Yeast cells expressing Hsp104-yEmCitrine (Hsp104Ci) and Pub1-mCherry (Pub1Ch) or GFP-Ssa1 (GSsa1) and Pub1Ch were exposed to heat shock. Single confocal sections are shown. The scale bar represents 1 μm. See also Figure S2.

(GSsa1; Figure S2A), and Hsp26-FLAG (Hsp26F) fusions [28]. Hsp104Ci, GSsa1, and Hsp26F showed diffuse localization in both the cytosol and nucleus at 30°C yet after 10 min heat shock at 46°C colocalized extensively with SG markers in heat-SGs (Figures 2 and S2B). We conclude that members of the Ssa1-Hsp104 bichaperone system and Hsp26 localize to mixed heat-SG-protein aggregate assemblies.

Association of SGs with Protein Aggregates Is Conserved from Yeast to Insects

The association of heat-SGs with protein aggregates could provide an efficient mechanism that coordinates translational activity with the folding status of the cytosolic proteome. To test whether coaggregation of SGs with heat-denatured proteins is evolutionary conserved, we examined stably expressed GLuc in *Drosophila* S2 cells, which normally grow at 25°C. Upon exposure to 38°C for 30 min, GLuc aggregates colocalized with heat-SGs, which were visualized by poly(A)-mRNA staining (Figure 3A) or mRFP fused to Rox8 (RRox8), the *Drosophila* homolog of the human TIA-1 protein (Figure 3B). This colocalization is specific to heat stress and does not occur for arsenite-induced SGs (Figures 3A and 3B).

We then explored the relation between protein aggregation and SG assembly in human U2OS osteosarcoma cells. A cytosolic luciferase variant GLuc^{cyt}DM, containing two point mutations (R188Q and R261Q) that render luciferase extremely thermolabile, formed multiple cytosolic aggregates at 44°C (Figure 3C). However, these aggregates did not colocalize with SGs visualized by poly(A)-mRNA staining, suggesting that misfolded proteins associate with heat-SGs in yeast and *Drosophila*, but not in human cells. This principal difference may be linked to the fact that translation repression during heat shock is independent of eIF2α phosphorylation in yeast and *Drosophila*, but not in mammalian cells [25, 26].

Heat-SG Components Are More Mobile Than Aggregated Proteins

Typically, protein components of SGs interact weakly with each other through low-complexity domains, allowing high rates of diffusion into and out of granules [14, 35]. Misfolded protein aggregates appear more stable, exchanging molecules with the cytosol slowly [28, 29]. Colocalization of SGs and protein aggregates in yeast and *Drosophila* cells raises the question whether the different components coaggregate passively in mixed assemblies or whether they retain their specific dynamic properties. To address this issue, we measured dynamics of GLuc and SG proteins by fluorescence loss in photobleaching (FLIP), fluorescence recovery after photobleaching (FRAP), and inverse FRAP (iFRAP). While these are powerful methods to detect molecule dynamics inside cells, they only allow for diffraction-limited bleaching making the analysis of subdiffraction-sized structures complicated. A clear distinction between SGs or luciferase aggregates to be bleached and the surrounding cytosolic fraction is difficult.

In heat-shocked yeast cells, the dynamics of GLuc and two SG RNA-binding proteins, Pab1G and Pub1-GFP (Pub1G), was assessed immediately after shift to 46°C by FLIP. We continuously bleached 20%–25% of the cell area and recorded the decrease in the mean fluorescence intensity of the entire cellular volume. Despite colocalization in the same granules, exchange of GLuc was distinctly slower (64% remaining signal after 15 min) than exchange of the SG proteins Pab1G and Pub1G (42% and 48% remaining signal; Figure 4A).

In heat-shocked *Drosophila* S2 cells, we measured dynamics of GLuc and the SG protein RRox8 by FLIP, FRAP, and iFRAP. For FLIP experiments, S2 cells coexpressing GLuc and RRox8 were continuously bleached for 22 min (Figure 4B; Movie S1). RRox8 lost 63% of its initial signal intensity, whereas GLuc intensity was reduced only by 21% at the end of the measurement. FRAP analysis in S2 cells expressing either GLuc or RRox8 also showed a pronounced difference in dynamics between the two. In cells exposed to 38°C, RRox8 moved into the bleached area within seconds, leading to almost complete (90%) recovery after 60 s. In contrast, the GLuc signal recovered only to about 70%, indicative of reduced molecule dynamics (Figure 4C). To determine whether RRox8 might be retained by GLuc aggregates, we performed FRAP analysis in S2 cells coexpressing both proteins. Interestingly, RRox8 dynamics was not affected by the presence of GLuc (Figure 4C, orange curve). For iFRAP, approximately 90% of the cell area was bleached, and fluorescence intensity of both reporters was monitored within the unbleached area. GLuc remained largely immobile over 5 min, whereas at least 40% of RRox8 shuttled out of SGs and associated with bleached heat-SGs (Figure S3, white arrows).

Our results demonstrate that SG components remain highly mobile when assembled with misfolded proteins in mixed granules. Each component appears to retain its distinct dynamics. We infer that colocalization of protein aggregates and SGs is therefore not due to coaggregation of misfolded SG components.

Chaperones Are Required for Efficient SG Disassembly and Regain of Translational Activity in *S. cerevisiae*

Colocalization of Hsp26, Ssa1, and Hsp104 with heat-SGs hinted that these chaperones might influence assembly or disassembly of SGs. To address this question, we analyzed heat-SG formation in yeast chaperone mutant strains. The kinetics of heat-SGs assembly or disassembly was not affected in the *Δhsp26Δhsp42* double mutant lacking the two small HSPs of *S. cerevisiae* (Figure S4A). Deletion of *hsp104* did not affect the formation of heat-SGs or luciferase aggregates. Strikingly, the disassembly of both misfolded protein

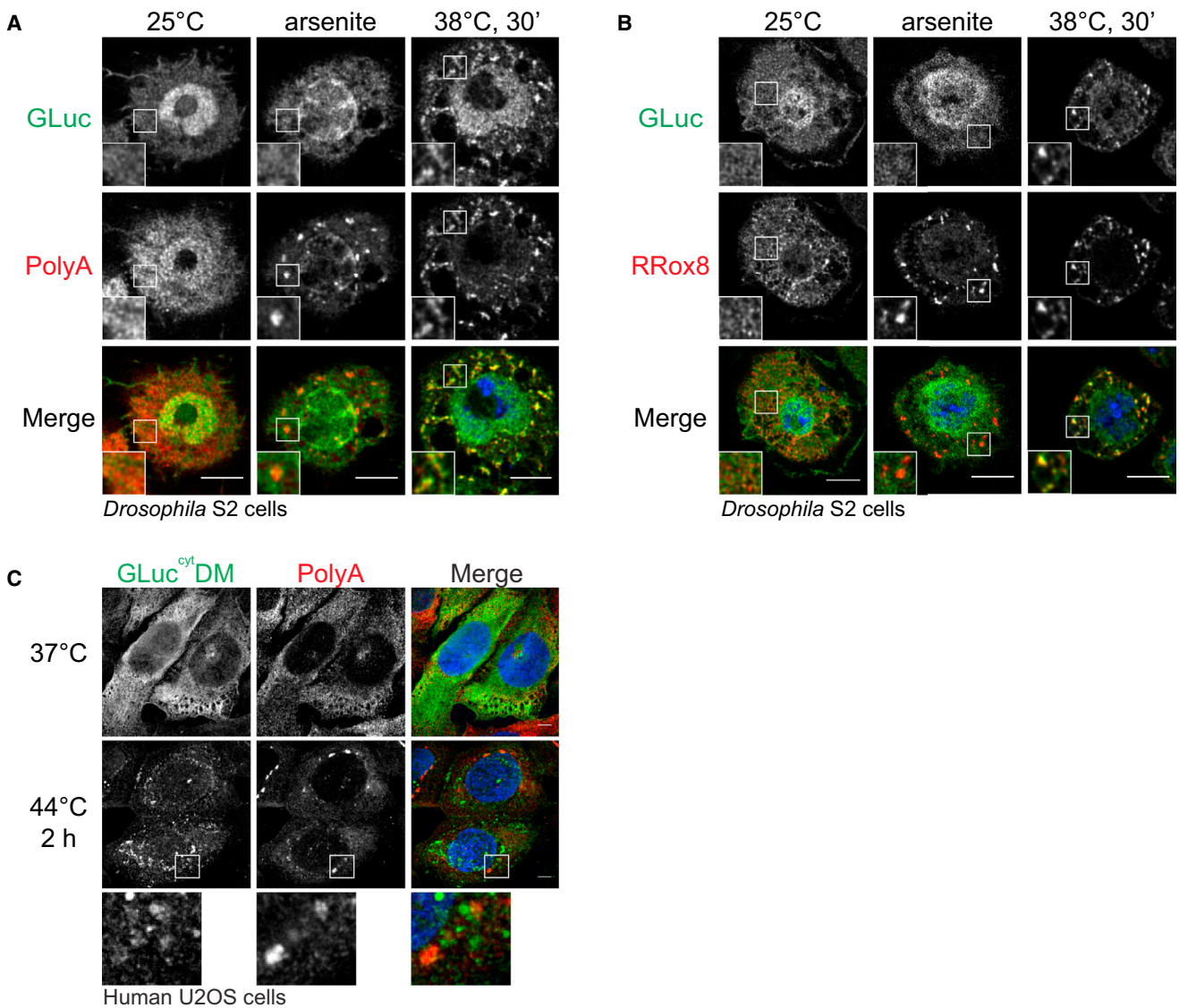**Figure 3. Colocalization of Heat-SGs and Thermolabile Proteins in _Drosophila_ Cells**

(A) S2 cells stably transfected with GLuc were left untreated, incubated with 500 μM arsenite for 1 hr, or subjected to heat shock. After fixation, poly(A)-mRNA was stained by FISH and imaged by confocal microscopy.

(B) S2 cells stably transfected with GLuc and RRox8 (mRFP-Rox8) were incubated with 500 μM arsenite for 1 hr at 25°C or exposed to 38°C for 30 m in (heat shock).

(C) Human U2OS cells expressing cytosolic thermolabile GFP-luciferase (GLuc^{cyt}DM) were incubated at 44°C for 2 hr (heat shock), fixed, and stained by FISH for poly(A)-mRNA.

Single confocal sections are shown. The scale bars represent 5 μm.

aggregates and SGs was strongly delayed in the _Δhsp104_ cells during recovery from heat shock (Figures 5A and S4B; Movies S2 and S3). We first verified that the _Δhsp104_ cells retain viability after the 10 min heat treatment at 46°C by a spot assay (Figure S4C). To address the solubility of the SG marker Pab1 as a measure for SG disassembly in the presence or absence of Hsp104 under these experimental conditions, we performed a protein aggregation assay (Figure 5B). While at 30°C, almost all Pab1G was present in the soluble fraction, 34%–43% of Pab1G became insoluble in both wild-type and _Δhsp104_ cells after severe heat shock. In wild-type cells, Pab1G largely regained solubility (95%) 2 hr after heat shock. Pab1G solubilization was delayed in _Δhsp104_ cells and required 4 hr of recovery for almost complete (92%) disaggregation (Figure 5B). Since

heat-SGs form as a consequence of translation arrest, we examined whether defective disassembly of heat-SGs in _Δhsp104_ cells would affect resumption of protein synthesis after stress removal. We recorded polysome profiles in cells exposed to a 10 min heat shock at 46°C and after 2 and 4 hr of recovery at 30°C (Figure 5C). Polysomal ribosomes from wild-type cells dropped from 83% of total ribosomes to 19% after heat shock but recovered almost completely (to 78%) within 2 hr. Polysomal ribosomes from _Δhsp104_ cells dropped to a similar extent (from 81% to 19%) upon heat shock but reassembled much more slowly during recovery (44% after 2 hr; 71% after 4 hr). This is consistent with our observation that disaggregation of the immobile Pab1 fraction is delayed in _Δhsp104_ cells and requires 4 hr of recovery for almost
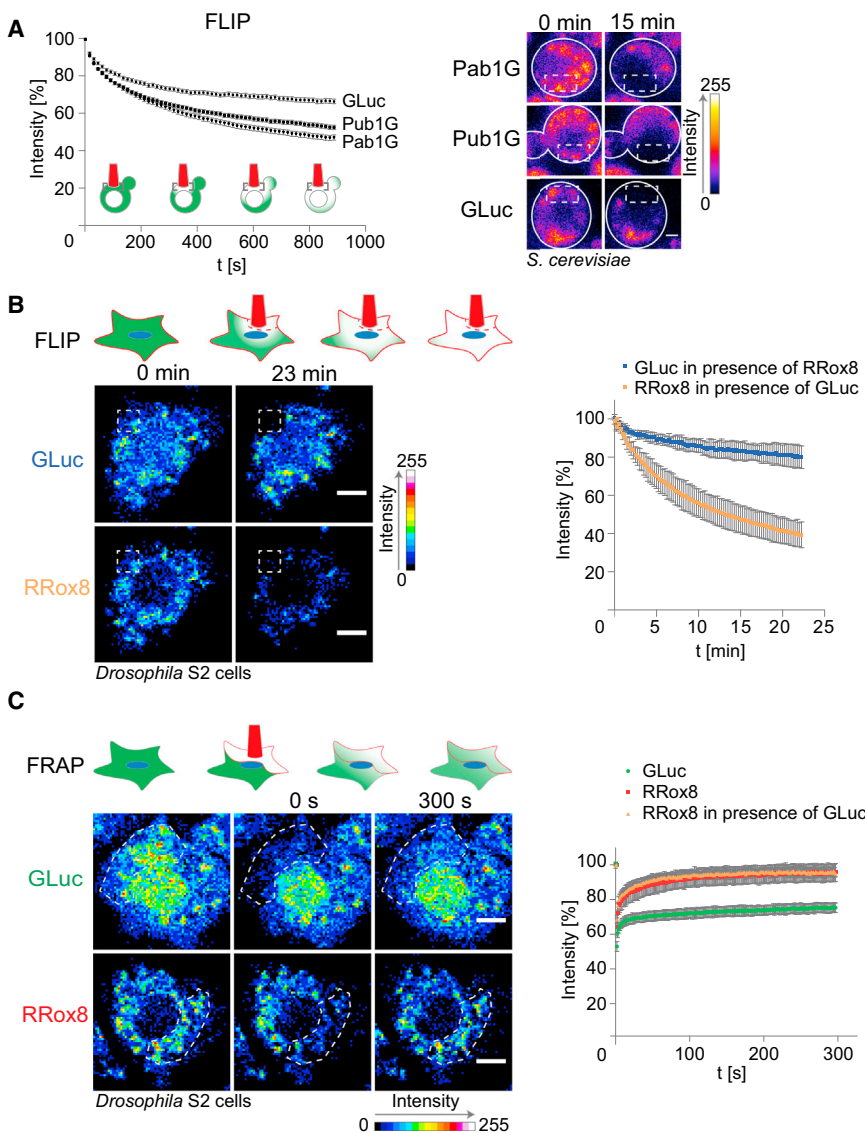
Figure 4. RNA-Binding SG Proteins Are More Mobile Than Misfolded Proteins in Mixed Assemblies

(A) Upon heat shock at 46°C for 10 min, yeast cells expressing either GLuc, Pab1G, or Pub1-GFP (Pub1G) were immediately subjected to FLIP analysis at room temperature (RT). Representative images are shown in the right panel. Average values (±SEM) were calculated from measurements on 32 (Pab1G, GLuc) and 36 (Pub1G) cells. The scale bar represents 1 μm.
(B) S2 cells expressing GLuc and RRox8 were exposed to heat shock and used immediately for FLIP analysis at RT. Average ± SD; n = 5.
(C) S2 cells expressing GLuc, RRox8, or both were exposed to heat shock and used immediately for FRAP analysis at RT. Average ± SD; n = 11 (GLuc), 14 (RRox8), and 10 (RRox8 in presence of GLuc).
Dashed lines indicate the area used for bleaching. The scale bars represent 5 μm. See also Figure S3 and Movie S1.

at 37°C, heat-SGs disassembled in the majority of *ssa1-wt* cells. In contrast, SGs persisted in the *ssa1-ts* cells at 37°C, indicating that SG disassembly requires Ssa1 function (Figures 5D, S4F, and S4G). When the recovery temperature was further reduced to 25°C, heat-SGs in the *ssa1-ts* strain began to disassemble, showing that the cells were not irreversibly damaged after heat shock (see also spot tests; Figure S4H). We note that in both *ssa1-wt* and *ssa1-ts* cells, heat-SGs disassembled prior to luciferase disaggregation (see also quantitative image representation; Figure S4G).

Together, these results provide compelling evidence that the Ssa1-Hsp104 bichaperone system enables yeast cell survival after extended exposure to severe heat treatment by promoting the release of the translation machinery trapped in SGs, which allows for reinitiation of protein synthesis.

complete solubilization (Figure 5B). We suggest that slow and partially Hsp104-independent solubilization of SGs is responsible for delayed translation reinitiation in *Δhsp104* cells.

To confirm the role of Hsp104 in SG disassembly, we treated GLuc- and Pab1Ch-expressing wild-type cells with low concentrations (3 mM) of guanidinium hydrochloride (Gnd), which reversibly inhibits Hsp104 in a specific manner [36], followed by a 46°C heat shock for 10 min. We washed half the cells with fresh medium, leaving the other half in Gnd, and let both cultures recover for 4 hr at 30°C. As in the *Δhsp104* strain, presence of Gnd during recovery prevented heat-SG disassembly (Figures S4D and S4E).

To assess the role of Ssa proteins, we investigated the triple deletion strain *Δssa2Δssa3Δssa4*, where the remaining canonical Hsp70 gene, *SSA1*, is expressed as wild-type or temperature-sensitive *ssa1-45* allele (referred to as *ssa1-wt* or *ssa1-ts*). The Ssa1-45 mutant protein is fully active at 30°C yet becomes reversibly inactivated at 37°C [37]. In both *ssa1-wt* and *ssa1-ts* strains grown at 25°C, Pab1G and ChLuc remained diffuse throughout the cell (Figure 5D) and aggregated to a similar extent upon shift to 46°C. After 2 hr recovery

Hsp70 Is Required for Efficient SG Disassembly and Regain of Translational Activity in *Drosophila*

Protein disaggregation in metazoa (which lack Hsp104 activity) is provided by the Hsp40, Hsp70, and Hsp110 system [38, 39]. We set out to investigate whether the central component of this system, Hsp70, is required for heat-SG disassembly and translation reinitiation in *Drosophila* cells during recovery from heat shock. For this purpose, we made use of VER-155008, a potent inhibitor of the intrinsic ATPase activity of Hsp70 [40].

In absence of the inhibitor, both GLuc aggregates and SGs were efficiently disassembled in S2 cells within 2–4 hr upon return to 25°C (Figure 6A; Movie S4). This was paralleled by regain of luciferase activity, a measure of protein folding to the native state, to 79% (Figure 6B). Complete reassembly of polysomes, however, required 8 hr of recovery at 25°C (Figure 6C). These results suggest that protein refolding, SG disassembly, and translation reinitiation proceed in a
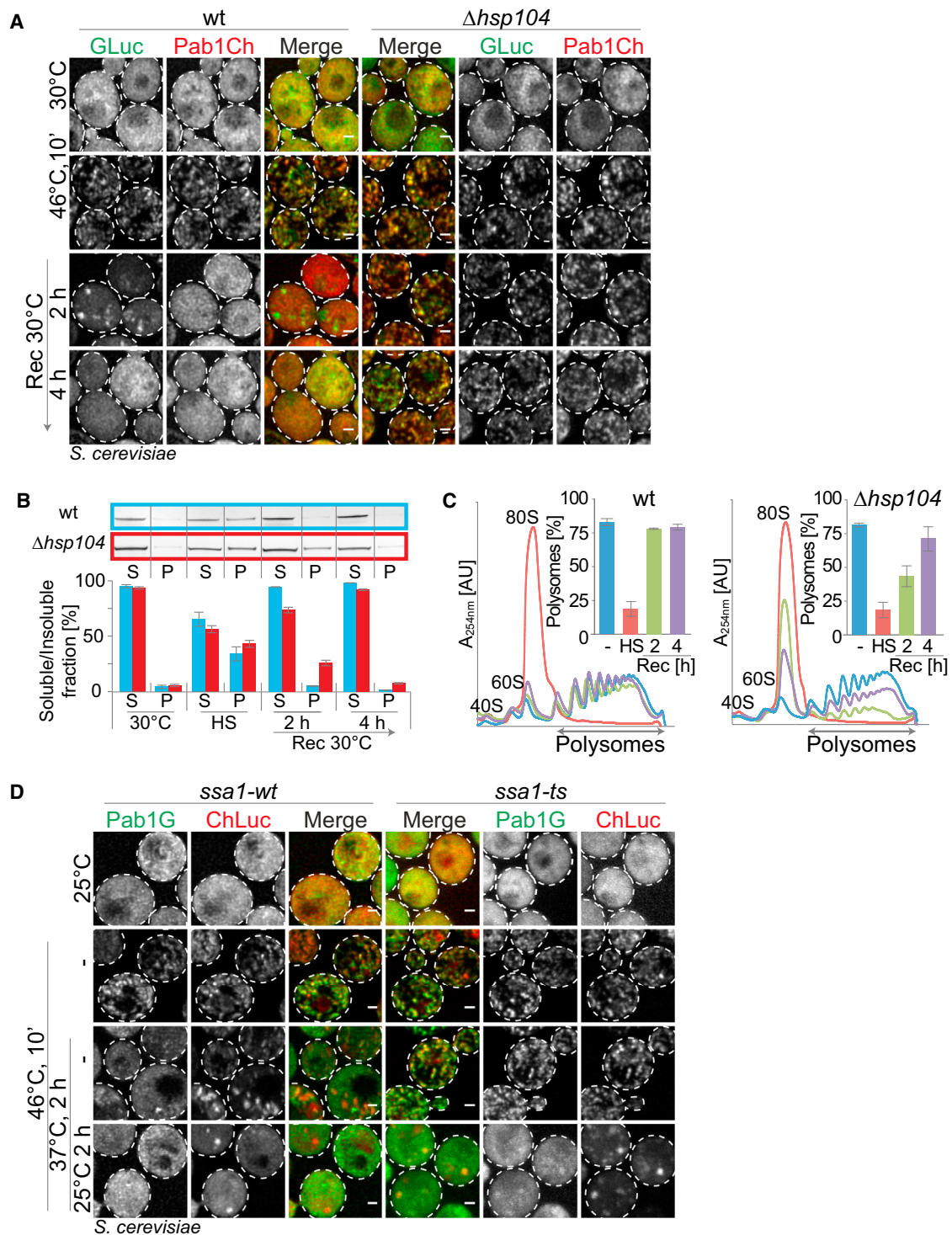
Figure 5. Molecular Chaperones Promote SG Disassembly and Translation Reinitiation during Recovery from Heat Stress

(A) Wild-type or *Δhsp104* yeast cells expressing GLuc and Pab1Ch were exposed to heat shock and subsequently incubated at 30°C for 2 or 4 hr.

(B) Wild-type and *Δhsp104* strains expressing Pab1G were treated as in (A). Samples were analyzed in a protein aggregation assay. Soluble and insoluble fractions of Pab1G were quantified from the western blot. For each fraction, the ratio between the respective fraction and the sum of soluble and insoluble fractions of the corresponding sample was calculated (average values; ±SD; n = 2). HS, heat shock.

(C) Polysome profiles from samples shown in (A) were recorded; the percentage of polysomal ribosomes ± SD (n = 3) is shown in the graph.

(D) *ssa1-wt* and *ssa1-ts* strains expressing Pab1G and ChLuc were exposed to heat shock and incubated at nonpermissive temperature (37°C) for 2 hr, followed by recovery at permissive temperature (25°C) for 2 hr.

Cells were fixed and imaged by confocal microscopy. Images show maximum intensity projections of all confocal sections. The scale bars represent 1 μm. See also Figure S4 and Movies S2 and S3.
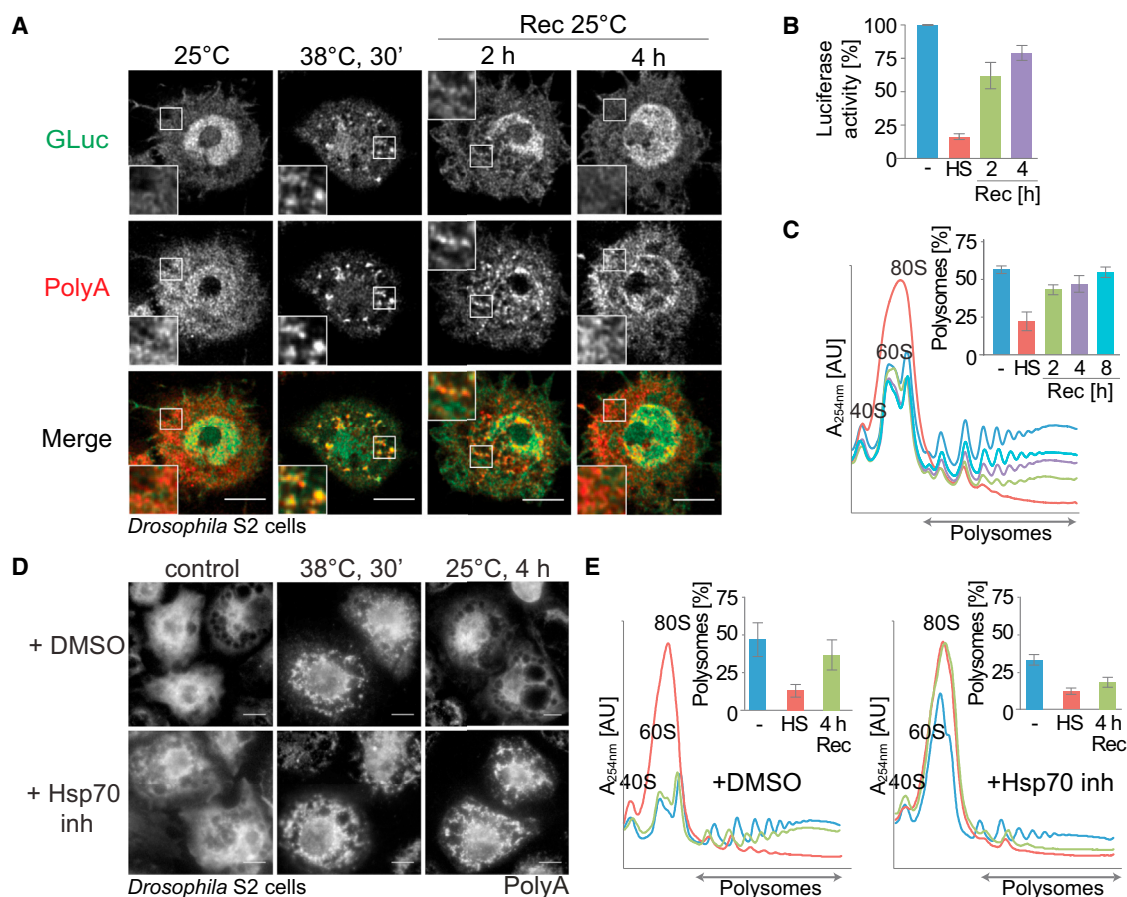
Figure 6. Hsp70 Promotes Heat-SG Disassembly and Translation Recovery in *Drosophila* Cells

(A) S2 cells expressing GLuc were exposed to heat shock at 38°C for 30 min, allowed to recover at 25°C for 2 or 4 hr, and stained by FISH for poly(A)-mRNA. Single confocal sections are shown.

(B) Luciferase activity ± SD (n = 3) was measured in cells treated as in (A).

(C) S2 cells expressing GLuc exposed to heat shock at 38°C for 30 min; allowed to recover at 25°C for 2, 4, or 8 hr; and subjected to polysome profile analysis. Average values ± SD (n = 3).

(D) S2 cells treated with 100 μM Hsp70 inhibitor VER-155008 for 1 hr, exposed to 38°C 30 min, allowed to recover at 25°C for 4 hr, and stained by FISH for poly(A)-mRNA. Maximum intensity projections are shown.

(E) S2 cells were treated as in (D) and subjected for polysome profile analysis. Average values ± SD (n = 3).

The scale bars represent 5 μm. See also Figure S5 and Movie S4.

coordinated manner during the recovery of *Drosophila* cells from heat shock.

To address the role of Hsp70 in disassembly of heat-SGs and regain of translational activity during recovery, S2 cells were treated with 100 μM Hsp70 inhibitor, exposed to heat shock and allowed to recover for 4 hr. Ver-155008 treatment efficiently inhibited Hsp70 activity as monitored by measurement of luciferase activity after the heat shock (Figure S5A). As compared to the DMSO-treated control cells, disassembly of heat-SGs and resumption of translation in inhibitor-treated cells were strongly delayed (Figures 6D, 6E, and S5B). Together, these findings indicate that chaperone-driven disaggregation of heat-SGs is required for the regain of translational activity in both *S. cerevisiae* and *Drosophila* cells.

Discussion

This study provides an analysis of the integrated consequences of high-temperature stress on two particularly vulnerable cellular targets: the translation process and protein

homeostasis. We demonstrate that granules containing stalled mRNA, formed rapidly in *S. cerevisiae* and *Drosophila* cells upon exposure to severe heat stress, colocalize with aggregates of misfolded proteins. These include thermolabile heterologous marker proteins but also endogenous yeast proteins (monitored by Hsp104 association). We also found that amyloid Htt103Q aggregates remain distinct from heat-SGs. This suggests that amorphous, but not amyloid, protein aggregates underpin mixed SG formation. The reported colocalization of SGs and amyloids in neuronal cells grown at physiological growth temperature seems to be a distinct phenomenon, likely involving RNA-binding properties of amyloids and associated proteins [41].

The molecular interrelationship between aggregating thermolabile proteins and heat-SGs is complex. Protein aggregation in the cytosol per se, during moderate heat stress ≤ 43°C in yeast, does not induce translation arrest or heat-SG formation. The successive increase in temperature from moderate (42°C) to severe (46°C) heat shock induces heat-SG formation in association with preformed protein aggregates.
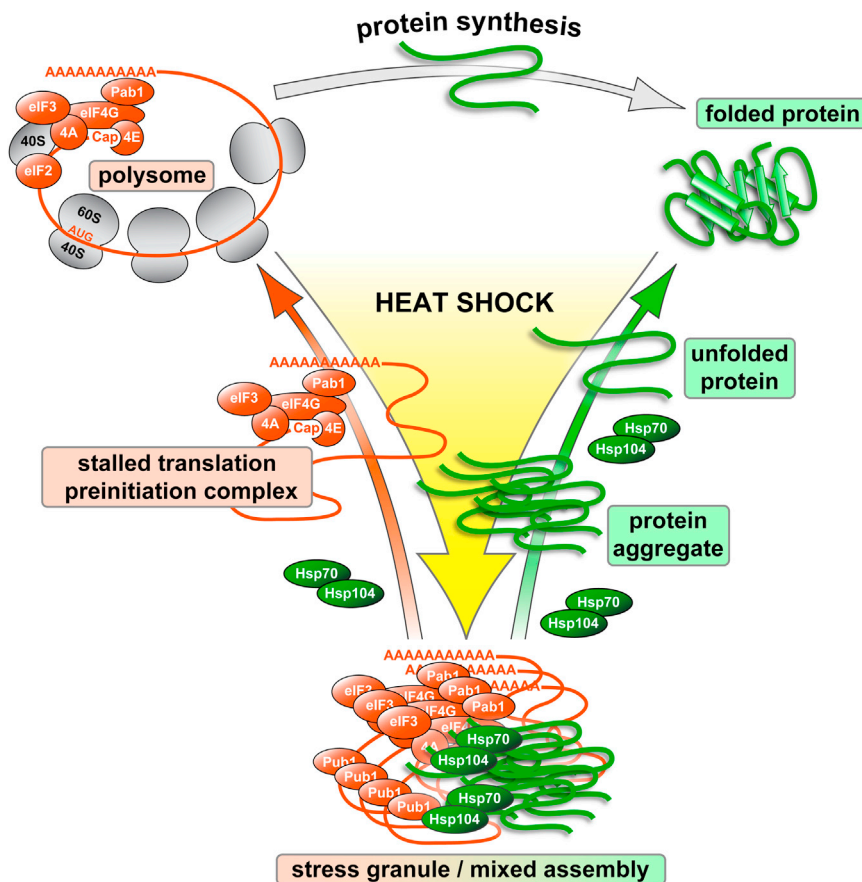
Figure 7. Model of Protein Homeostasis Maintained through Sequestration of Translation Machinery in Protein Aggregates

Heat-labile proteins aggregate after a modest rise in temperature and provide scaffolds for assembly of stalled translation preinitiation complexes at higher temperatures. This leads to the formation of mixed assemblies of protein aggregates and heat-SGs. Chaperone induction and return to lower temperature reduces the load of aggregated proteins, which in turn liberates preinitiation complexes from mixed assemblies and enables translation reinitiation.

mixed heat-SG assemblies. However, we were not able to detect 40S ribosomal subunits in these assemblies. This suggests that heat shock in yeast and *Drosophila* cells may arrest translation initiation at an early stage, prior to 40S recruitment to the mRNA and upstream of the eIF2α-dependent stress-signaling pathway [43, 44]. In contrast, mammalian cells activate the eIF2α pathway during heat shock [25] and form heat-SGs considered to contain 40S subunits. These differences correlate with our observation that protein aggregates do not localize with heat-SGs at elevated temperatures in human U2OS cells. Formation and composition of heat-SGs may therefore differ substantially between mammalian and other eukaryotic cells.

However, we did not observe increased kinetics of heat-SG formation within cells containing preformed protein aggregates. This argues for initially independent, noncooperative assembly processes for aggregating proteins and heat-SGs and against cross-seeding as observed for amyloids [35]. Our data indicate that protein aggregates serve as scaffolds for the docking of stalled translation initiation complexes, implying the existence of microdomains within mixed aggregates.

This idea is further supported by the dynamics of molecules within the mixed aggregates. FRAP, iFRAP, and FLIP analyses (measuring aggregate dissociation and association of molecules) consistently revealed that heat-denatured luciferase trapped in inclusions is immobile, in agreement with earlier reports [42], whereas the RNA-binding protein Pub1/Rox8 (TIA-1 in mammalian cells) shuttled faster into and out of yeast and *Drosophila* heat-SGs. Heat-SGs therefore do not simply constitute aggregates of misfolded components of the translation machinery. Instead, they retain the dynamic features of transient storage forms of preinitiation complexes described for RNA granules formed by different types of stress [14]. We speculate that protein aggregates may form a nucleus around which heat-SG components assemble. This is supported by the observation that heat-SGs form only at very severe heat-stress conditions, while protein aggregates form already at much milder stress conditions. We assume that the resulting mixed aggregates are heterogeneous in organization and size and that the dynamics of heat-SG components may depend on their position within the SG.

Our experiments show that poly(A)-mRNA, multiple translation initiation factors, and RNA-binding proteins localize to

Extending the intricate functional links between misfolded protein aggregation and heat-SG formation, we show for the first time that Hsp26, Hsp70, and Hsp104 chaperones directly associate with heat-SGs in yeast. These chaperones are not essential for the assembly process, since chaperone-deficient yeast mutants show normal formation of mixed heat-SGs. However, yeast mutants depleted for cytosolic Hsp70 (Ssa) activity were inefficient at solubilizing the mixed assemblies during recovery from heat shock. *Δhsp104* mutants showed an even larger delay in recovery, leading to the persistence of mixed SG assemblies for ≥4 hr and compromised translation reinitiation. We infer that the central disaggregase of yeast, the Hsp104-Ssa1 bichaperone system, has an important function in liberating stalled translation initiation complexes from heat-SGs. We obtained conceptually similar results for *Drosophila* cells, where chemical inactivation of Hsp70 also impairs disassembly of heat-SGs during recovery. These findings are in agreement with the earlier observation that, in mammalian cells, Hsp70 overproduction decreases the aggregation of the prion-related domain of TIA-1 [17].

Together, our findings demonstrate that protein disaggregation is essential for efficient translation reinitiation during recovery of yeast cells from heat shock. The thermotolerance defects previously described for *Δhsp104* and *ssa* mutants [37, 45] may therefore also result from the inability to extract essential components of the translation machinery from heat-SGs.

The requirement of disaggregating chaperones for heat-SG disassembly reveals a molecular mechanism by which the protein-folding status in the cytosol of yeast and *Drosophila* cells controls the translation machinery (Figure 7). Heat-labile

proteins aggregate early during a modest rise in temperature and act as scaffolds for the association of stalled translation preinitiation complexes at higher temperatures. We envision this involves misfolded proteins and aggregation-prone domains within SG proteins, which need to be disaggregated during recovery from heat shock to allow for reinitiation of translation. They might act as molecular thermometers to ensure that translation is stalled at severe heat-stress temperatures to limit the influx of newly synthesized proteins into the proteome. Recent findings show that transient low-affinity interactions of RNA-binding components with prion-like, low-complexity domains are responsible for RNA granule formation [35]. We speculate that such proteins undergo thermally induced conformational transitions leading to the collapse of translation and the assembly of heat-SGs. This would trap translation preinitiation complexes in mixed SG assemblies, preventing de novo protein synthesis. The induction of chaperones and/or a drop in temperature would in turn reduce the amount of aggregated proteins, releasing preinitiation complexes from mixed assemblies to re-enter the translation cycle. We therefore propose that the presence of misfolded proteins in mixed assemblies serves as a timer that coordinates reinitiation of translation with elimination of unfolded proteins in the cytosol, both involving chaperone disaggregase activities. We conclude that coaggregation of translation factors, mRNAs, and heat-labile proteins brings together two essential arms of the protein quality control system, ensuring cell survival by making protein synthesis mechanisms directly responsive to changes in protein refolding, aggregation, and degradation levels during heat stress. This provides a robust, self-calibrating system by which protein homeostasis in the cytosol is maintained over a broad range of temperatures.

Experimental Procedures

The complete details of the experimental procedures are provided in the Supplemental Experimental Procedures.

Photobleaching Experiments

For photobleaching experiments in *Drosophila*, S2 cells were plated on concanavalin A-coated glass bottom microwell dishes (MatTek) and incubated for 30 min at 38°C before proceeding with microscopy. For FLIP experiments in yeast, cells attached to a concanavalin A-coated microwell dish were shifted to 46°C for 10 min. Regions of interest of the same size were repeatedly bleached using 488 nm laser.

Polysome Profile Analysis

For quantifications of polysome profiles, a profile of the lysis buffer alone was subtracted from "sample gradient" values, and the areas under the curve corresponding to the amount of polysomal and total ribosomes were determined by integration. The amounts of polysomal ribosomes were divided by total ribosomes as a measure for the translation activity.

Protein Aggregation Assay

Yeast cells were grown to mid-log phase, treated as indicated, harvested by centrifugation, resuspended in 50 mM Tris-HCl pH 8.5/500 mM NaCl/1 mM phenylmethylsulfonyl fluoride (PMSF)/EDTA-free protease inhibitors (Roche), and frozen in liquid nitrogen. Cells were pulverized by mixer milling (MM 400; Retsch), precleared by centrifugation at 3,000 × g and centrifuged at 16,000 × g for 20 min. The supernatant contained the soluble fraction. The pellet was washed in 50 mM Tris-HCl pH 8.5/150 mM NaCl/1 mM PMSF/EDTA-free protease inhibitors (Roche), centrifuged at 16,000 × g for 20 min, and resuspended in 8 M urea/2% SDS/50 mM TRIS pH 8.5/150 mM NaCl/1 mM PMSF/2 mM dithiothreitol/EDTA-free protease inhibitors (Roche). After centrifugation at 16000 × g for 5 min, the supernatant contained the insoluble protein fraction. Same volumes of soluble and insoluble fractions were used for quantitative western blotting. To calculate the soluble or insoluble fraction of a protein, the signal of the supernatant or pellet was divided by the total signal of the supernatant and pellet of the same sample.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, five figures, two tables, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.09.058.

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