Stress-dependent relocalization of translationally primed mRNPs to cytoplasmic granules that are kinetically and spatially distinct from P-bodies

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Stress-dependent relocalization of translationally primed mRNPs to cytoplasmic granules that are kinetically and spatially distinct from P-bodies

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Cytoplasmic RNA granules serve key functions in the control of messenger RNA (mRNA) fate in eukaryotic cells. For instance, in yeast, severe stress induces mRNA relocalization to sites of degradation or storage called processing bodies (P-bodies). In this study, we show that the translation repression associated with glucose starvation causes the key translational mediators of mRNA recognition, eIF4E, eIF4G, and Pab1p, to resediment away from ribosomal fractions. These mediators then accumulate in P-bodies and in previously unrecognized cytoplasmic bodies, which we define as EGP-bodies. Our kinetic studies highlight the fundamental difference between EGP- and P-bodies and reflect the complex dynamics surrounding reconfiguration of the mRNA pool under stress conditions. An absence of key mRNA decay factors from EGP-bodies points toward an mRNA storage function for these bodies. Overall, this study highlights new potential control points in both the regulation of mRNA fate and the global control of translation initiation.

Introduction

Control of translation initiation allows a rapid and dynamic cellular response to stress. We have previously shown the dramatic inhibitory impact of stress on translation initiation in yeast (Ashe et al., 2000, 2001; Holmes et al., 2004). This global inhibition has an immediate effect on protein levels and, thus, conserves resources. Additionally, this regulation affords the cell a respite period, allowing a redirection of resources toward stress survival (Natarajan et al., 2001; Smirnova et al., 2005).

Two established regulatory mechanisms target distinct steps in translation initiation. First, the selection of mRNAs can be regulated. eIF4E and Pab1p select mRNA via interaction with the 5′ cap and 3′ poly(A) tail, respectively. eIF4G interacts with both factors, promoting a closed loop messenger RNP (mRNP) complex (Sachs, 2000). The formation of the closed loop mRNP complex can be inhibited either by eIF4E-binding proteins (4E-BPs) or by eIF4E homologous proteins (4EHPs).

4E-BPs competitively inhibit the eIF4G–eIF4E interaction, thereby preventing translation initiation either in a global or mRNA-specific manner. In higher eukaryotes, inactivation of the mammalian target of rapamycin pathway activates 4E-BPs, leading to a general down-regulation of 5′ cap–dependent protein synthesis (Richter and Sonenberg, 2005). Alternatively, 4E-BPs such as Maskin in *Xenopus laevis* or Cup in *Drosophila melanogaster* are targeted to specific mRNAs by interaction with mRNA-binding proteins (Richter and Sonenberg, 2005). *Saccharomyces cerevisiae* has two 4E-BPs, Caf20p and Eap1p, which translationally regulate some mRNAs yet are unlikely to act as global translational regulators (Altmann et al., 1997; Ibrahimo et al., 2006).

4EHPs have the capacity to interact with the mRNA cap structure but do not interact efficiently with eIF4G (Cho et al., 2005). Therefore, the closed loop mRNP complex may be compromised by 4EHP competition with eIF4E for the 5′ cap structure. For instance, this mechanism explains the translational regulation of *Caudal* mRNA in *Drosophila*, in which 4EHP is specifically targeted to the mRNA 3′ untranslated region (Cho et al., 2005). A similar mechanism has recently been proposed to explain the impact of Argonaute proteins on translation initiation. Here, micro-RNAs target Argonaute protein to specific transcripts, where they interact with the 5′ cap structure to inhibit translation initiation (Kiriakidou et al., 2007).

A second regulated step in the translation initiation pathway involves activation of the stress-responsive eIF2α kinases. The initiator methionyl tRNA (Met-tRNA$_i$) forms a ternary complex (TC) with eIF2-GTP and is recruited to the 40S ribosome.

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Abbreviations used in this paper: mRNP, messenger RNP; P-body, processing body; SG, stress granule; TC, ternary complex.

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GTP hydrolysis generates eIF2-GDP as a by product of translation initiation, and this is recycled to eIF2-GTP by a guanine nucleotide exchange factor, eIF2B. Phosphorylation of eIF2 by the eIF2α kinases inhibits this recycling to reduce the level of TC, which ultimately limits translation initiation (Kapp and Lorsch, 2004). The yeast eIF2α kinase Gcn2p responds in this manner to stresses such as amino acid starvation (Hinnebusch, 2005).

In a variety of experimental systems, pools of mRNA are specifically sequestered into cytoplasmic granules or bodies during periods of translational inactivity (Anderson and Kedersha, 2006). In germ cells, specific germ cell granules are sites where repressed mRNAs congregate during oogenesis (Strome and Wood, 1982; Mahowald, 2001). After stress in mammalian cells, translationally repressed mRNAs assemble into particles called stress granules (SGs; Kedersha and Anderson, 2002). In neuronal cells, mRNAs that are in transit to their dendritic site of translation are held in neuronal-specific RNA granules (Anderson and Kedersha, 2006). Finally, in yeast and mammalian systems, RNA processing bodies (P-bodies) have been defined as cytoplasmic bodies that harbor many of the enzymes involved in the 5′ to 3′ pathway of mRNA decay (Sheth and Parker, 2003; Cougot et al., 2004). A diverse array of functions has been ascribed to these RNA granules, including roles in mRNA localization, degradation, and storage as well as in the micro-RNA pathway (Anderson and Kedersha, 2006).

P-bodies harbor mRNA along with various components of the mRNA metabolic machinery (Parker and Sheth, 2007). The earliest described P-body components in yeast were the mRNA degradation components Dcp1p, Dcp2p, Dhh1p, Pat1p, Lsm1p, and Xrn1p (Sheth and Parker, 2003). These components are highly conserved and can be thought of as core components. Less well-conserved components include micro-RNA repressor factors, nonsense-mediated decay enzymes (Upf1-3p), and viral factors (for review see Parker and Sheth, 2007). In yeast, translation factors were described as absent from P-bodies, although eIF4E was found in human P-bodies (Andrei et al., 2005; Brengues et al., 2005; Ferraiuolo et al., 2005; Teixeira et al., 2005).

Translational shut-off induced either genetically or via environmental stress causes P-bodies to increase in both size and abundance. P-bodies return to normal levels after a restoration of translation (Brengues et al., 2005; Teixeira et al., 2005). It has been suggested that P-bodies represent accumulations of translationally silent mRNPs undergoing degradation or storage (Parker and Sheth, 2007). A decrease in global translation would increase free mRNP concentration and, thus, increase flux into P-bodies. Mutation of mRNA degradation and P-body components (such as Dcp1p and Xrn1p) also increases P-body size and abundance, in this case inhibiting degradative flux through the P-body (Sheth and Parker, 2003, 2006).

An inhibition of translation initiation by eIF2α phosphorylation induces SGs in higher eukaryotes but not in yeast (Anderson and Kedersha, 2006). SGs harbor ribosomal proteins and early translation initiation factors such as eIF2, eIF3, eIF4E, and eIF4G. Therefore, they are thought to contain abortive translation initiation complexes (Kedersha et al., 1999). SGs are compositionally and functionally distinct from P-bodies, yet several factors populate both structures in vivo (Cougot et al., 2004; Anderson and Kedersha, 2006). It has been suggested that P-bodies dock with SGs and degrade designated mRNAs, whereas the SGs remain a site of mRNA triage (Kedersha et al., 2005).

In this study, we provide important insight into mRNP fate in translationally repressed cells. This work highlights which step in the yeast translation initiation pathway is inhibited by glucose depletion. Moreover, we show that this translational shutdown causes eIF4E, eIF4G, and Pab1p to redistribute away from ribosomal subunits to cytoplasmic granules. Quantitative analyses of dual-tagged strains indicate a partial colocalization with P-body components. However, a substantial population of the translation factor granules do not contain core P-body components. Critically, de novo formation of such granules demonstrates a kinetic and spatial distinction from P-bodies. We term these granules EGP-bodies and propose that they are sites where mRNAs are stored during periods of translational inactivity. This would suggest that EGP-bodies share at least functional analogy with higher eukaryotic SGs.

Results

Glucose depletion causes a severe but reversible inhibition of translation initiation by an unidentified mechanism (Ashe et al., 2000). Formaldehyde cross-linking of cells before polysome analysis has been used to provide mechanistic insight into translational regulation (Nielsen et al., 2004). Using this methodology, we compared glucose starvation with amino acid starvation, a stress that inhibits translation initiation by activating the eIF2α kinase Gcn2p to give lower TC levels (Hinnebusch, 2005). Importantly, the abundance of the translation factors tested in this analysis is not appreciably altered by glucose starvation (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200707010/DC1). Both glucose and amino acid starvation cause an accumulation of 80S monosomes via polysome run-off (Fig. 1). This is characteristic of the inhibition of translation initiation (Ashe et al., 2000) and is accompanied by a movement of the ribosomal proteins Rps3p and Rpl35p toward the 80S fractions (Fig. 1).

Both eIF3 and eIF2 interact with the 40S ribosomal subunit in a 43S complex before mRNA recruitment (Kapp and Lorsch, 2004) and are therefore detected in 40S fractions (Fig. 1). Additional protein is detected in polysomal regions and likely represents the formation of initiation complexes on heavily translated polysome-bound mRNAs. After amino acid starvation, a reduced level of eIF2α was observed cosedimenting with the 40S subunit (Fig. 1 B). This is consistent with the translational consequence of amino acid starvation: reduced TC levels, which would ultimately produce 40S ribosomal subunits with decreased associated eIF2 (Hinnebusch, 2005). In contrast, eIF3 is still associated with the 40S subunit after amino acid starvation (Fig. 1 B). eIF3 is also maintained on the 40S ribosome after eIF2 depletion (Nielsen et al., 2004). Therefore, it seems that a partial 43S complex can still form when eIF2 function has been abrogated.

After glucose starvation, we observed maintained or even slightly increased levels of eIF3 and eIF2 with the 40S

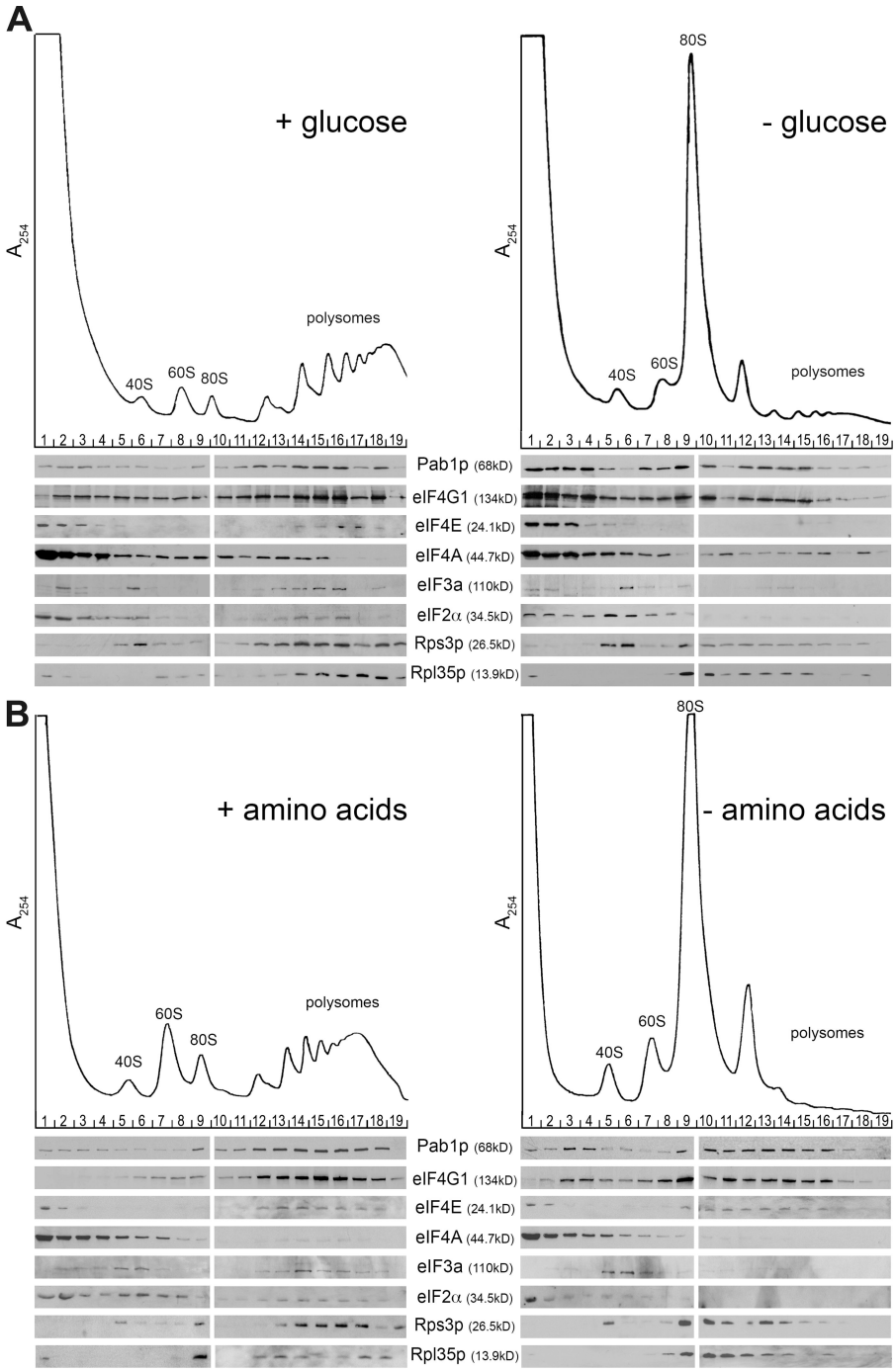

Figure 1. **Glucose starvation caused the resedimentation of specific translation factors.** (A and B) Sucrose density gradient analysis on extracts from strain yMK36 grown in YPD (A) or SCD (B) and resuspended in YPD (+glucose), YP (−glucose; A), SCD (+amino acids), or SC (−amino acids; B) for 30 min. Immunoblots on gradient fractions probed using antibodies against the indicated proteins are shown below the traces.

ribosomal subunit accompanied by decreases in polysomal fractions (Fig. 1 A). Therefore, in contrast to amino acid starvation, glucose starvation does not decrease the level of eIF2 with the 40S ribosomal subunit. Any increase in eIF2 and eIF3 with the 40S subunit after glucose starvation is likely to result from the stress-induced polysome run-off. This causes polysomal 43S complexes in unstressed cells to relocate to the 40S region.

The most striking observation from the polysome analysis is that after glucose starvation, there is a marked increase in the levels of eIF4G and Pab1p sedimenting in submonosomal fractions (Fig. 1 A, fractions 1–3; +/− glucose). Similarly, the proportion of eIF4E in these fractions becomes enriched, although submonosomal eIF4E in glucose replete extracts somewhat masks this effect (Fig. 1 A). This demonstrates a reduced association of these factors with ribosomes and contrasts noticeably with the sedimentation of these factors after amino acid starvation (Fig. 1 B). Therefore, the key translational components present in the closed loop mRNP appear to resediment away from ribosomal fractions after glucose starvation. Given that there is a low affinity interaction between eIF4A and eIF4G in yeast (Neff and Sachs, 1999) and that eIF4A is one of the most abundant translation initiation factors (present at roughly 40 times the number of eIF4G molecules; von der Haar and McCarthy, 2002), it is unsurprising that the sedimentation pattern of eIF4A

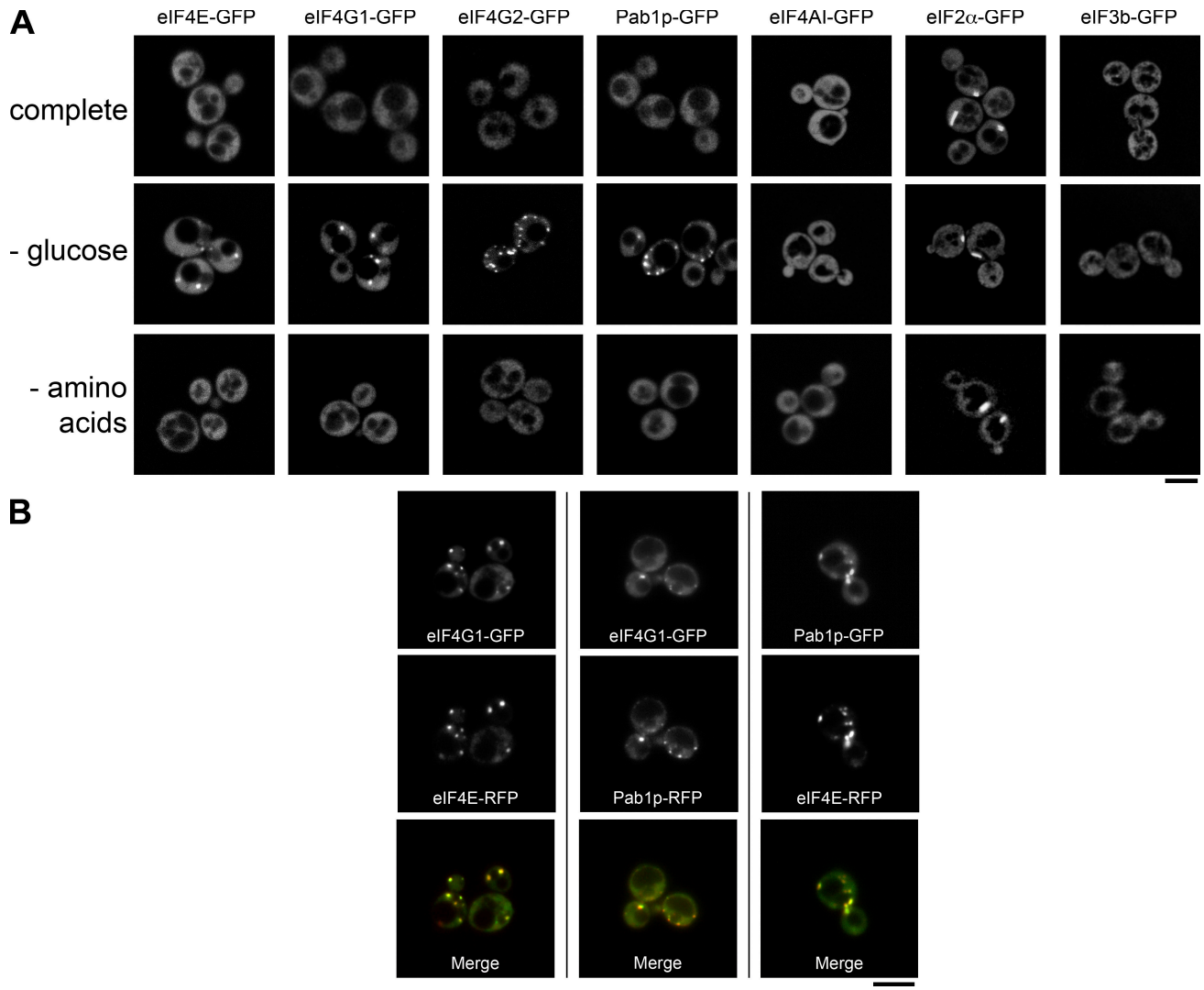

Figure 2. **Glucose starvation induces cytoplasmic granules of eIF4E, eIF4G1, eIF4G2, and Pab1p.** (A) Confocal microscopic images of yMK strains 885 (eIF4E-GFP), 1172 (eIF4G1-GFP), 1214 (eIF4G2-GFP), 1185 (Pab1p-GFP), 1170 (eIF4AI-GFP), 883 (eIF2α-GFP), or 881 (eIF3b-GFP) in which exponential cells were preincubated for 30 min in SCD (complete), SCD lacking glucose (−glucose), or SCD lacking amino acids (−AAs). (B) Epifluorescent microscopic images of glucose-starved cells of yMK strains 1299 (eIF4G1-GFP and eIF4E-RFP), 1304 (eIF4G1-GFP and Pab1p-RFP), and 1305 (eIF4E-GFP and Pab1p-RFP). Bars, 5 μm.

remains submonosomal regardless of the stress condition (Fig. 1). Even if a weak eIF4A–eIF4G interaction persisted after stress, the considerable level of free eIF4A would mask changes in ribosome-associated material.

Overall, these data suggest a reorganization of the mRNP closed loop translation complex after glucose starvation, whereby the cosedimentation of eIF4E, eIF4G, and Pab1p with ribosomal complexes is compromised. Amino acid starvation produces a contrasting effect, which is consistent with a fundamentally different mode of action. Indeed, the observed decrease in eIF2 cosedimentation with the 40S ribosomal subunit agrees with the established model for amino acid starvation.

As part of our analysis, we assessed the localization of translation initiation factors in response to both glucose and amino acid starvation. We show that glucose starvation induces genomic GFP fusions of eIF4E, eIF4G1, eIF4G2, and Pab1p to accumulate as cytoplasmic granules (Fig. 2 A). In contrast, the localization of eIF3b, eIF4AI, eIF2α, or eIF2Bγ is unaffected by glucose starvation. Previously, we have shown that eIF2α and eIF2Bγ localize to a large cytoplasmic focus (Campbell et al., 2005). Irrespective of the stress condition, we still observe this defined localization.

Strikingly, those factors forming stress-induced granules (eIF4E, eIF4G1/2, and Pab1p) are identical to those with altered gradient sedimentation properties (Fig. 1). The translation initiation factor granules occur with an approximate frequency of four to five granules per cell (see Fig. 4 C). In correlation with the cosedimentation analysis, amino acid starvation failed to induce cytoplasmic granules for any of these factors after 30 min (Fig. 2 A).

It is intriguing that glucose starvation causes eIF4E, eIF4G, and Pab1p to relocalize to cytoplasmic bodies, whereas amino acid starvation does not elicit this response or induce the marked resedimentation of eIF4E, eIF4G, and Pab1p.

We attribute this to either variation in the severity of the imposed stresses or fundamental differences in their modes of action. Interestingly, an exposure of cells to 10 min of glucose starvation induced a shift in eIF4E, eIF4G, and Pab1p of lower magnitude than that observed for cell extracts from cells starved for 30 min. In accordance with this observation, 10 min of glucose starvation also failed to induce cytoplasmic granules of eIF4E, eIF4G, and Pab1p (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200707010/DC1). Collectively, these data imply a requirement for the large-scale release of eIF4E, eIF4G, and Pab1p from polysomes to induce visible cytoplasmic aggregations. Short periods of glucose starvation are insufficient to induce such an effect as a result of an apparent lag in factor release. Amino acid starvation induces minimal eIF4E, eIF4G, and Pab1p resedimentation and aggregation into cytoplasmic granules after either 30 or 60 min (Figs. 1, 2, and S2). These data further corroborate the fundamental difference between these nutritional stresses and their mechanism of inhibition of translation initiation.

An obvious question is whether eIF4E, eIF4G, and Pab1p localize to the same cytoplasmic bodies after glucose starvation. To investigate this, we generated dual-tagged strains bearing specific genomic GFP and RFP fusions. As shown in Fig. 2 B, we compared eIF4G1-GFP with eIF4E-RFP, eIF4G1-GFP with Pab1p-RFP, and eIF4E-GFP with Pab1p-RFP. For all of these experiments, we observed >90% colocalization (Fig. 2 B and see Fig. 4 B).

As eIF4E, eIF4G, and Pab1p colocalize to the same cytoplasmic granules after glucose starvation, a key question is whether they relocate to these granules as mRNPs (i.e., bound to mRNA). To test the ability of eIF4E, eIF4G, and Pab1p to interact with each other as well as the cap and poly(A) tail structures after glucose starvation, we used cap and poly(A) affinity chromatography approaches (Fig. 3 A). The levels of eIF4E and copurifying eIF4G on the cap affinity column remain largely unchanged in extracts from glucose-starved or unstarved cells (Fig. 3 A, left). Similarly, glucose starvation does not alter the level of Pab1p, eIF4G, or eIF4E associated with the poly(A) column (Fig. 3 A, right). Therefore, the interaction of these factors with mRNA is likely to persist after glucose starvation. We also used a strategy that enables a specific mRNA to be followed in live cells (Brodsky and Silver, 2000; Teixeira et al., 2005). Here, the *PGK1* mRNA has multiple U1A-binding sites inserted into the 3′ untranslated region and can be visualized using fluorescence microscopy when coexpressed with a plasmid bearing a U1A-GFP fusion. When this mRNA localization system is used during the translational repression induced by glucose starvation, *PGK1* transcripts formed distinct cytoplasmic GFP foci, which precisely colocalize with the eIF4E-RFP granules (Fig. 3 B, top). Controls using either the *PGK1* reporter plasmid or the U1A-GFP fusion plasmid in isolation generate no GFP foci.

These results suggest that the dramatic effect of glucose starvation on translation initiation does not result from a reduced capacity to form mRNA closed loop complexes. Furthermore, the combination of live cell imaging and affinity chromatography experiments suggests that mRNA accompanies eIF4E, eIF4G,

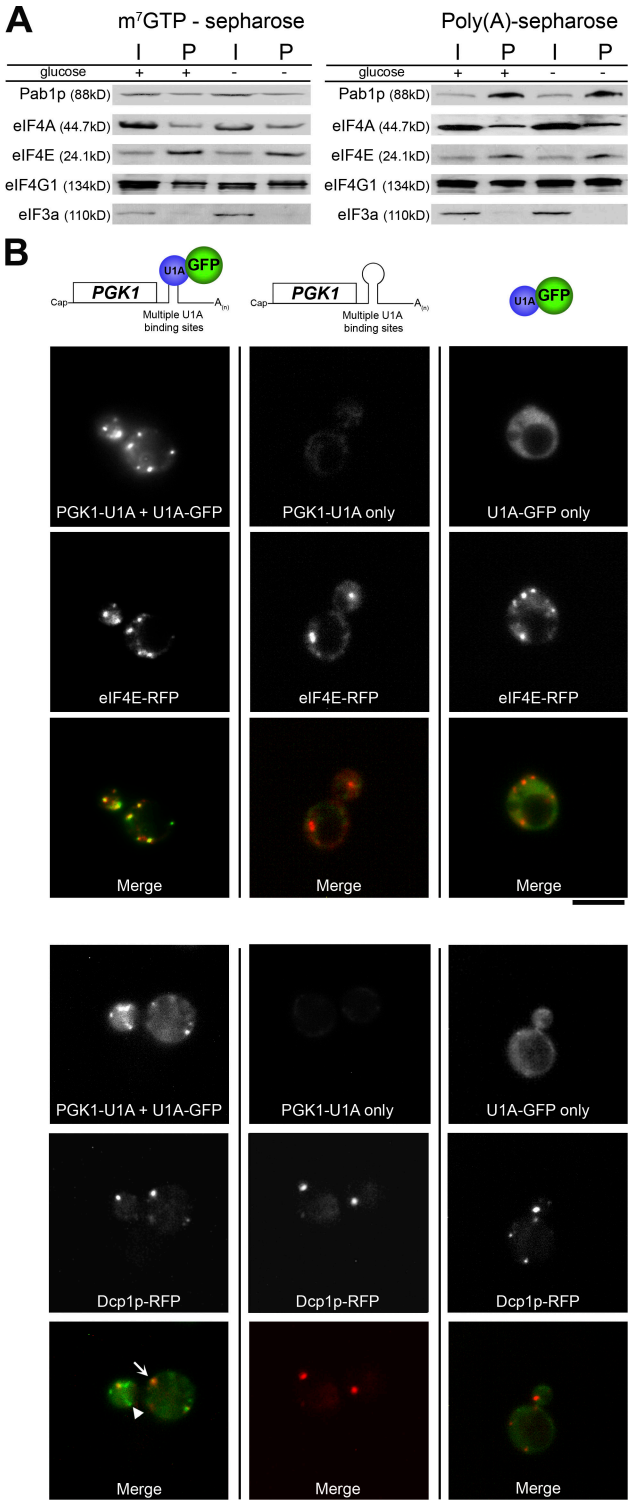

Figure 3. **mRNA colocalizes with the translation initiation factor granules.** (A) Immunoblots on affinity purifications of extracts from glucose-starved (−glucose) or unstarved (+glucose) cultures. 7-methyl-GTP-Sepharose (left) and poly(A)-Sepharose (right) input (I) and pellet (P) fractions were probed using specific antibodies. (B) Epifluorescent microscopic images of yMK1307 (eIF4E-RFP; top) or yMK1366 (Dcp1p-RFP; bottom) starved for glucose and coexpressing *PGK1-U1A* mRNA (pPS2037) and U1A-GFP protein (pRP1187) to allow analysis of *PGK1* mRNA localization. Controls expressing either the *PGK1-U1A* mRNA or U1A-GFP protein are shown. The arrow indicates a granule bearing both Dcp1p and *PGK1* mRNA, whereas the arrowhead indicates a granule harboring just the *PGK1* mRNA. Bars, 5 μm.

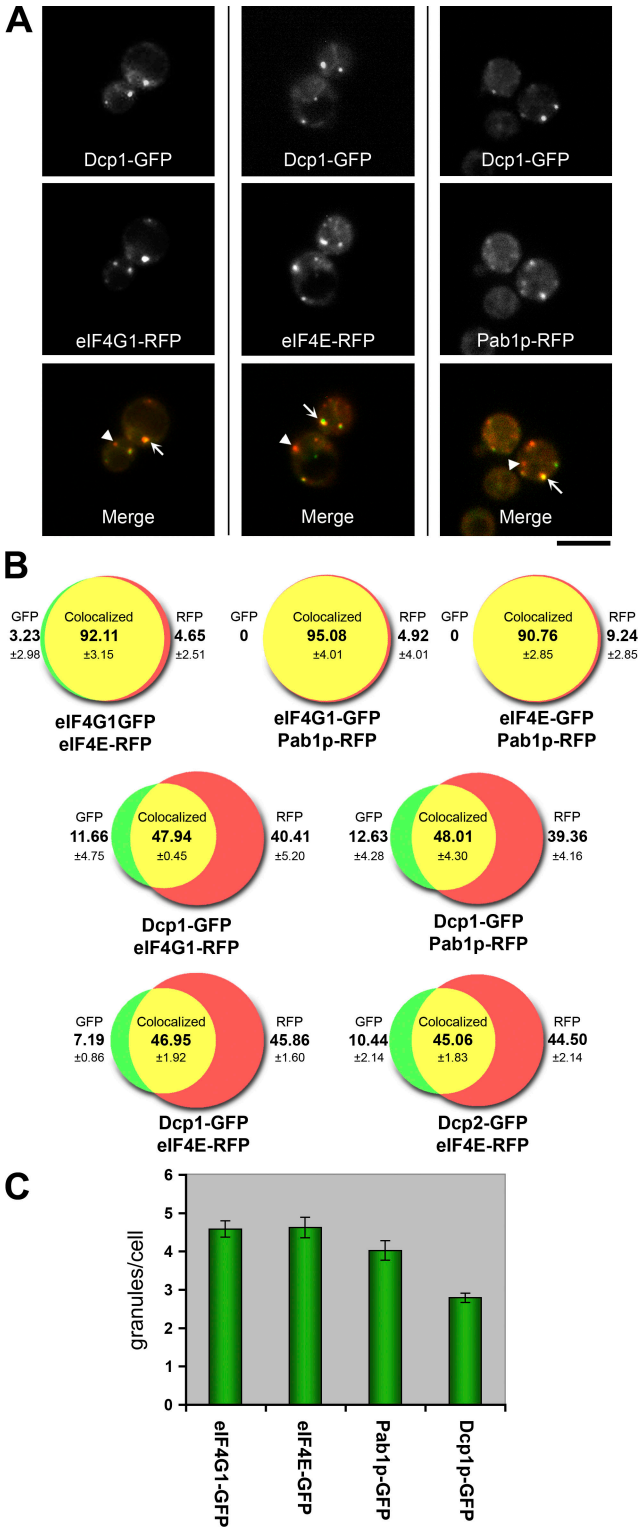

Figure 4. **EGP-bodies represent a distinct subpopulation of mRNP foci.** (A) Epifluorescent microscopic images of glucose-starved yMK strains 1302 (Dcp1p-GFP and eIF4G1-RFP), 1303 (Dcp1p-GFP and eIF4E-RFP), and 1344 (Dcp1-GFP and Pab1p-RFP). Arrowheads identify bodies containing only the translation initiation factor, whereas arrows indicate bodies where Dcp1p colocalizes with the translation initiation factor. (B) Area-proportional Venn diagrams derived from the quantitation of three biological replicates (>50 fluorescent foci each) for the glucose-starved strains yMK1299, 1304, 1305, 1302, 1303, 1344, and 1359. Errors are ±1 SD. (C) Bar chart depicting a count of granules per cell for strains bearing the indicated GFP-tagged factor. 12-image merged z-stacks generated from >75 cells starved for glucose were counted manually using ImageJ. Error bars indicate ±1 SD. Bar, 5 μm.

and Pab1p to cytoplasmic granules. Thus, it appears that there is a relocalization of closed loop mRNP complexes to cytoplasmic granules after severe translational repression.

After stresses such as glucose starvation, mRNA can become localized to P-bodies (Sheth and Parker, 2003). Originally in yeast, translation initiation factors were not identified in P-bodies (Brengues et al., 2005; Teixeira et al., 2005). However, while this manuscript was in preparation, this question was reevaluated, and the authors now suggest that eIF4E, eIF4G, and Pab1p do enter P-bodies (Brengues and Parker, 2007). In our experiments, we also investigated the relationship between P-bodies and the granules containing eIF4E, eIF4G1, and Pab1p. We simultaneously visualized eIF4E-, eIF4G1-, or Pab1p-RFP versus Dcp1p-GFP as a marker for P-bodies in living cells. After a 30-min glucose depletion, eIF4E, eIF4G1, and Pab1p were found in most Dcp1p-containing bodies (Fig. 4, A and B). In addition, Dcp1p cosediments with these translation factors across sucrose gradients, and there is an increase in eIF4G, eIF4E, and Pab1p coimmunoprecipitation with Dcp1p in glucose-starved versus unstarved extracts (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200707010/DC1). Strikingly, however, Dcp1p was not always found in the translation initiation factor granules (Fig. 4, A and B). Furthermore, we identified a mean of four to five granules per cell for each of the translation initiation factors, which is approximately twice that observed for Dcp1p (Fig. 4 C). This value for Dcp1p correlates well with the two to three granules per cell previously published for another core P-body component, Dhh1p (Sheth and Parker, 2003).

Overall, these data suggest that after glucose depletion for 30 min, a mean of two to three P-bodies form per cell, which also contain eIF4E, eIF4G1, and Pab1p. Intriguingly, a mean of approximately two additional bodies form per cell that contain eIF4E, eIF4G1, and Pab1p but do not harbor Dcp1p. Similar results were obtained when a second marker for P-bodies, Dcp2p, was used (Fig. 4 B and not depicted). This is consistent with observations that Dcp1p colocalization with *PGK1* mRNA is incomplete in that *PGK1* mRNA granules exist that do not contain Dcp1p (Fig. 3 B, bottom). Overall, these results suggest that previously unrecognized cytoplasmic bodies exist that contain the translation initiation factors eIF4E, eIF4G, and Pab1p but do not contain the key P-body markers Dcp1p and Dcp2p. We have termed these bodies EGP-bodies, as thus far their only known protein constituents are eIF4E, eIF4G, and Pab1p.

Distinct possibilities exist as to the mode of EGP-body formation, which have important functional implications. First, EGP-bodies may form via a P-body maturation process in which core P-body components such as Dcp1p and Dcp2p are lost. Alternatively, EGP-bodies could arise de novo having never contained core P-body components.

To investigate these possibilities, we undertook detailed time course experiments following individual cells after glucose starvation, simultaneously visualizing eIF4E-RFP as a marker for EGP-bodies and Dcp1p-GFP as a marker for P-bodies (Fig. 5).

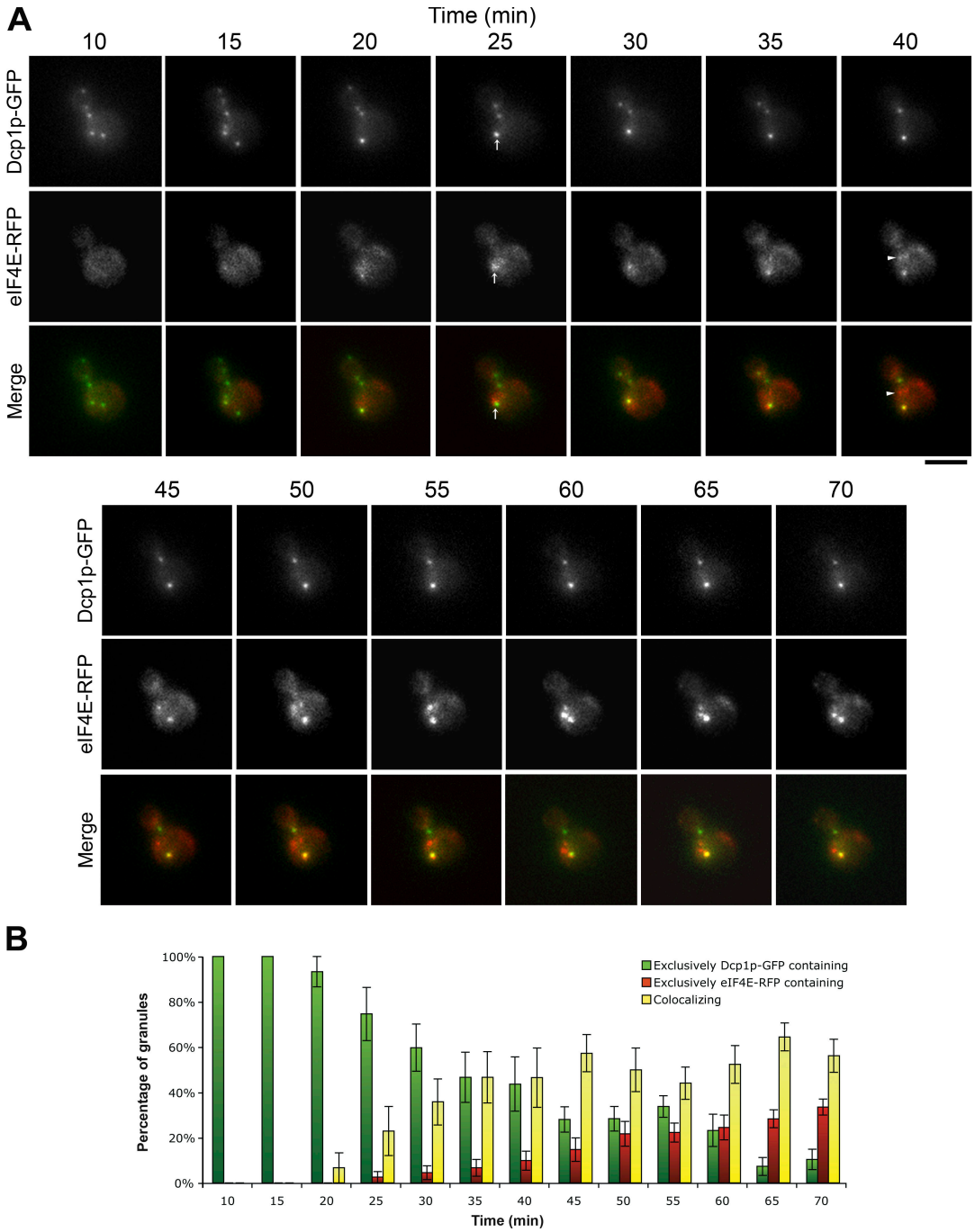

Figure 5. **EGP-bodies form independently of P-bodies.** (A) Epifluorescent real-time 2D deconvolved projections generated from continuous z-sweep acquisition of a representative glucose-starved cell of strain yMK1303 (Dcp1p-GFP and eIF4E-RFP). Exponential cells were washed and resuspended in SC and visualized at 5-min time points for 70 min. The arrowheads identify an emergent body that contains only eIF4E and has never contained Dcp1p. The arrows indicate a body in which eIF4E is recruited to an existing Dcp1p focus. (B) A bar chart showing proportions of granules per cell from images of 10 cells, generated as described in A. Granules were categorized as those containing only Dcp1-GFP, those containing both Dcp1p-GFP and eIF4E-RFP (colocalizing), and those granules exclusively containing eIF4E-RFP, which arise independently of Dcp1p-GFP foci. Error bars are ±1 SEM. Bar, 5 μm.

The complex experimental setup dictates that the earliest possible time point for image acquisition is 10 min after glucose starvation. At this time point, it can be seen that in accordance with previously published data, Dcp1p has accumulated in P-bodies (Fig. 5 A; Teixeira et al., 2005). In contrast, eIF4E enters P-bodies after 20–25 min of glucose starvation, and, ultimately, most P-bodies recruit eIF4E (Fig. 5 B). Perhaps the most striking observation from these time course experiments concerns the EGP-bodies. The EGP-bodies harboring just eIF4E and not Dcp1p always arise spontaneously in the cell and have never been observed to accumulate as a result of the loss of Dcp1p from eIF4E-containing P-bodies (Fig. 5 A). This defined population of de novo EGP-bodies is quantified in Fig. 5 B, in which there is a gradual increase in the formation of EGP-bodies starting

after 20–25 min. Overall, therefore, we conclude that P-bodies recruit eIF4E after prolonged glucose starvation but do not mature into EGP-bodies by losing core P-body components. In fact, EGP-bodies arise spontaneously as independent entities within the cell.

Discussion

Glucose starvation causes the most severe stress-induced inhibition of translation initiation in *S. cerevisiae* that has yet been characterized (Holmes et al., 2004). Paradoxically, the mechanism for such a catastrophic alteration in protein synthesis is unknown. In this study, we demonstrate that the mode of regulation is distinct from the well-studied impact of amino acid starvation on TC levels. We also show that the capacity of different translation initiation factors to recognize the mRNA is unaltered after glucose starvation. Indeed, the mRNA and a core of mRNA-interacting translation initiation factors relocalize to cytoplasmic granules as a consequence of this stress.

Glucose starvation also does not alter the level of eIF2 or eIF3 associated with the 40S ribosomal subunit. Accordingly, mutants that are incapable of phosphorylating eIF2α and so do not elicit an amino acid starvation response still respond to glucose starvation to give wholesale translational repression (Ashe et al., 2000). Equally, glucose starvation does not rapidly induce established responses to amino acid starvation such as eIF2α phosphorylation or *GCN4* derepression (Fig. S1 and not depicted). Therefore, our data are inconsistent with a model in which glucose regulates translation by altering TC levels or affecting the interaction of eIF2 or eIF3 with the 40S ribosomal subunit. Regulation at steps further downstream in the translation initiation pathway can be ruled out, as the closed loop mRNP relocalizes away from the ribosomal pool to cytoplasmic granules. This pinpoints recruitment of the 43S complex to mRNA as the regulated step in the translation pathway after glucose starvation. There are precedents for regulatory mechanisms targeting this step. For instance, positioning of the RNA cis-acting iron response element to a cap-proximal position sterically prevents 43S complex binding to mRNA (Gray and Hentze, 1994; Muckenthaler et al., 1998). More recently, phospo-L13a has been shown to inhibit 43S subunit recruitment to γ interferon inhibitor of translation element–containing mRNAs (Kapasi et al., 2007). In these examples, the inhibition of translation initiation is exerted at the level of a specific mRNA, whereas glucose starvation elicits a global translational repression (Ashe et al., 2000), and, thus, the regulatory mechanism studied here represents a new means by which to dramatically attenuate protein synthesis.

In this study, we also show that eIF4E, eIF4G, and Pab1p localize to P-bodies after glucose starvation. Previously, mRNA has been shown to enter P-bodies during translational shutoff, where it can either be degraded by virtue of the mRNA decay factors present within the P-body or stored (Sheth and Parker, 2003; Brengues et al., 2005). The mechanism by which mRNA could be stored in the presence of high local concentrations of mRNA decay factors has yet to established. The factors eIF4E, eIF4G, and Pab1p could be viewed as the minimal requirement

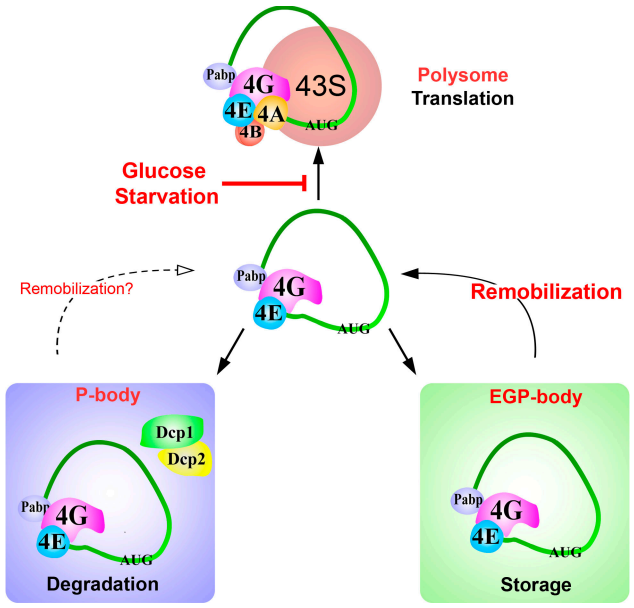

Figure 6. **A model for mRNA sorting in yeast.** A closed loop mRNP enters the translational pool via interaction with the 43S complex. Glucose starvation inhibits this process, leaving the mRNP two potential fates: redistribution to P-bodies, in which decapping facilitates mRNA degradation, or transfer to EGP-bodies, in which a lack of decapping factors dictates mRNA storage.

to protect the mRNA 5′ and 3′ ends from degradation (Sachs, 2000; Schwartz and Parker, 2000). We observe a recruitment of eIF4E to P-bodies after ∼25 min of glucose starvation, concomitant with resedimentation of eIF4E, eIF4G, and Pab1p away from ribosomal fractions of a sucrose density gradient. This is consistent with a model in which closed loop mRNPs are recruited to P-bodies in a manner kinetically distinct from the translation inhibition and P-body induction that occurs in <10 min.

In contrast, after extended periods of translational inhibition induced by amino acid starvation, we observe little or no P-body induction and no increase in mRNP relocalization. Once again, this highlights fundamental differences in the impact of the glucose and amino acid starvation stresses on translation initiation. Interestingly, in the case of amino acid starvation, it is particularly important for cellular adaptation that translation initiation events still occur on mRNAs such as *GCN4* (Hinnebusch, 2005). This may provide a physiological explanation as to why mRNA does not appear to be at liberty to transit through P-bodies. That is, translation initiation events can still occur on mRNA after this stress even though these may be partial or nonproductive in many cases.

After glucose starvation, we show that there is a kinetic separation of P-body formation and closed loop mRNP redistribution. This poses interesting questions as to the relationship between these two events. Cycloheximide prevents P-body accumulation by freezing mRNAs in polysomes (Teixeira et al., 2005). This denotes a polysomal origin for mRNAs that enter P-bodies. The resedimentation and relocalization kinetics of eIF4E, eIF4G, and Pab1p suggest that after glucose starvation, several mRNAs are retained in polysomes as closed loop mRNPs, whereas others are relocalized to P-bodies without associated

eIF4E, eIF4G, and Pab1p. The mechanism by which the cell distinguishes between these populations or initiates the secondary redistribution of closed loop mRNPs requires further study.

As well as P-body–localized eIF4E, eIF4G, and Pab1p, we observe cytoplasmic bodies bearing these factors that do not contain the key decapping enzymes (and P-body markers) Dcp1p and Dcp2p. These EGP-bodies may be sites for the long-term storage of mRNA and translation initiation factors under specific stress conditions (Fig. 6). Furthermore, as translation initiation factors remain associated with mRNAs under conditions of translational repression in EGP-bodies, the rapid resumption of translation initiation on these mRNAs would be favored should conditions improve (Fig. 6). Thus, although lacking in 43S complex components, these EGP-bodies could be viewed as at least functionally analogous to the SGs that are found in higher cells (Anderson and Kedersha, 2006). A particularly intriguing possibility is that gene regulation might occur when mRNA fate is decided such that transcripts that are still valuable to the cell can be stored in EGP-bodies, whereas those that are not required would be targeted to P-bodies. The closed loop complex is perceived to be associated with high levels of translation initiation and, therefore, is a target for translational regulation (Richter and Sonenberg, 2005). However, the aforementioned results are particularly striking, as they suggest that the closed loop complex exists on mRNAs that are translationally repressed. Therefore, as well as identifying a new strategy for translational regulation, these results challenge the notion that the formation of a closed loop complex predisposes an mRNA to translation initiation.

Materials and methods

Strains and plasmids

Strain genotypes are listed in the Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200707010/DC1). Proteins were C-terminally tagged and verified by both PCR and immunoblotting using a previously described method (Janke et al., 2004), and plasmid reagents were provided by G. Pereira (German Cancer Research Centre, Heidelberg, Germany). Anti-Rps3p and Rpl35p were gifts from M. Pool (University of Manchester, Manchester, UK), and anti-eIF2α and eIF2Bδ were gifts from G. Pavitt (University of Manchester, Manchester, UK). Anti-eIF3a was generated against an in vitro–synthesized peptide (Genosphere). The 2μ RNA localization construct plasmids pPS2037 (Brodsky and Silver, 2000) and pRP1187 (Teixeira et al., 2005) were gifts from R. Parker (Howard Hughes Medical Institute, University of Arizona, Tucson, AZ).

Strain growth and extract preparation

Cells were grown to OD_{600} 0.6 in rich media containing yeast extract, peptone, and glucose (YPD; Burke et al., 2000). Cells were harvested, and, for starvation experiments, cell pellets were resuspended in 50 ml of either YPD or YPD lacking glucose (YP) and incubated for the indicated times at 30°C. Extracts were prepared by centrifugation and immediate freezing of cell pellets in liquid N_2 before hand grinding to a powder. The powder was resuspended in buffer A (2 mM MgOAc and 30 mM Hepes, pH 7.5, 100 mM KOAc, 1 mM PMSF, and 1× Complete Mini-EDTA free protease inhibitors [Roche]) and centrifuged for 10 min at 800 *g*. Supernatant was isolated, and protein content was assayed by Bradford reagent assay (Bio-Rad Laboratories). Western blotting was conducted as described previously (Ashe et al., 2000).

Affinity purification

For cap and poly(A) affinity chromatography assays, 3 mg of protein was preincubated with 4B Sepharose (GE Healthcare) for 30 min before incubation with 25 μl 7-methyl-GTP Sepharose (GE Healthcare) or poly(A) Sepharose (GE Healthcare) for 2 h. Beads were washed three times in buffer A before elution in 50 μl Laemmli buffer at 95°C for 2 min. In the case of the cap-binding assay, an additional 1-h wash step in the presence of 0.1 mM GTP was conducted. For Western analysis, 10 μl of the input was loaded for comparison with 10 μl of pellet fractions.

Formaldehyde sucrose density gradient analysis

Cells were grown as described in Strain growth and extract preparation and were fixed by the addition of 1% (vol/vol) formaldehyde to media. They were incubated on ice for 1 h before the addition of 0.1 M glycine and lysed with glass beads. 7.5 OD_{260} U of extract was loaded onto 15–50% sucrose gradients and centrifuged at 40,000 *g* for 2.5 h. 500-μl gradient fractions were collected while the Abs_{254} was continuously measured. Individual fractions were precipitated with 10% TCA, washed with 300 μl acetone, and resuspended in 50 μl Laemmli buffer. Proteins were analyzed by SDS-PAGE and immunoblotting. Profiles and Western blots are shown from a representative experiment repeated at least three times

Microscopy and quantitation

Cells were grown to an OD_{600} of 0.6 in synthetic complete glucose media (SCD), washed twice, and resuspended in media lacking either glucose (SC) or amino acids (SCD-AA; Burke et al., 2000). After 30 min of incubation, cells were observed. Confocal images were taken at room temperature by a confocal microscope (SP5; Leica) using a 63× 0.6–1.40 NA plan Apo oil objective (Leica). Images were acquired using Application suite 1.6.3 (Leica). For densitometric analysis, a merged 12-image z-series was taken; for clarity, the images shown are single planes. Dual-label microscopy was conducted at room temperature using a microscope (Eclipse E600; Nikon) with a 100× 0.5–1.3 NA planFluor oil immersion objective (Nikon) and camera (Axiocam MRm; Carl Zeiss MicroImaging, Inc.). Images were acquired using Axiovision 4.5 software (Carl Zeiss MicroImaging, Inc.). Representative cells are shown from experiments repeated at least three times. Images were quantified using ImageJ (National Institutes of Health). >10 cells were analyzed for densitometric analysis of factor abundance in the granules. For counts of granules/cell, >75 cells were analyzed. For the colocalization scoring, three biological replicates, each with 50 cells, were used.

For single-cell kinetic experiments, cells grown in SCD were washed in SC and immediately applied to a poly-lysine–coated glass slide. Real-time 2D deconvolved projections generated from continuous z-sweep acquisition were captured at room temperature using a microscope system (DeltaVision RT; Applied Precision) with a 100× 1.40 NA differential interference contrast oil plan Apo objective (Olympus) and camera (CoolSNAP HQ; Roper Scientific) using Softworx 1.1 software (Applied Precision). Images were taken over the course of 1 h at 5-min intervals, allowing 10 min initially for experimental set-up. A representative cell is shown from an experiment repeated at least 10 times. For colocalization scoring per cell, 10 biological replicates were analyzed using ImageJ (National Institutes of Health).

Online supplemental material

Table S1 details yeast strains used in this study and their genotypes. Fig. S1 shows that the abundance of translation factors and eIF2α phosphorylation are unaffected by glucose starvation for periods of at least 45 min. Fig. S2 shows by sucrose density gradient sedimentation analysis and confocal microscopy that after either 10 min of glucose starvation or 1 h of amino acid starvation, Pab1p, eIF4E, and eIF4G are not induced to resediment or form cytoplasmic bodies. Fig. S3 shows sucrose density gradient sedimentation analysis of Dcp1p, which is concentrated in submonosomal fractions (A). It also shows an induction of eIF4G1, eIF4E, and Pab1p association with Dcp1p-TAP (tandem affinity purification epitope) after glucose starvation by Western blotting against tandem affinity purification outputs (B). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707010/DC1.

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