

Processing Body and Stress Granule Assembly Occur by Independent and Differentially Regulated Pathways in *Saccharomyces cerevisiae*

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ABSTRACT A variety of ribonucleoprotein (RNP) granules form in eukaryotic cells to regulate the translation, decay, and localization of the encapsulated messenger RNA (mRNAs). The work here examined the assembly and function of two highly conserved RNP structures, the processing body (P body) and the stress granule, in the yeast *Saccharomyces cerevisiae*. These granules are induced by similar stress conditions and contain translationally repressed mRNAs and a partially overlapping set of protein constituents. However, despite these similarities, the data indicate that these RNP complexes are independently assembled and that this assembly is controlled by different signaling pathways. In particular, the cAMP-dependent protein kinase (PKA) was found to control P body formation under all conditions examined. In contrast, the assembly of stress granules was not affected by changes in either PKA or TORC1 signalling activity. Both of these RNP granules were also detected in stationary-phase cells, but each appears at a distinct time. P bodies were formed prior to stationary-phase arrest, and the data suggest that these foci are important for the long-term survival of these quiescent cells. Stress granules, on the other hand, were not assembled until after the cells had entered into the stationary phase of growth and their appearance could therefore serve as a specific marker for the entry into this quiescent state. In all, the results here provide a framework for understanding the assembly of these RNP complexes and suggest that these structures have distinct but important activities in quiescent cells.

EUKARYOTIC cells contain a number of membrane-bound compartments that partition the cytoplasm into distinct functional units. Proteins that act in similar pathways are often localized to the same compartment whereas those with competing activities are sequestered within different environments. Interestingly, recent data suggest that particular proteins and RNAs are also concentrated in what can be thought of as nontraditional compartments that lack a boundary membrane. These ribonucleoprotein (RNP) complexes, or granules, are more dynamic in nature and are found in both the nucleus and the cytoplasm of the cell (Anderson and Kedersha 2006; Mao *et al.* 2011; Weber and Brangwynne 2012). The formation of these granules can be induced by a variety of cues, including an exposure to stress or specific

developmental transitions. In some cases, the underlying reasons for this reorganization of protein and RNA are known. For example, the polar granules present in germ cells store maternal mRNAs that are translated following fertilization (Schisa *et al.* 2001; Leatherman and Jongens 2003). However, for most RNP granules, the physiological role of the larger aggregate-like structures remains unclear. Nonetheless, the prevalence and evolutionary conservation of these complexes suggests that they serve important functions in the cell.

Two of the better-characterized cytoplasmic RNPs are the processing bodies (P bodies) and stress granules that form in response to a variety of stress conditions. These particles contain translationally repressed messenger RNA (mRNAs) and a partially overlapping set of protein constituents (Kedersha and Anderson 2002; Anderson and Kedersha 2009; Balagopal and Parker 2009). Since a number of factors important for protein translation are also found in stress granules, these structures have been suggested to be sites of mRNA storage (Yamasaki and Anderson 2008). In contrast, P bodies were originally identified as cytoplasmic foci containing proteins important for mRNA decay (Sheth and

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Parker 2003; Eulalio *et al.* 2007a). Although this initially led to speculation that these foci were sites of mRNA turnover, more recent studies have found that this decay proceeds normally in cells lacking the larger P body complexes (Stoecklin *et al.* 2006; Decker *et al.* 2007; Eulalio *et al.* 2007b). As a result, the biological functions associated with P body foci remain unclear. However, an intriguing possibility has been suggested by studies demonstrating that P bodies contain proteins that do not appear to have a direct role in mRNA decay. These proteins include the phosphatase, calcineurin, and the catalytic subunits of the cAMP-dependent protein kinase (PKA) (Tudisca *et al.* 2010; Kozubowski *et al.* 2011). P bodies may therefore carry out specific functions that are dictated by the particular proteins present within these cytoplasmic structures. These functions may vary depending upon the particular cell type and stress condition used to induce the foci.

A significant body of work has linked the induction of both P bodies and stress granules to the inhibition of protein synthesis, but less is known about the mechanisms regulating the subsequent formation of the larger aggregate-like assemblies (Franks and Lykke-Andersen 2008). These latter structures appear to form by a self-assembly process that involves the prion-like domains present in a number of granule proteins (Gilks *et al.* 2004; Decker *et al.* 2007; Reijns *et al.* 2008). Some insight into the regulation of this latter process was provided by a recent study of the P bodies that form in response to glucose deprivation in *Saccharomyces cerevisiae* (Ramachandran *et al.* 2011). This work showed that the inactivation of the PKA signaling pathway was both a necessary and a sufficient condition for P body foci formation. PKA directly phosphorylates *Pat1*, a conserved core constituent of these RNP structures, and thereby disrupts *Pat1* interactions with a number of P body components, including the RNA helicase *Dhh1* (Ramachandran *et al.* 2011). In contrast, defects in other nutrient-sensing pathways, including those involving the TORC1 or *Snf1* protein kinases, did not have a significant effect upon P body formation. This work also suggested that P body foci were important for the long-term survival of cells that had entered into the stationary phase of growth. In particular, mutants that were defective for foci formation lost viability more rapidly during this period of quiescence (Ramachandran *et al.* 2011). This latter result is of interest in light of other work indicating that as much as 20% of the yeast proteome might relocalize to cytoplasmic foci when cells enter into this G₀-like resting state (Narayanaswamy *et al.* 2009; Noree *et al.* 2010). Since this phenomenon might not be restricted to yeast (An *et al.* 2008; Noree *et al.* 2010), the concentration of material at discrete sites in the cytoplasm may be generally important for the biology of the quiescent cell. Determining the underlying reasons for this relocalization of protein will therefore be critical for a complete understanding of the physiology of the eukaryotic cell.

In *S. cerevisiae*, PKA is an essential component of one of the key signaling pathways responsible for coordinating cell growth with nutrient availability (Bahn *et al.* 2007; Zaman *et al.* 2008). This pathway also involves the GTP-binding Ras

proteins *Ras1* and *Ras2* and is thought to respond, either directly or indirectly, to the levels of glucose present within cells (Santangelo 2006; Slattery *et al.* 2008; Zaman *et al.* 2009). The active, GTP-bound forms of the Ras proteins interact with the adenylyl cyclase *Cyr1* and stimulate the production of cAMP (Field *et al.* 1990; Suzuki *et al.* 1990). This cyclic nucleotide is then bound by *Bcy1*, the regulatory subunit of the PKA enzyme, leading to the subsequent release of the active catalytic subunits; the basal state of PKA is an inactive heterotetramer made up of two catalytic and two regulatory subunits (Uno *et al.* 1982; Toda *et al.* 1987a; Taylor *et al.* 2008). These catalytic subunits are then free to phosphorylate their respective targets and thereby influence cell growth (Budovskaya *et al.* 2005). The existing genetic data suggest that this Ras/PKA pathway might play an important role in regulating the entry into stationary phase. For example, mutants that inactivate this pathway result in a growth arrest that resembles stationary phase (Iida and Yahara 1984; Schnepfer *et al.* 2004). Conversely, cells with constitutively elevated levels of PKA activity fail to arrest normally in stationary phase when nutrients are limiting (Broek *et al.* 1985; Broach 1991). The above results with *Pat1* suggest that the PKA-mediated control of P body formation is one important component of this regulation of stationary-phase biology.

In this study, we examined the regulation of P body and stress granule assembly in response to a variety of environmental cues, including several that can induce both of these RNP foci. This work demonstrated that the PKA pathway has a general role in the regulation of P body foci formation as mutants with constitutive PKA signaling were defective for P body assembly in all conditions tested. In contrast, stress granule formation was not influenced by changes in either PKA or TORC1 signalling activity. The results here also demonstrate that both types of RNP foci are present in stationary-phase cells and provide further support for a role for P bodies in the long-term survival of these quiescent cells. Finally, we show that P bodies and stress granules form at different times during batch culture growth and that stress granules in particular appear only after cells enter into stationary phase. Therefore, stress granule formation could serve as a useful marker for cell entry into this quiescent state. In all, this work indicates that P bodies and stress granules form independently of one another and that each assembly pathway is regulated by distinct signaling mechanisms.

Materials and Methods

Yeast strain construction and growth conditions

The yeast strains used are listed in Table S1. Standard *Escherichia coli* and yeast growth conditions and media were used throughout this study. The yeast rich growth medium, Yeast extract- Peptone- Adenine- Dextrose (YPAD), and the minimal Yeast Minimal (YM) and SC media have been described (Kaiser *et al.* 1994; Chang *et al.* 2001; Ramachandran and Herman 2010). The carbon starvation experiments were performed in SC- or YPA-based media lacking glucose for strains with

and without plasmids, respectively. For ethanol stress, cells were grown in synthetic complete dextrose (SCD) medium and then transferred to SCD containing 6 or 15% ethanol. Similar transfers were made for the other stress conditions used in this study, including 0.5% NaN₃, 3 mM H₂O₂, and 1 M KCl. The incubation time for each stress was typically 10–15 min for the induction of P bodies and 30 min for stress granules. As noted previously, stress granules were not induced by an exposure to 1 M KCl or 3 mM H₂O₂, and P bodies did not form following a 46° heat stress (our unpublished observations; Teixeira *et al.* 2005; Buchan *et al.* 2008, 2010, 2011; Grousl *et al.* 2009). To assess P body or stress granule formation in stationary phase, cells were inoculated into either YPAD or SCD media and then grown with agitation at 30° for the indicated number of days.

Strains carrying the *MET3-RAS2^{val19}* allele were grown in medium containing 500 μM methionine to repress expression from the *MET3* promoter. Expression from this promoter was induced by transferring cells to a medium that lacked methionine. For constructs under the control of the *CUP1* promoter, expression was induced by adding CuSO₄ to a final concentration of 100 μM. Expression from the *GAL1* promoter was induced by growing cells overnight in SC medium with 2% raffinose and then shifting cells to SC medium containing 1% raffinose and 2% galactose for 3–4 hr. To generate strains with integrated GFP-tagged versions of Pat1-SS, Pat1-AA, and Pat1-EE, the respective integrative plasmids were linearized within the *HIS3* locus and transformed into a *pat1Δ* strain. The *PAT1* locus was deleted where indicated with a PCR-based strategy (Baudin *et al.* 1993). The presence of the null allele in each of the commercial deletion strains was confirmed with a PCR analysis.

Plasmid construction

The plasmids used in this study are listed in Table S2. The *MET3-RAS2^{val19}* plasmids, pPHY795 and pPHY796, have been described (Howard *et al.* 2001; Ramachandran and Herman 2010). Plasmids expressing Dcp2-GFP (pRP1175), Edc3-mCherry (pRP1574), Edc3-mCherry/Pbp1-GFP (pRP1944), Pab1-GFP/Edc3-mCherry (pRP1657), and Pab1-GFP/Dcp2-mCherry (pRP1660) constructs were kindly provided by Roy Parker (Coller and Parker 2005; Decker *et al.* 2007). The GFP-*PAT1* fusion constructs were made in the vector pRS413 (Ramachandran *et al.* 2011). The *PAT1* variants described in the study were constructed by site-directed mutageneses performed with the Transformer mutagenesis kit (Clontech). The *HIS3*-marked, single-copy plasmids with the *PAT1* locus were constructed in pRS413; the encoded protein was tagged with a single *myc* epitope at its N terminus. The plasmid vectors used for the copper-inducible expression of the HA epitope-tagged versions of the Tpk proteins have been described (Deminoff *et al.* 2006).

Fluorescence microscopy

Cells expressing the appropriate fusion construct(s) were grown to early log phase (0.3–0.6 OD₆₀₀ units/ml) before

exposure to the indicated stress regimen or drug treatment. The cells were then collected by centrifugation, resuspended in the appropriate medium, and spotted onto microscope slides (Budovskaya *et al.* 2005). The samples were then imaged with a ×100/1.4 numerical aperture Plan-Apo objective lens on a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) equipped with a Nikon cooled digital camera DS-QI1 and appropriate filter sets (DIC, GFP, mCherry). For the quantitation shown, the data in all cases represent the average of two or more experiments; in each of these experiments at least 100 cells were examined. For the colocalization experiments, the final merged images were created with the Image J software package. For the analysis of RNP foci in stationary phase, cells were incubated in either YPAD or SCD media for the indicated number of days.

Stationary-phase viability assays

Cells were grown in YM glucose minimal medium for the appropriate number of days, collected by centrifugation, and resuspended in water at a final concentration of 5 OD₆₀₀ equivalents/ml. The growth assays were performed by spotting 5 μl of this cell suspension, and fivefold serial dilutions thereafter, onto the appropriate growth medium and incubating the plates at 30° for 2–3 days.

Protein analysis

Protein samples for Western blotting were prepared with a glass-bead lysis method, separated on 7.5–10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes as described (Budovskaya *et al.* 2002, 2004). The membranes were then probed with the appropriate primary and secondary antibodies. The Supersignal chemiluminescent substrate (Pierce) was subsequently used to detect the reactive bands. Cell extracts for immunoprecipitation were prepared by resuspending log-phase cells in lysis buffer [25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 0.1% Tween-20, 1 mM PMSF] and lysing by agitation with glass beads. Myc- and GST-tagged proteins were immunoprecipitated with the appropriate monoclonal antibodies (Cell Signaling), and the immunoprecipitates were collected on protein A-Sepharose beads (GE Healthcare). To assess the PKA phosphorylation status of Pat1 *in vivo*, this protein was immunoprecipitated from cell extracts prepared under denaturing conditions as described (Budovskaya *et al.* 2005; Deminoff *et al.* 2009). Protease and phosphatase inhibitors were present at all steps. The level of PKA phosphorylation was then assessed by Western blotting with the anti-PKA substrate antibody (Cell Signaling) as described (Chang *et al.* 2004; Deminoff *et al.* 2006).

In vitro kinase assays

The substrate proteins were immunoprecipitated, treated with λ phosphatase (NEB), and then washed three times with wash buffer as described (Deminoff *et al.* 2009; Ramachandran *et al.* 2011). The precipitated substrate was then incubated with the indicated Tpk protein and 10 μCi [γ-³²P]ATP in a 40-ml reaction (50 mM potassium phosphate, 5 mM NaF,

10 mM MgCl₂, 4.5 mM DTT, and both protease and phosphatase inhibitors). The proteins were separated by SDS-polyacrylamide gel electrophoresis, and the level of phosphorylation was assessed by autoradiography.

Results

Presence of PKA signaling activity is sufficient to trigger P body disassembly

Our goal here was to test whether the ectopic presence of Ras/PKA signaling activity was sufficient to trigger P body disassembly in the absence of glucose addition. For this analysis, we engineered cells to express the dominant *RAS2^{val19}* allele in glucose-free conditions. The protein encoded by this allele exhibits diminished GTPase activity and results in constitutively elevated levels of PKA activity (Toda *et al.* 1985). For these experiments, *RAS2^{val19}* was placed under the control of the inducible promoter from the *MET3* gene; expression from this promoter is induced when methionine is absent from the growth medium (Cherest *et al.* 1987; Howard *et al.* 2002). Log-phase cells expressing *Edc3*-mCherry and/or *Pat1*-GFP reporters were transferred to a glucose-free medium for 10 min to allow P body foci to form. The cells were then moved to a medium that also lacked methionine to elicit expression from the *RAS2^{val19}* locus. Under these conditions, we found that the majority of pre-existing P bodies were disassembled after *RAS2^{val19}* induction (Figure 1, A and B). In contrast, the transfer of the wild-type strain to this methionine-minus medium did not have a significant effect upon P body number. Since our previous work identified *Pat1* as a critical target for PKA, we tested whether *Pat1* phosphorylation was required for the observed P body disassembly. For this analysis, the inducible *MET3-RAS2^{val19}* allele was introduced into strains expressing GFP-tagged versions of either the wild-type *Pat1* (*Pat1*-SS) or the nonphosphorylatable *Pat1*-AA variant. The *Pat1*-AA protein has alanine residues replacing the two serines that are phosphorylated by PKA, Ser-456 and Ser-457 (Ramachandran *et al.* 2011). These cells also contained the *Edc3*-mCherry reporter. As above, we found that the P bodies in cells with the wild-type *Pat1* were efficiently dispersed following the induction of *RAS2^{val19}* expression (Figure 1C). However, this disassembly was effectively blocked in cells containing the *Pat1*-AA variant (Figure 1, C and D). Therefore, the presence of PKA activity was sufficient to trigger the disassembly of already formed P bodies in a *Pat1*-dependent manner.

All three PKA isoforms influence P body formation

S. cerevisiae has three genes, *TPK1*–*3*, that encode a catalytic subunit of the PKA enzyme (Toda *et al.* 1987b). Although each of these genes is sufficient for viability, there are data suggesting that the different isoforms have distinct functions (Robertson and Fink 1998; Pan and Heitman 2002; Ptacek *et al.* 2005). Moreover, a recent study has indicated that the *Tpk2* and *Tpk3* proteins are themselves recruited to P body foci in response to glucose deprivation (Tudisca *et al.* 2010).

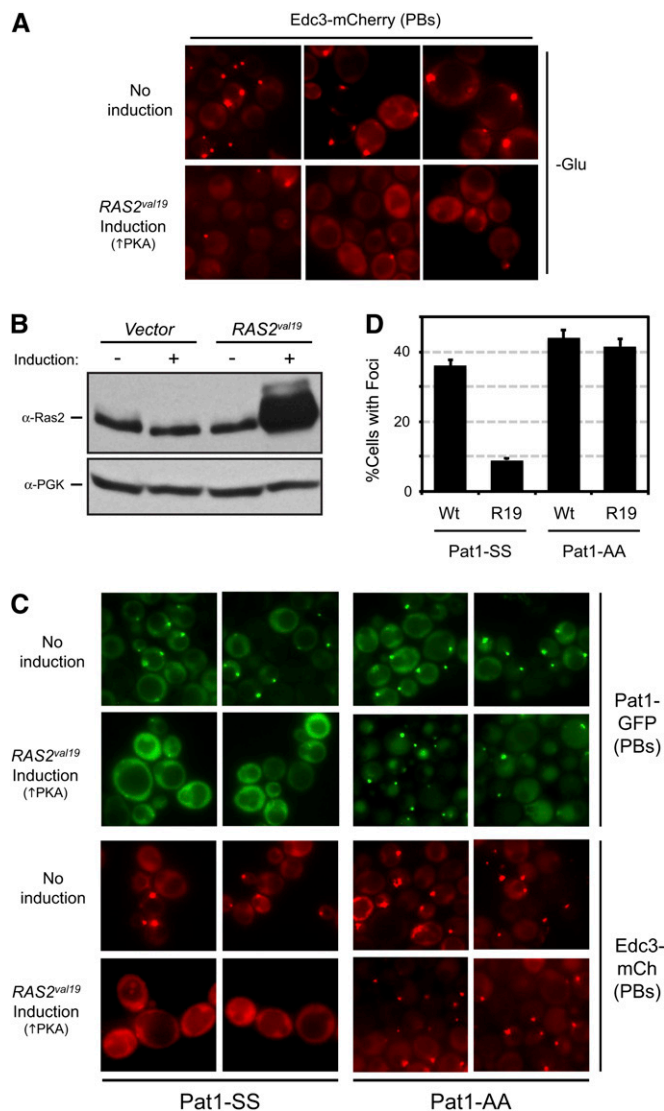


Figure 1 The presence of Ras/PKA signaling activity was sufficient to trigger P body disassembly. (A) The ectopic expression of the constitutively active *Ras2^{val19}* protein resulted in the dispersal of *Edc3*-containing foci. Cells expressing the *Edc3*-mCherry protein were transferred to a glucose-minus medium containing 250 mM methionine for 10 min to induce P body formation. The cells were then moved to a medium that also lacked methionine for 2 hr to induce expression from the *MET3-RAS2^{val19}* locus and then were examined by fluorescence microscopy. Three representative images are shown for both the control and the induced conditions. (B) Increased *Ras2* protein expression was detected in cells containing the *MET3-RAS2^{val19}* locus. Protein extracts were prepared from cells containing either a control vector or a *MET3-RAS2^{val19}* plasmid, and the level of *Ras2* protein was assessed by Western blotting with an anti-*Ras2* antibody. (C and D) The *RAS2^{val19}*-triggered disassembly of P bodies was blocked in cells containing the nonphosphorylatable variant *Pat1*-AA. The disassembly of preformed P bodies was assessed as described above in cells expressing GFP-tagged versions of either the wild-type *Pat1*-SS or the nonphosphorylatable *Pat1*-AA. The microscopy data are shown in C, and the quantitation of these data in D. The data shown in the graph represent the average of at least two experiments ($n = 100$) with the *Edc3*-mCh reporter.

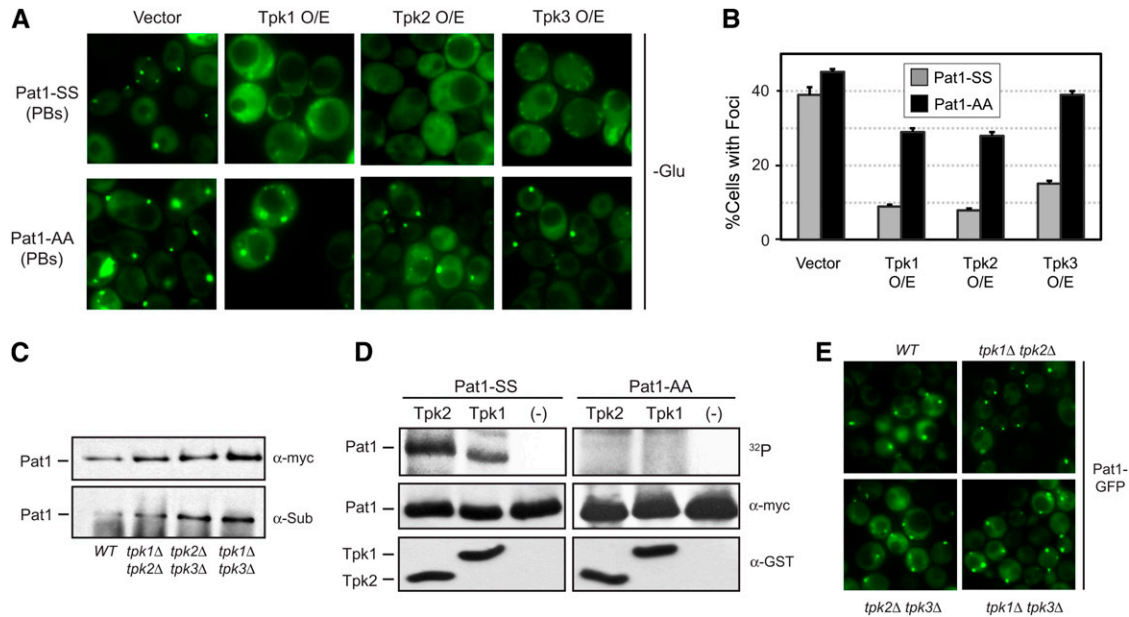


Figure 2 All three isoforms of the *S. cerevisiae* PKA were able to influence P body formation. (A and B) The overexpression of any one of the three Tpk proteins was sufficient to inhibit P body formation. Cells containing either the control vector or a plasmid with the indicated *TPK* gene were transferred to a glucose-minus medium for 10 min to induce P body formation. The cells also expressed either the Pat1-SS or Pat1-AA protein, as indicated. The microscopy data are presented in A and the quantitation of these data in B. (C) The PKA site in Pat1 was phosphorylated in cells lacking any two of the three Tpk isoforms. Pat1 was immunoprecipitated from log-phase cells with the indicated genotypes, and the level of *in vivo* PKA phosphorylation was assessed by Western blotting with an antibody that specifically recognizes the phosphorylated form of Pat1 (α -Sub). (Top) Western blot showing the total amount of the *myc*-tagged Pat1 present. (D) Pat1 was phosphorylated *in vitro* by the Tpk1 and Tpk2 isoforms of PKA. The *myc*-tagged Pat1 proteins were immunoprecipitated from log-phase cells, treated with phosphatase, and then incubated with the indicated GST-tagged Tpk protein and [γ - 32 P]ATP. The bottom two panels are Western blots indicating the total amount of the respective proteins present in the reaction mixtures. “(-)” indicates the “no kinase” control. (E) P body formation in mutants containing only a single Tpk isoform. Cells with the indicated genotypes were transferred to a medium lacking glucose for 10 min, and P body formation was then assessed by fluorescence microscopy.

Therefore, we tested here whether each of the isoforms could influence P body assembly. Consistent with this possibility, we made the following observations. First, we found that the overexpression of any single Tpk protein was able to efficiently inhibit P body formation (Figure 2, A and B). This inhibition was suppressed in each case by the introduction of the nonphosphorylatable Pat1-AA. Second, we found that Pat1 was phosphorylated in mutants lacking any two Tpk isoforms, suggesting that all three Tpk proteins were capable of phosphorylating Pat1 (Figure 2C). This *in vivo* phosphorylation was assessed with an antibody that specifically recognizes the PKA phosphorylated form of Pat1 (Chang *et al.* 2004; Ramachandran *et al.* 2011). Third, we found that both Tpk1 and Tpk2 were able to phosphorylate the Pat1 protein *in vitro* (Figure 2D). The epitope-tagged Tpk3 protein had very low enzymatic activity, and we were unable to assess its ability to phosphorylate Pat1 in these assays. Finally, we found that P bodies were able to form in double-mutant cells lacking any two of the Tpk proteins (Figure 2E). In all, these results indicated that each of the three Tpk enzymes was able to phosphorylate Pat1 and thereby regulate P body assembly in *S. cerevisiae*.

PKA activity inhibits the P body formation induced by a variety of stress conditions

P body foci form in response to a number of stress conditions in addition to glucose deprivation (Teixeira *et al.* 2005;

Izawa *et al.* 2007; Yoon *et al.* 2010; Buchan *et al.* 2011). Although previous work indicated that PKA activity is influenced by glucose availability, it is not clear how the other conditions affect the Ras/PKA signaling pathway (Santangelo 2006; Zaman *et al.* 2008; Ramachandran and Herman 2010). Therefore, we tested whether the presence of the Ras2^{val19} protein would inhibit the P bodies induced by these other stresses. We first examined the foci that form in response to a hyperosmotic stress. For these experiments, we examined cells after a 10-min exposure to media containing 1 M KCl. The foci were visualized with three different P body reporters, Edc3, Dcp2, and Xrn1. Dcp2 is the catalytic subunit of the main mRNA decapping enzyme in eukaryotes, and Xrn1 is the primary exonuclease in the 5'-to-3' mRNA decay pathway (Bashkirov *et al.* 1997; Ingelfinger *et al.* 2002; Van Dijk *et al.* 2002; Eystathioy *et al.* 2003; Sheth and Parker 2003; Cougot *et al.* 2004). For each reporter, we found that the localization to cytoplasmic foci was greatly diminished in the presence of the Ras2^{val19} protein (Figure 3A). To test whether this inhibition was dependent upon the phosphorylation of Pat1, we introduced the RAS2^{val19} allele into cells expressing GFP-tagged versions of either the wild-type Pat1 (Pat1-SS) or Pat1-AA. Although the presence of RAS2^{val19} inhibited foci formation with the wild-type protein, this inhibition was effectively suppressed in cells containing Pat1-AA (Figure 3, B

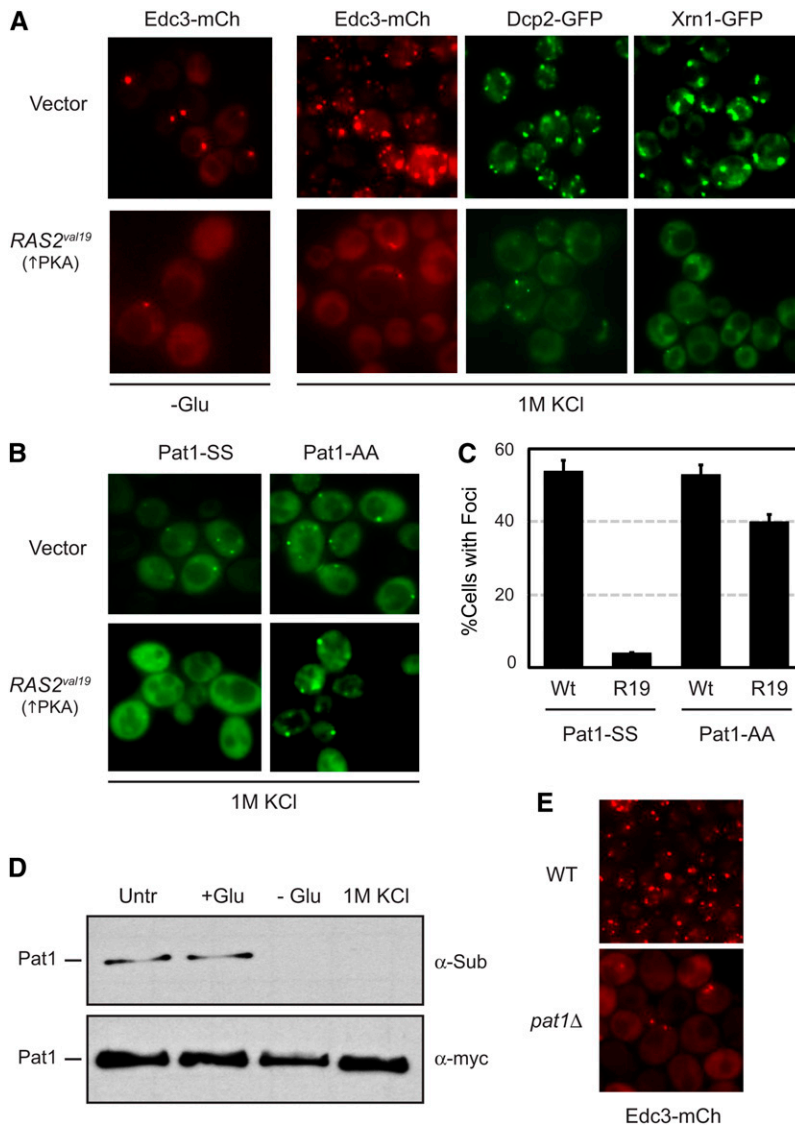


Figure 3 Ras/PKA signaling activity inhibited P body formation in response to hyperosmotic stress. (A) Osmotic stress-induced P body formation was inhibited in cells expressing the constitutively active Ras2^{val19} protein. Cells transformed with either a control vector or a plasmid containing the RAS2^{val19} locus were grown to mid-log phase and transferred to a medium either lacking glucose (–Glu) or containing 1 M KCl for 10 min. P body formation for each of the indicated reporters was then assessed by fluorescence microscopy. (B) The PKA-mediated inhibition of P body formation was suppressed by the presence of the nonphosphorylatable Pat1-AA. P body formation in response to 1 M KCl was assessed as above for *pat1Δ* cells expressing GFP-tagged versions of either Pat1-SS or Pat1-AA. (C) Quantification of the data shown in B. The graph indicates the fraction of cells with Pat1-containing foci after a 10-min exposure to 1 M KCl. (D) The PKA-dependent phosphorylation on Pat1 was rapidly diminished upon exposure to 1 M KCl. Cells expressing a *myc*-tagged Pat1 were grown to mid-log phase and then transferred for 10 min to the same medium (SC glucose; +Glu) or media either lacking glucose (–Glu) or containing 1 M KCl. The level of PKA phosphorylation was assessed by Western blotting with the anti-PKA substrate antibody (α-Sub). Untr, the extract prepared from the untreated control culture. (E) The frequency of P body formation following an osmotic stress was diminished in cells lacking Pat1. Wild-type and *pat1Δ* cells expressing the Edc3-mCh reporter were exposed to a medium containing 1 M KCl for 10 min and then examined by fluorescence microscopy.

and C). In addition, we found that the level of PKA phosphorylation on Pat1 was dramatically reduced following a 10-min exposure to 1 M KCl (Figure 3D). This reduction was similar in magnitude to that observed upon glucose starvation and did not occur in cells containing the RAS2^{val19} allele (Figure 3D; Supporting Information, Figure S1). Consistent with a role for Pat1, we also found that the number of P body foci induced by this hyperosmotic stress was reduced in *pat1Δ* cells (Figure 3E).

To determine if PKA generally inhibited P body formation, we examined the effects of expressing the Ras2^{val19} protein in cells exposed to 3 mM H₂O₂, 6 and 15% ethanol, and 0.5% NaN₃. All of these stress conditions have been shown to induce P body formation. In each case, we found that the number of P bodies was significantly diminished in the presence of elevated PKA activity (Figure 4, A–C). Moreover, these PKA inhibitory effects were effectively suppressed by the presence of the Pat1-AA variant (Figure 4C; Figure S2A). Finally, the number of Edc3-mCherry foci in-

duced by these different conditions was greatly reduced in cells lacking the Pat1 protein (Figure 4D; Figure S2B). Altogether, these data indicate that PKA signaling activity has a general role in the regulation of P body foci formation in *S. cerevisiae*.

Assembly of stress granules is not influenced by PKA-signaling activity

A second evolutionarily conserved RNP complex, known as the stress granule, is also induced by several of the above conditions (Hoyle *et al.* 2007; Buchan *et al.* 2008, 2011; Grousl *et al.* 2009; Kato *et al.* 2011). Here, we tested whether stress granule assembly was also controlled by PKA activity. For these studies, stress granules were induced by a 30-min exposure to either 15% ethanol, 0.5% NaN₃, a 46° heat stress, or glucose deprivation. GFP-tagged versions of Pab1 and Pbp1 were used to mark stress granule foci. Pab1 is the yeast poly(A)-binding protein, and Pbp1 is the homolog of the human ataxin-2 protein that is the

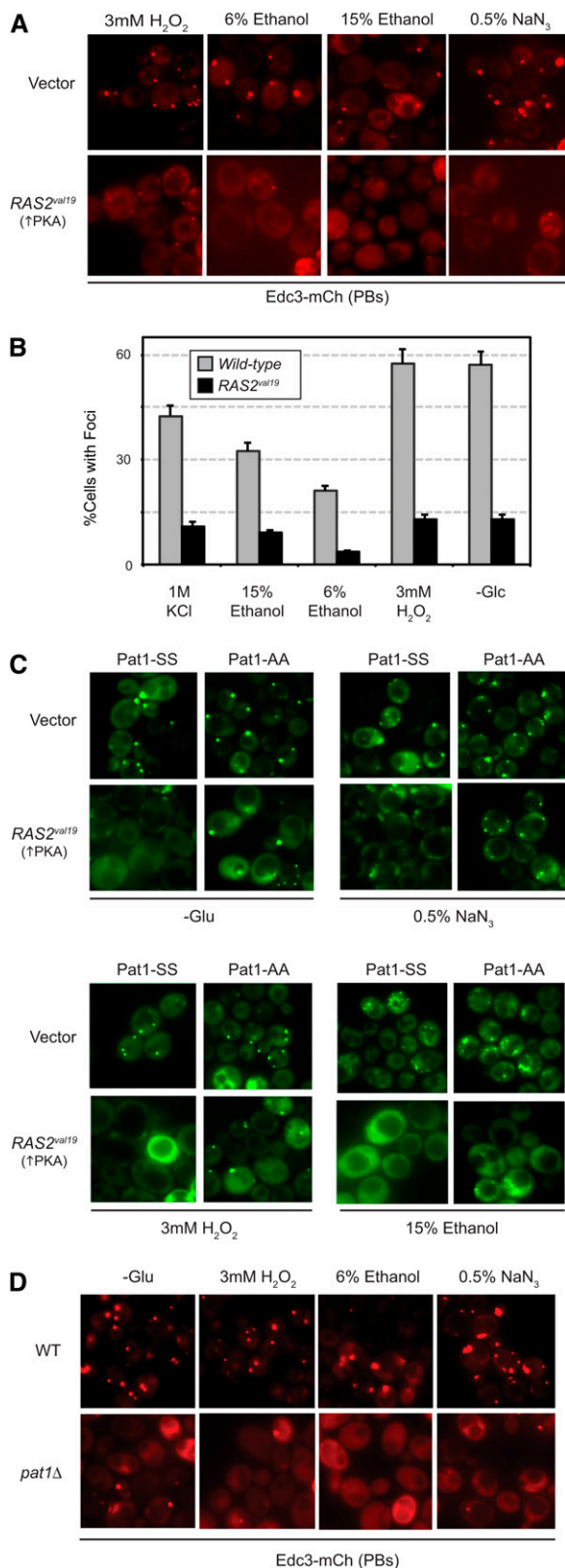


Figure 4 The presence of elevated PKA signaling activity inhibited P body induction in response to a variety of stress conditions. (A and B) Expression from the *RAS2^{val19}* locus inhibited the P body formation brought on by a number of stress conditions. Wild-type cells expressing the Edc3-mCh reporter were transformed with either a *RAS2^{val19}* plasmid or a control

product of the spinocerebellar ataxia type 2 gene, *SCA2* (Sachs *et al.* 1986; Imbert *et al.* 1996; Ralser *et al.* 2005). An induction of stress granules was observed for each of these conditions, but the final gross morphology of the fluorescent RNP structures varied for each stress (Figure 5, A and B). However, in contrast to our results with P bodies, these stress granules were present in similar numbers in cells either expressing *RAS2^{val19}* or lacking *Bcy1*, the regulatory subunit of the PKA enzyme (Figure 5, A and B; Figure S3). In addition, stress granules were not induced by the inactivation of either the Ras/PKA or the TORC1 signalling pathways (Figure 5, C and D). To inactivate the PKA pathway, we used a strain that expressed an analog-sensitive version of *Tpk1* as its sole source of PKA activity (Bishop *et al.* 2001; Stephan *et al.* 2009). The encoded *Tpk1^{as}* protein is rapidly inactivated by the membrane-permeable inhibitor 1NM-PP1 (Stephan *et al.* 2009). As noted previously, the inactivation of the PKA pathway resulted in the formation of P body foci (Figure 5C) (Ramachandran *et al.* 2011). To inactivate the TORC1 pathway, the cultures were treated with the macrolide rapamycin. Finally, we found that stress granules were able to form at essentially wild-type levels in cells lacking the *Pat1* protein for all inducing conditions except NaN₃ (Figure 5E). In this latter case, we found that *pat1Δ* cells had ~20–30% fewer foci than the wild-type strain (see also Buchan *et al.* 2011). Altogether, these results indicated that stress granule assembly is not regulated by PKA signaling activity in *S. cerevisiae*.

Presence of P body foci is correlated with the long-term survival of stationary-phase cells

Our previous work indicated that P body foci formation might be required for the long-term survival of stationary-phase cells (Ramachandran *et al.* 2011). This survival has been used as a model for the study of eukaryotic aging, and the length of time that the quiescent yeast cells remain viable has been referred to as the chronological life span, or CLS (Kennedy 2008). To examine this link with P bodies further, we asked whether the presence of a phosphomimetic form of *Pat1*, *Pat1-EE*, would affect foci formation and/or the survival of stationary-phase cells. *Pat1-EE* has glutamic acid residues replacing the two serines that are normally

vector and exposed to the indicated stress for 10 min. P body formation was then assessed by fluorescence microscopy. The microscopy data are presented in A and the quantitation of these data in B. The 1 M KCl microscopy data are shown in Figure 3A. (C) The presence of the non-phosphorylatable *Pat1-AA* diminished the inhibitory effects of the *RAS2^{val19}* protein. P body foci formation was assessed after a 10-min exposure to the indicated stress in *pat1Δ* cells expressing GFP-tagged *Pat1-SS* or *Pat1-AA*. The cells contained either a control vector or a *RAS2^{val19}* plasmid, as indicated. A graph showing the quantitation of these data are presented in Figure S2A. (D) P body formation in response to a variety of stresses required the presence of the *Pat1* protein. P body formation was assessed in wild-type and *pat1Δ* cells after a 10-min exposure to the indicated conditions. A graph showing the quantitation of these data is presented in Figure S2B.

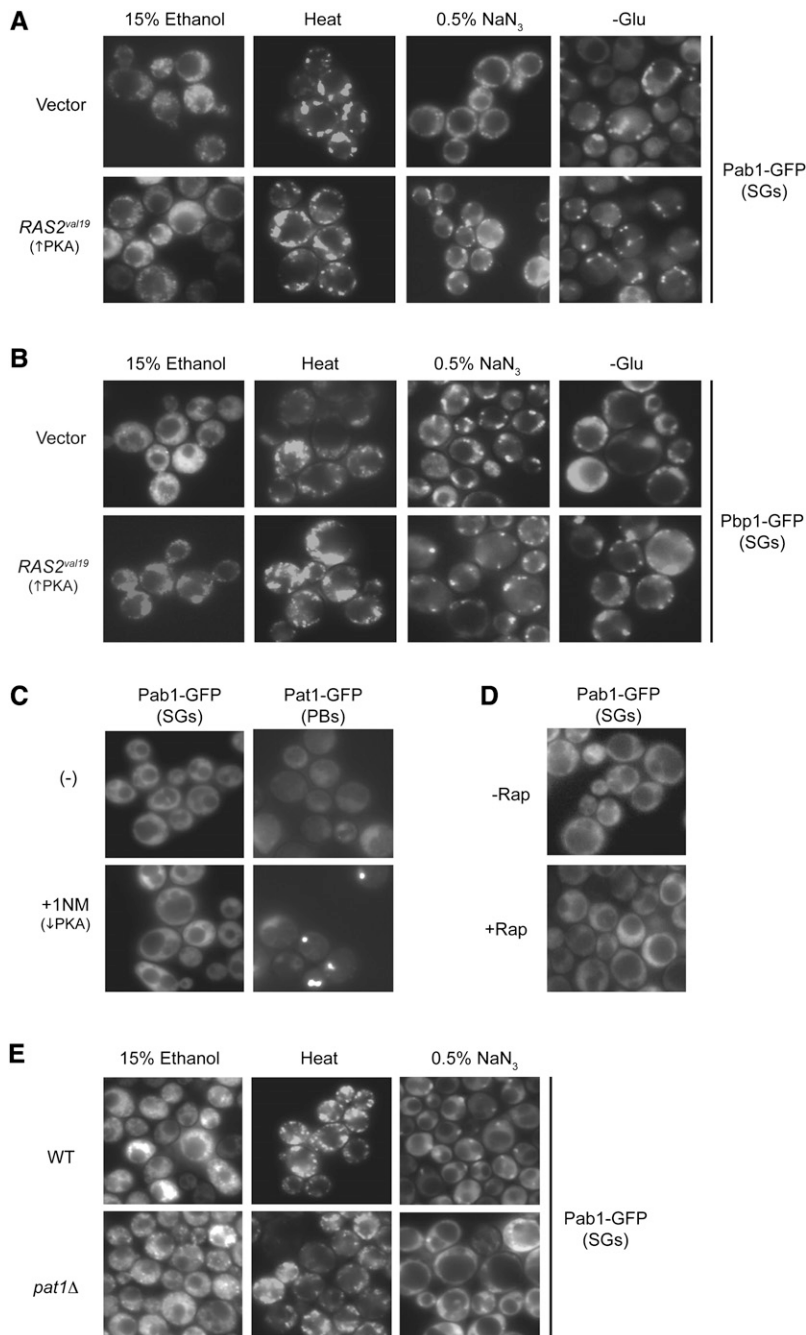


Figure 5 Stress granule formation was not influenced by Ras/PKA signaling activity. (A and B) Elevated Ras/PKA activity did not inhibit stress granule formation in response to a number of inducing conditions. Wild-type cells expressing either Pab1-GFP (A) or Pbp1-GFP (B) were transformed with either a control vector or a *RAS2^{val19}* plasmid. Stress granule formation was induced by a 30-min exposure to a 46° heat stress (Heat) or media either lacking glucose or containing 15% ethanol or 0.5% NaN_3 , as indicated. (C) The down-regulation of PKA signaling did not result in stress granule formation. Plasmids with GFP-tagged versions of either Pab1 or Pat1 were introduced into a yeast strain (PHY4697) with an analog-sensitive version of *TPK1*, *tpk1^{as}*. The cells were then treated for 30 min with 5 μM 1NM-PP1 to inhibit the encoded Tpk1^{as} protein, and foci formation was assessed by fluorescence microscopy. (D) Stress granule formation was not induced upon inactivation of the TORC1 signalling pathway. Wild-type cells expressing the Pab1-GFP fusion protein were treated for 20 min with 200 ng/ml rapamycin. (E) Stress granule formation in *pat1* Δ cells. Wild-type and *pat1* Δ cells were exposed to the indicated stresses for 30 min, and Pab1-GFP foci were visualized by fluorescence microscopy.

phosphorylated by PKA (Ramachandran *et al.* 2011). The presence of a glutamic acid can functionally substitute for a phosphorylated serine residue (Thorsness and Koshland 1987; Pearlman *et al.* 2011), and Pat1-EE could therefore act as a constitutively phosphorylated version of Pat1. Consistent with this latter possibility, we found that cells containing the Pat1-EE variant had significantly fewer P body foci than wild-type cells after 5 days of growth (Figure 6, A and B; Figure S4A). The presence of Pat1-EE also resulted in fewer P body foci in response to all of the other stress conditions tested (Figure S4, B and C). Moreover, the foci that were present in the Pat1-EE cells were generally much

smaller than those in the wild type. Strikingly, the decrease in foci number correlated with a diminished rate of stationary-phase survival as the cells containing Pat-EE rapidly lost viability during this period of quiescence (Figure 6C). Two observations indicated that these results were not due to Pat1-EE being simply a misfolded and nonfunctional polypeptide. First, a Western blot analysis showed that the Pat1-EE variant was present at wild-type levels in cell extracts (Figure S4D). Second, we had shown previously that the over-expression of Pat1-EE results in a growth arrest that is similar to that seen with the wild-type protein (Ramachandran *et al.* 2011). Pat1 has been shown to act as a translational repressor,

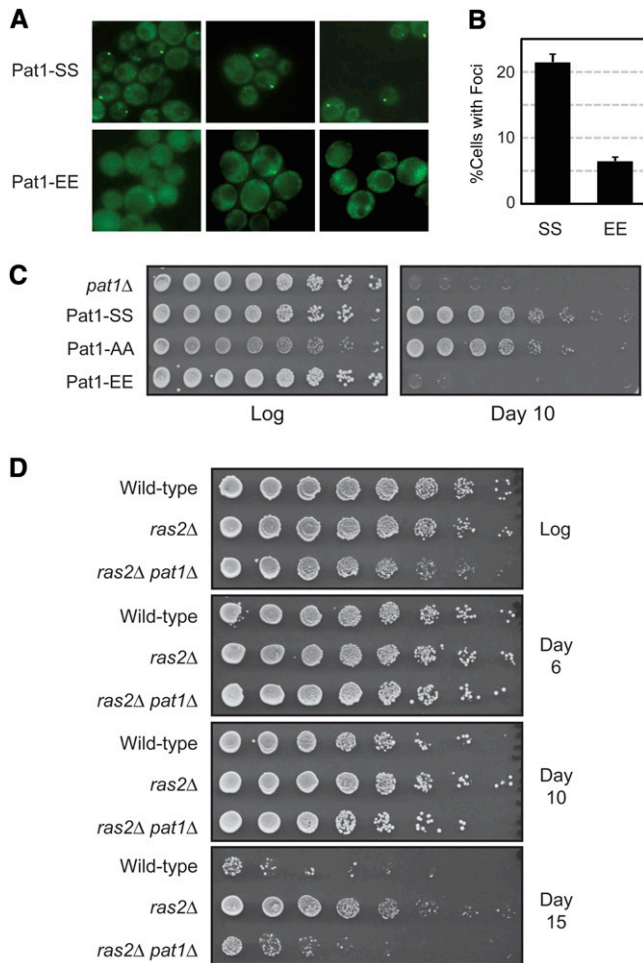


Figure 6 The presence of the phosphomimetic Pat1-EE resulted in fewer P body foci and a reduced survival in stationary phase. (A and B) Cells with the Pat1-EE variant had fewer P body foci. The number of P body foci after 5 days of growth in YPAD medium was assessed for cells expressing GFP-tagged versions of either Pat1-SS or Pat1-EE. Three representative images are shown for cells containing either Pat1-SS or Pat1-EE. The microscopy data are shown in A and the quantitation of these data in B. (C) Cells expressing the Pat1-EE variant had a diminished CLS. The number of viable cells present during log-phase growth and after 10 days in a minimal YM glucose medium was determined by plating out increasing dilutions of these cultures. (D) Deletion of the *PAT1* locus suppressed the extended CLS of a *ras2Δ* mutant. Serial dilutions of strains with the indicated genotypes were plated out at the indicated times of culture growth in a YM glucose minimal medium.

and this overexpression result therefore indicates that Pat1-EE retains at least some functions of the wild-type protein (Coller and Parker 2005). Therefore, the presence of the Pat1-EE variant resulted in both a diminished number of P body foci and a decreased rate of survival during the stationary phase of growth.

We also tested whether Pat1 was required for the extended CLS observed for mutants with diminished levels of Ras/PKA signaling activity. For these experiments, we used a *ras2Δ* yeast strain that remains viable because of the presence of the wild-type *RAS1* locus. As reported previously, we found that this strain survived significantly longer than the

isogenic wild type when in stationary phase (Figure 6D) (Longo 2003). However, this increased survival was largely lost upon deletion of the *PAT1* locus (Figure 6D). Cumulatively, these data are consistent with a role for Pat1, and perhaps for P body foci, in the long-term survival of stationary-phase cells.

P body foci persist throughout the stationary phase of growth

Despite their potential role in stationary-phase survival, it was not known whether P bodies were present in these quiescent cells. Previous studies have most likely examined cells that had not yet entered into stationary phase (Teixeira *et al.* 2005; Brengues and Parker 2007). To address this question, we examined cells expressing various P body markers at different times during a 4-week period of growth. The fraction of cells containing foci at any time was assessed by fluorescence microscopy. With each reporter, P bodies were initially detected after ~24 hr of growth and were found to persist throughout the entire incubation period (Figure 7, A and B; Figure S5, A and B). The time of P body induction observed is consistent with previous reports indicating that these RNP complexes begin to form when glucose is almost depleted from the medium (see below) (Teixeira *et al.* 2005). As with the other stress conditions, we found that the number of stationary-phase foci was severely decreased in both *RAS2^{val19}* and *pat1Δ* mutant cells (Figure 7, C–G). These data therefore indicated that P body foci are present throughout the stationary phase of growth and that the induction of these RNP granules is regulated by the PKA signaling pathway.

Stress granule foci form specifically upon entry into stationary phase

We also examined the temporal pattern of stress granule appearance in cells that were grown into stationary phase on glucose-based media. Under these batch culture conditions, yeast cells proceed through a number of distinct growth transitions (Figure 8A). During the initial or logarithmic phase, the cells grow rapidly via fermentation of the available glucose. This fermentative growth occurs even though oxygen is available to the cells and continues for ~24 hr (Diaz-Ruiz *et al.* 2009; Castelli *et al.* 2011). When the glucose is depleted, the cells alter their metabolism and subsequently grow by respiration on the ethanol that was produced during the prior period of fermentation. This switch in carbon source utilization is known as the diauxic shift and the time of respiratory growth as the post-diauxic shift growth period (PDS) (Figure 8A) (Gray *et al.* 2004). This latter period of slower growth continues for another 4–5 days until the ethanol is also used up. At this time, the cells enter the true stationary phase of growth (Werner-Washburne *et al.* 1993; Herman 2002).

Using a variety of GFP-tagged reporters, we found that stress granules were present in stationary-phase cells and that these foci appeared to persist for as long as the cells

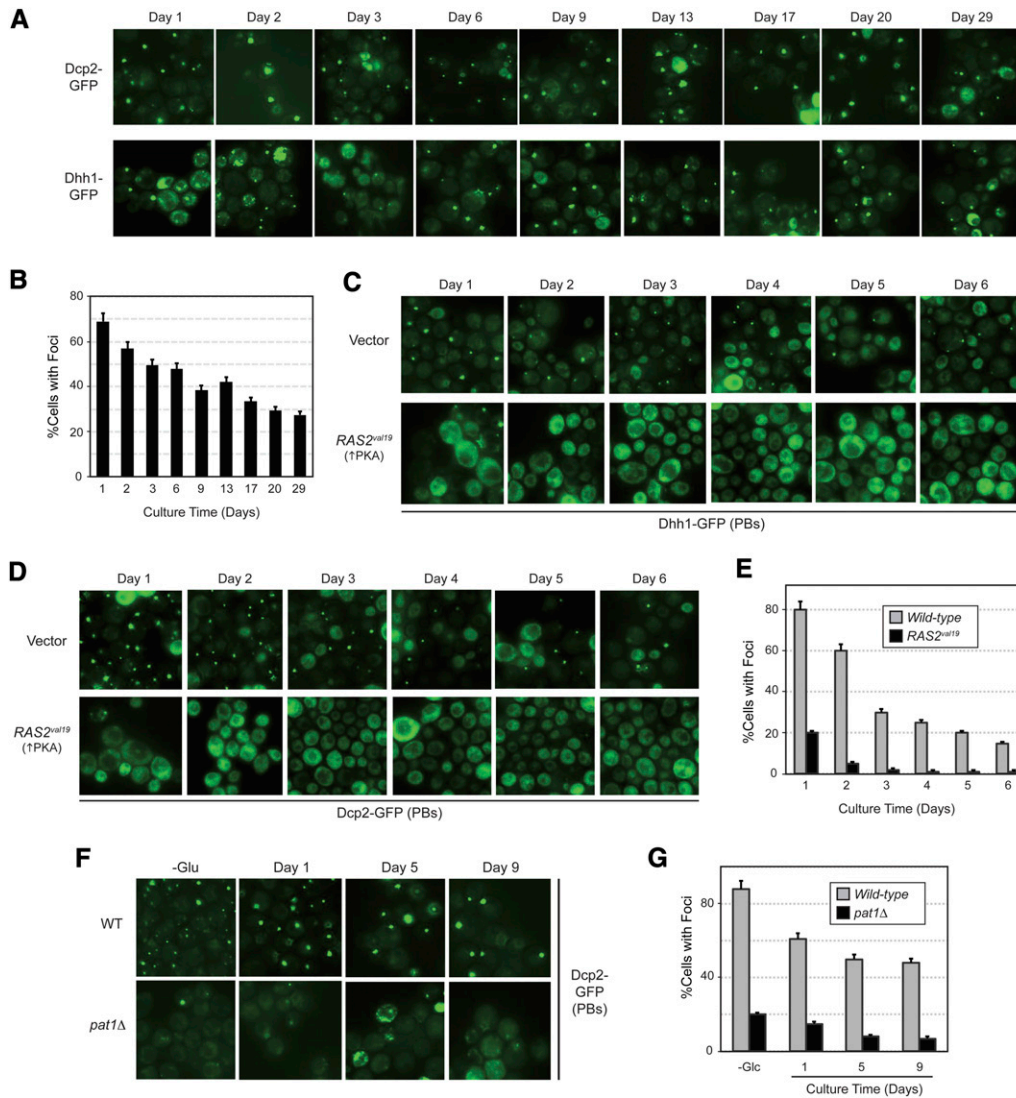


Figure 7 P body foci are present and persist throughout the stationary phase of growth. (A) P body foci are present in stationary-phase cells. The frequency of P body formation was determined for cells with the integrated Dcp2-GFP and Dhh1-GFP reporters at the indicated times of growth in YPAD cultures. (B) A graph showing the number of Dcp2 foci at the indicated culture times; the relevant microscopy data are presented in A. (C and D) The presence of the constitutively active Ras2^{val19} protein resulted in fewer P body foci. P body foci were visualized by fluorescence microscopy after the indicated number of days of growth for wild-type cells with either a control vector or a Ras2^{val19} plasmid. (E) A graph showing the quantitation of the Dcp2-GFP microscopy data presented in D. (F and G) The presence of the Pat1 protein was required for the formation of stationary-phase P body foci. The fraction of cells with P body foci was determined for wild-type and *pat1Δ* cells after the indicated number of days of growth in YPAD. The results for a 10-min starvation for glucose are shown for comparison purposes (–Glc). The microscopy data are shown in F and the quantitation of these data in G.

remained in this quiescent state (Figure 8B). As with the other inducing conditions, the number of these stress granules was not significantly diminished by either the presence of the Ras2^{val19} protein or the absence of Pat1 (Figure 8C; Figure S6A). In addition, the stress granule reporters tested, Pbp1, Pbp4, and Lsm12, did not colocalize with the Edc3-mCh marker for P bodies, suggesting that these two foci were distinct structures in these resting cells (Figure S6B). In all, we found that the stress granule and P body reporters failed to colocalize in 92.0% ($\pm 7.1\%$) of those cells that had both types of these foci. However, the important point to emphasize here is that the stress granule foci did not appear in significant numbers until after 6–7 days of culture incubation, a time when the cells appear to have already entered into the stationary phase of growth (Figure 8, D and E; Figure S6C). This temporal pattern of localization is illustrated here with the stress granule constituent Pbp1. Whereas Pbp1 is distributed throughout the cell during the fermentative, or log phase, of growth, this protein is found predominantly in the nucleus during the PDS growth period

(Figure 8, D and F). Upon the cessation of growth, this protein relocates to discrete foci in the cytoplasm of the cells (Figure 8, D and E). Other stress granule reporters, including Pab1, Pbp4, Pub1, and Lsm12, were also found in foci after day 6 or 7 of culture incubation (Figure 8D; Figure S6C). These data therefore indicate that stress granules form specifically after cells enter into the stationary phase of growth.

We also examined the CLS of strains lacking different stress granule constituents, including the *pbp1Δ*, *pbp4Δ*, *lsm12Δ*, and *pub1Δ* mutants (Buchan and Parker 2009). Of these strains, only the *pub1Δ* mutant exhibited a significantly shorter CLS than the wild-type control (Figure 9A). This survival phenotype was apparently not due to a defect in stress granule formation as all of these mutant strains had wild-type levels of Pab1-containing foci (Figure 9B). However, we found that *pub1Δ* cells contained a dramatically reduced number of P body foci, a defect that was specific to this particular mutant (Figure 9, C and D). This latter result was somewhat surprising as *pub1Δ* mutants were

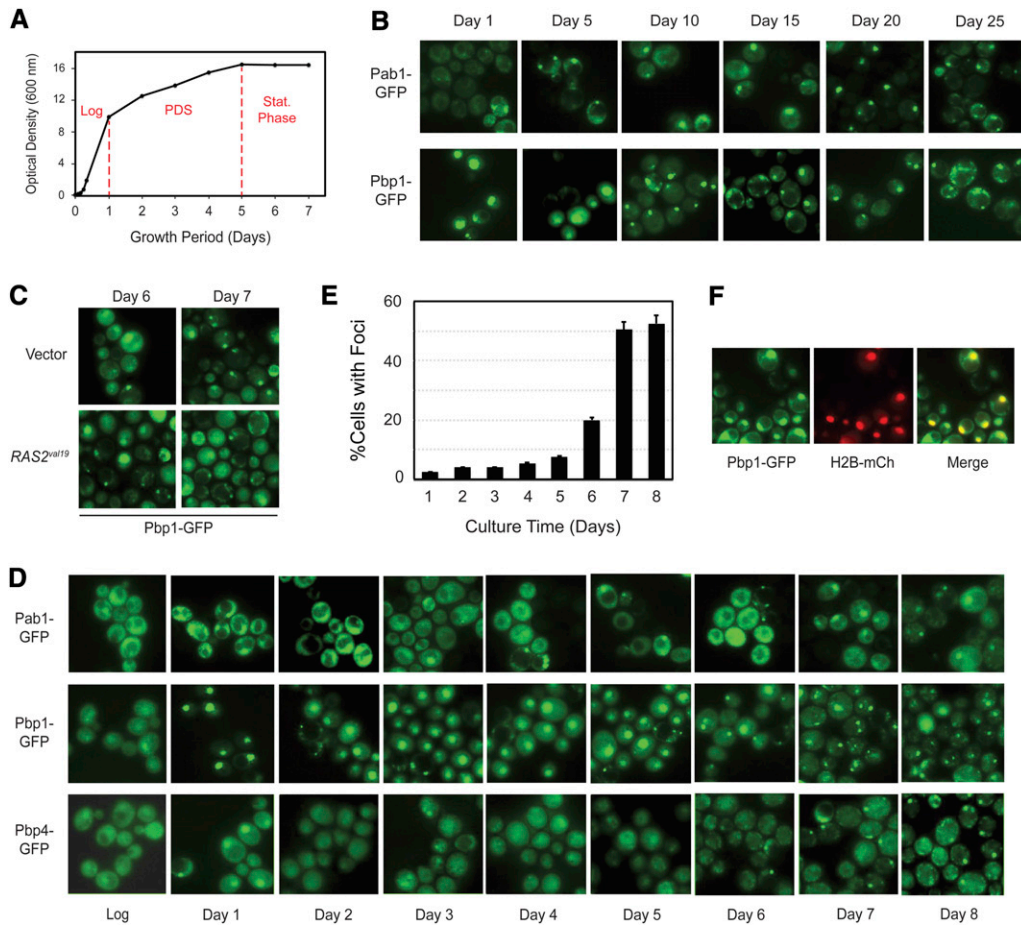


Figure 8 Stress granules appeared in cells after entry into stationary phase. (A) A representative growth curve for a wild-type strain with the integrated Pbp1-GFP reporter. The red lines delimit the log, PDS, and stationary phases of growth. (B) Stress granule foci were present in stationary-phase cells. The subcellular localization of Pab1-GFP and Pbp1-GFP was assessed by fluorescence microscopy after the indicated days of growth in the rich growth medium YPAD. (C) Stress granule formation was not affected by the presence of the *RAS2^{val119}* locus. The subcellular localization of a Pbp1-GFP reporter was assessed after the indicated number of days of growth for cells containing either a control vector or a *RAS2^{val119}* plasmid. (D and E) Stress granule foci did not appear in significant numbers until after cells had entered into the stationary phase of growth. The subcellular localization of the indicated reporters was assessed in cells at daily intervals of growth in a rich medium. The graph in E shows the fraction of cells with a Pbp1-GFP focus after the indicated days of growth. (F) Pbp1 was

found in the nucleus during the PDS period of growth. Cells expressing Pbp1-GFP were transformed with a plasmid encoding the nuclear marker, histone H2B-mCh, and the colocalization of the two reporters was assessed after 3 days of culture growth.

not defective for P body formation under other induction conditions, including glucose deprivation (Buchan *et al.* 2008, 2011) (Figure S7). The defect observed here suggests that Pub1 may have additional functions in stationary-phase cells, and it will be interesting to determine the nature of this activity. In any case, the findings with the *pub1Δ* mutant were consistent with the above assertion that P body foci play an important role in the long-term survival of stationary-phase cells.

Discussion

In this study, we examined the assembly and function of two representative RNP complexes, the P body and stress granule, in *S. cerevisiae*. These complexes are structurally related and are induced by similar types of stress conditions (Kedersha *et al.* 2005; Buchan and Parker 2009). However, despite these similarities, the data here indicate that these two RNP granules are independently assembled and that this assembly is controlled by different signaling pathways. In particular, we found that the PKA pathway has a general role in the regulation of P body foci formation. Constitutive PKA signaling inhibited P body formation under all conditions tested, and, in each case, this inhibition was reversed

by the presence of the nonphosphorylatable Pat1-AA variant. All three isoforms of the PKA enzyme, Tpk1–3, were found to inhibit P body formation. Consistent with a key role for Pat1, we also found that P body assembly was less efficient in cells carrying the phosphomimetic variant Pat1-EE. Finally, the ectopic presence of PKA activity was found to be sufficient to trigger the disassembly of preformed P bodies in glucose-starved cells. In all, these data point to a key regulatory role for the PKA-mediated phosphorylation of Pat1 in the P body assembly process. For each stress condition, this control could be due to changes in the activity of either PKA itself or the particular phosphatase(s) responsible for the dephosphorylation of Pat1. Ongoing efforts in the laboratory aim to discern between these two possibilities for each of the induction conditions.

In contrast to these results with P bodies, we found no evidence of a role for PKA in the control of stress granule assembly. Neither the presence of constitutive PKA activity nor the inactivation of this pathway had any significant effect upon stress granule formation. This was true for all conditions tested, including those that can induce both types of RNP foci. Thus, stress granule assembly is apparently regulated by other, as-yet-unidentified, signaling pathways. The results also point out that stress granules, for the most

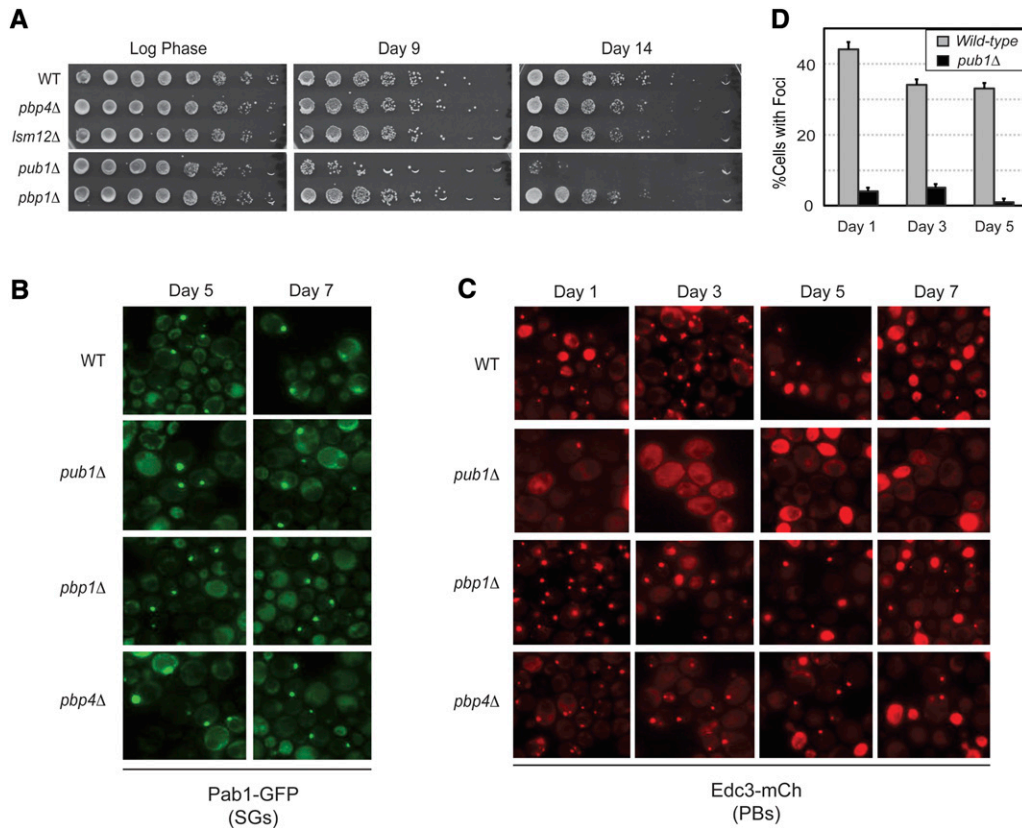


Figure 9 Mutants lacking the stress granule protein Pub1 have a diminished CLS and fewer stationary-phase P body foci. (A) *pub1Δ* mutants exhibited a decreased rate of survival during stationary phase. The number of viable cells present at the indicated times of growth was determined by plating out increasing dilutions of the cultures as described in *Materials and Methods*. (B and C) The subcellular localization of a stress granule (B: Pab1-GFP) or P body (C: Edc3-mCh) marker was assessed by fluorescence microscopy after the indicated days of growth. (D) Quantitation of the P body foci data for the wild-type and *pub1Δ* strains shown in C.

part, form normally in cells, such as the *RAS2^{val19}* and *pat1Δ* mutants, that are defective for P body assembly. Stress granule formation therefore was not generally dependent upon a pre-existing P body template, an observation that is consistent with studies of these granules in mammalian cells (Ohn *et al.* 2008; Anderson and Kedersha 2009). However, it should be noted that our results conflict with a prior study that found that the presence of stress granules in yeast cells was dependent upon P body formation in glucose-limiting conditions (Buchan *et al.* 2008). Although we do not yet know the underlying reason for these different observations, it is relevant to point out that glucose deprivation was the least robust condition for stress granule formation. Subtle changes in media composition and strain background had a significant effect on the efficiency of stress granule induction, and either could be responsible for the differences reported in these two studies (Nissan and Parker 2008; Takahara and Maeda 2012). Nonetheless, the key points here are that stress granule formation is not regulated by PKA activity and that stress granule and P body assembly appear to occur independently in *S. cerevisiae*.

The data here also show that P body and stress granule foci are present in stationary-phase cells and that these complexes persist throughout this period of quiescence. However, each of these RNP structures is induced at a very different time. P bodies form as glucose is depleted from the growth medium, and these foci are then present throughout both the PDS and stationary phases of growth. The presence of these foci in the PDS period indicates that P bodies can

form in yeast cells that are actively dividing. In contrast, stress granules did not appear in significant numbers until the cells had stopped dividing and entered into stationary phase. The induction of these stress granules was again not dependent upon the prior presence of P body foci. In addition, we found that reporters for P body and stress granule foci did not colocalize in stationary-phase cells, a result consistent with a recent study of stationary-phase RNP foci containing the *Hos2* deacetylase enzyme (Liu *et al.* 2012). In all, these results emphasize again the independent nature of the two RNP assembly pathways and further point out that the appearance of stress granules might serve as a marker for cell entry into stationary phase. There are very few useful reporters for this transition, and it will be important to determine the underlying reasons for the induction of stress granule foci that occurs at this time.

The final point that we would like to make concerns one of the key outstanding questions in the RNP field, that of the physiological function of the larger P body assemblies. Interestingly, the data here provide further support for a model positing that the presence of P bodies is important for the long-term survival of stationary-phase yeast cells (Ramachandran *et al.* 2011). In particular, this work has identified a direct correlation between the number of P body foci and the length of time that cells survive in this quiescent state. For example, we found here that cells either carrying the phosphomimetic variant *Pat1-EE* or lacking the stress granule protein *Pub1* had fewer P body foci and a correspondingly shorter CLS. In addition, previous work showed

that the introduction of the nonphosphorylatable Pat1-AA was able to restore both P body foci and a more-normal CLS to mutants harboring the constitutively active *RAS2^{val19}* allele (Ramachandran *et al.* 2011). The underlying reasons for this correlation are not yet clear as the precise physiological activity of the larger P body foci in quiescent cells has not been determined. The foci could be responsible for the removal of particular deleterious activities from the cytoplasm or for the storage of proteins (or RNAs) necessary for the subsequent resumption of growth. Finally, it is interesting to speculate that stress granules are also important for stationary-phase survival as these RNP granules appear specifically upon entry into this quiescent state. Unfortunately, we were unable to test this possibility as all of the mutants examined here were proficient for stress granule formation. Obtaining answers to these and related questions are major goals for the field at large and should provide important insights into the physiological roles of the various RNP foci that are found in quiescent cells.

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GENETICS

Supporting Information

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Processing Body and Stress Granule Assembly Occur by Independent and Differentially Regulated Pathways in *Saccharomyces cerevisiae*

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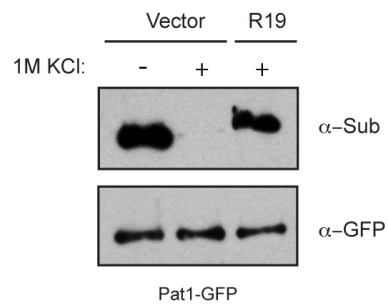


Figure S1 The presence of the *RAS2^{val19}* allele resulted in elevated levels of PKA phosphorylated Pat1. The level of PKA phosphorylation on Pat1 was assessed by Western blotting with the anti-phosphosubstrate antibody (α -Sub). The protein extracts were prepared from cells either before (-) or after a 10 min exposure to a 1M KCl salt stress (+). The cells contained either a control vector or a *RAS2^{val19}* plasmid as indicated. The bottom panel is a loading control that shows the total amount of the Pat1-GFP protein present in the cell extracts.

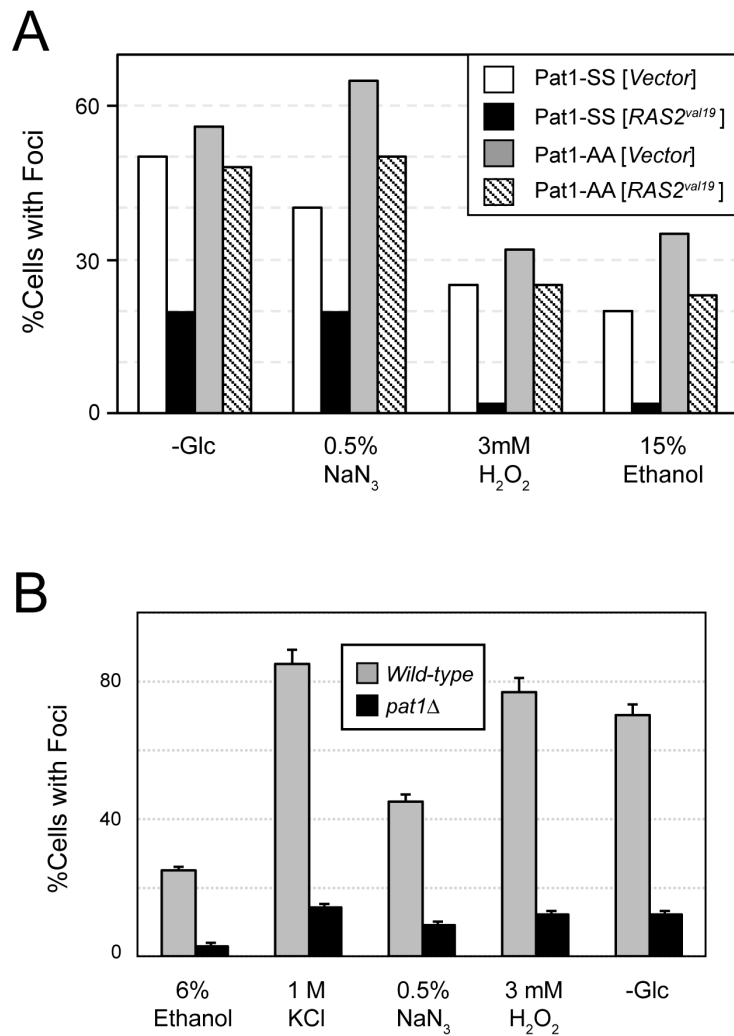


Figure S2 Quantifying the effects of *RAS2^{val19}* and the loss of Pat1 on P body foci formation in response to different stimuli. (A) Quantitation of the microscopy data shown in Figure 4C. The presence of the Pat1-AA variant suppresses the inhibitory effect that *RAS2^{val19}* has on P body foci formation. P body foci were indicated by GFP-tagged versions of either Pat1-SS or Pat1-AA, as indicated. (B) Quantitation of the microscopy data shown in Figure 4D. The fraction of cells containing an Edc3-mCh focus was assessed in wild-type and *pat1Δ* cells after a 10 min exposure to the indicated stress condition.

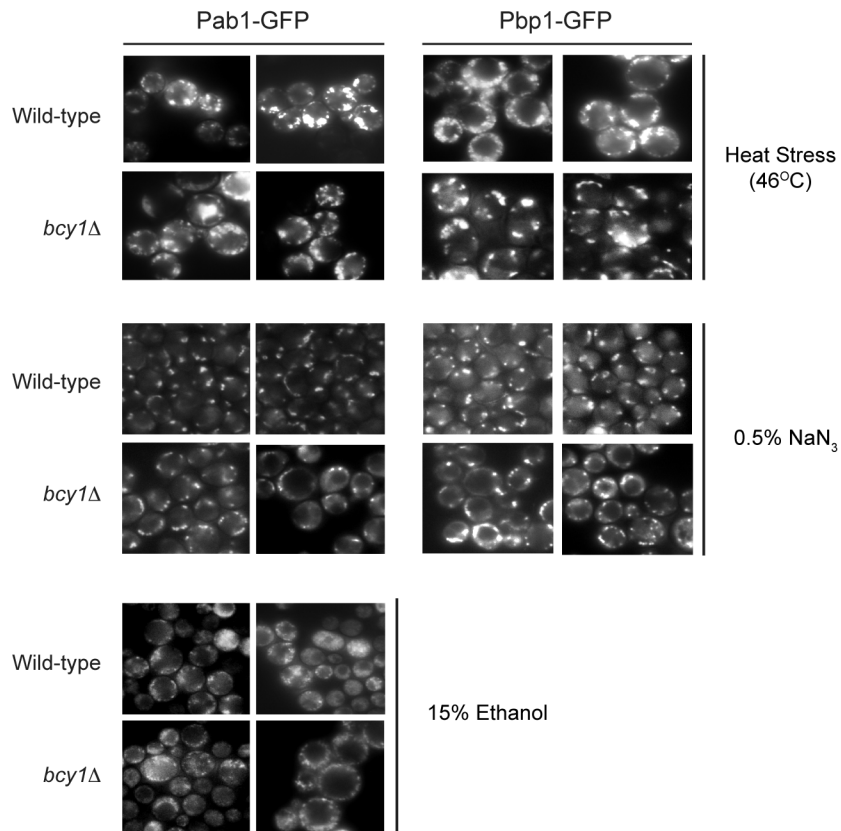


Figure S3 Stress granule foci form in normal numbers in *bcy1Δ* mutant cells. Wild-type or *bcy1Δ* cells expressing either a Pab1- or Pbp1-GFP reporter were examined by fluorescence microscopy after a 30 min exposure to the indicated stress condition.

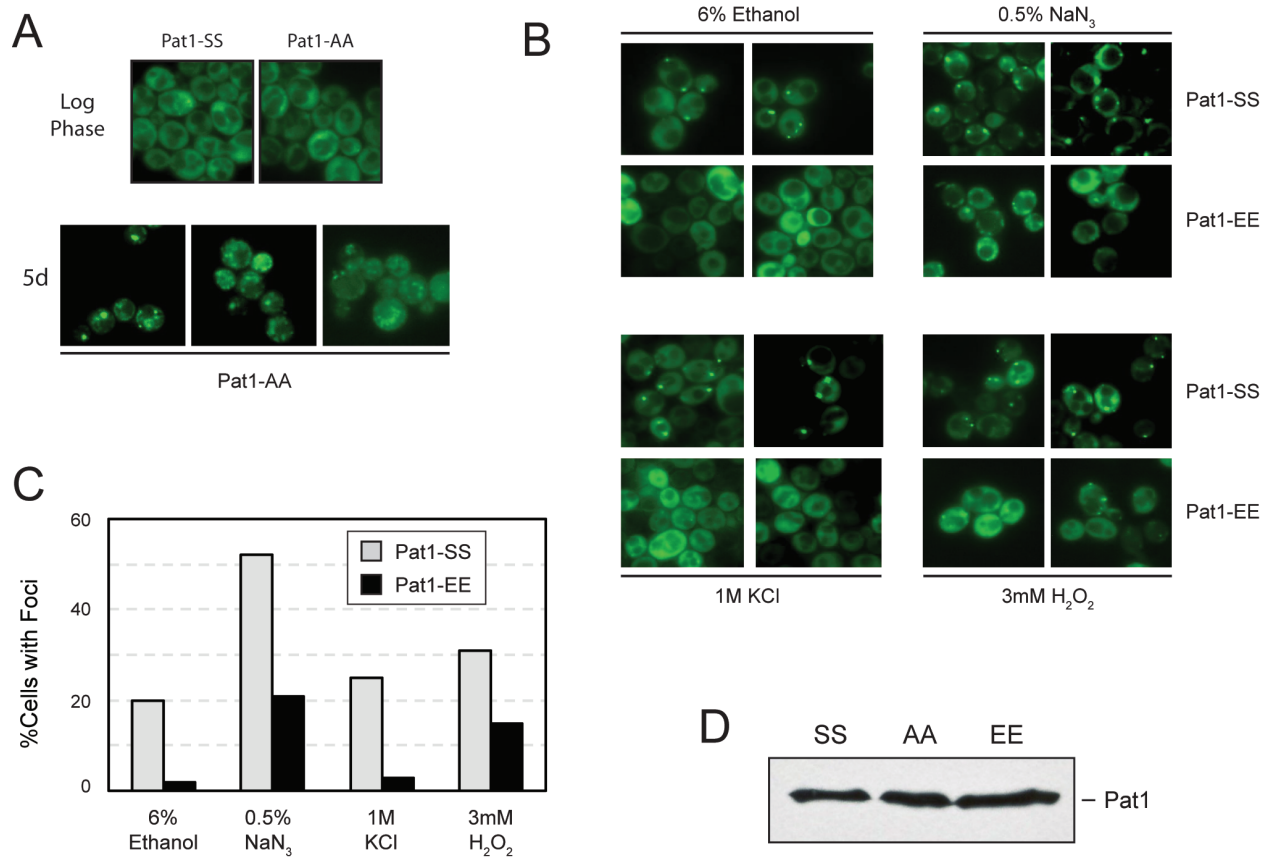


Figure S4 P body foci formation was diminished in cells expressing the phosphomimetic Pat1-EE variant. (A) P body foci formation in cells expressing the nonphosphorylatable Pat1-AA. Cells expressing the wild-type, Pat1-SS, or the Pat1-AA variant were examined by fluorescence microscopy during either log phase growth or after 5 days of incubation. (B) The presence of Pat1-EE resulted in fewer P body foci in response to all stress conditions tested. Cells expressing either Pat1-SS or Pat1-EE were examined by fluorescence microscopy after at 10 min exposure to the indicated stress conditions. (C) A graph showing the quantitation of the microscopy data presented in panel B. (D) Levels of the indicated Pat1 variants in log phase cells were determined by Western blotting with an α -GFP antibody.

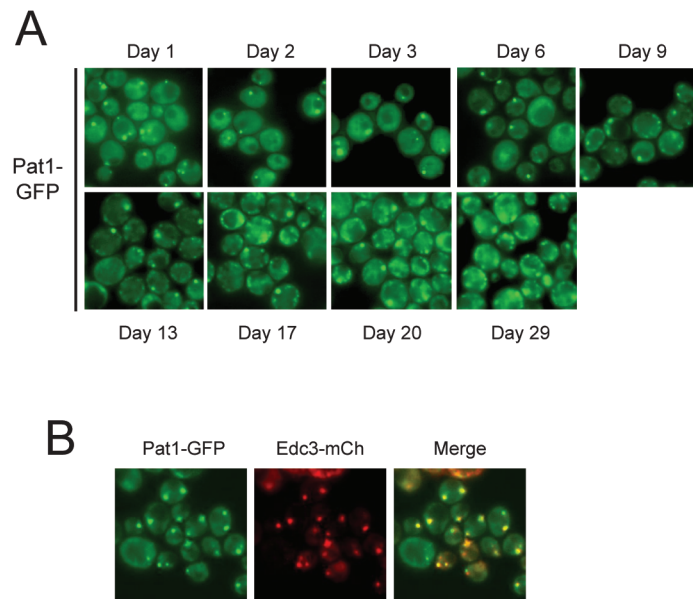


Figure S5 P body foci were present in stationary phase cells. (A) The localization of a Pat1-GFP reporter was assessed by fluorescence microscopy after the indicated days of growth. (C) The localization patterns of the Pat1-GFP and Edc3-mCh reporters is shown for cells after one day of growth in SC glucose medium.

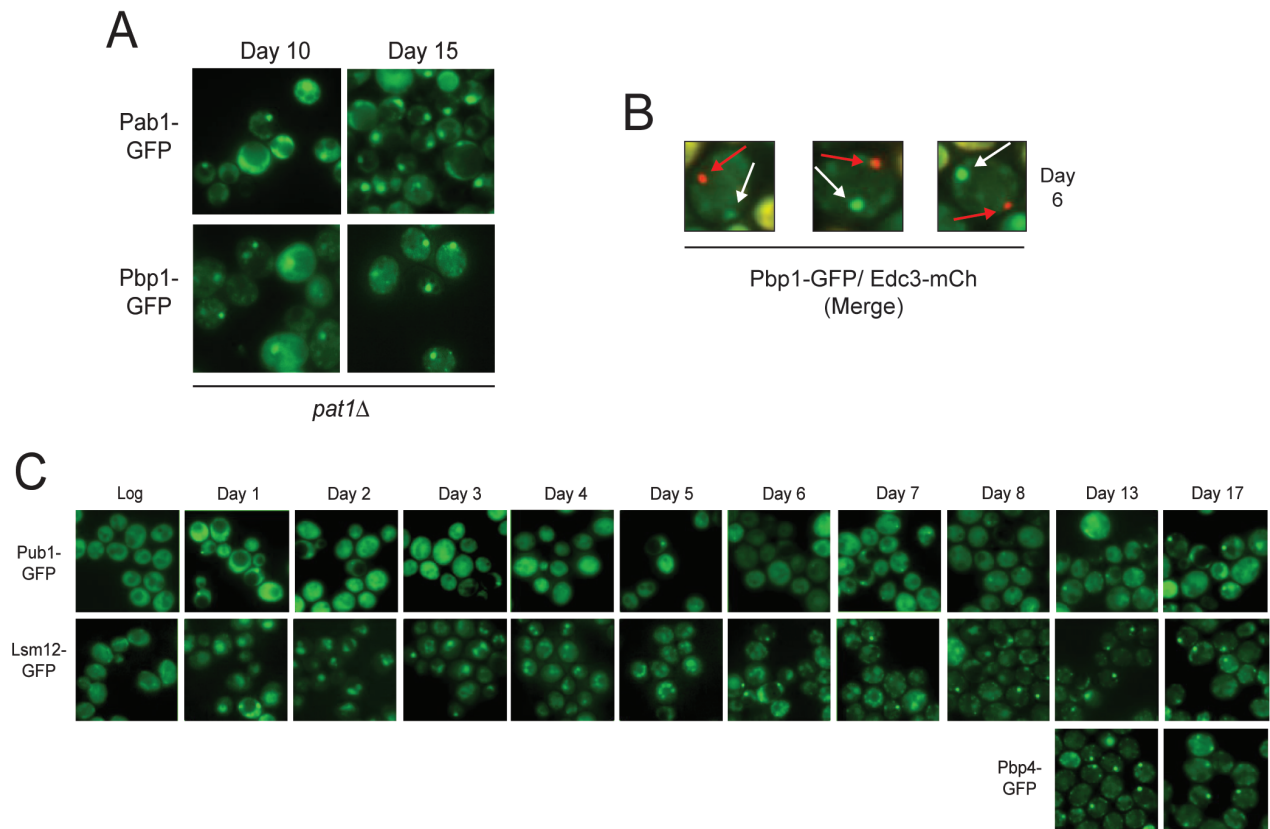


Figure S6 Stress granule foci were present in stationary phase cells. (A) The number of stress granule foci was not reduced in *pat1Δ* cells. The localization of the Pab1- and Pbp1-GFP reporters in wild-type and *pat1Δ* cells was assessed by fluorescence microscopy at the indicated times of growth. (B) P body and stress granule reporters did not co-localize in cells after 6 days of growth in an SC-glucose medium. Representative images of three cells containing both Pbp1-GFP (indicated by the white arrows) and Edc3-mCh (red arrows) foci are shown. Quantitation of the data are indicated in the text. (C) The localization of the indicated GFP-tagged stress granule reporters was assessed by fluorescence microscopy at the indicated days of growth.

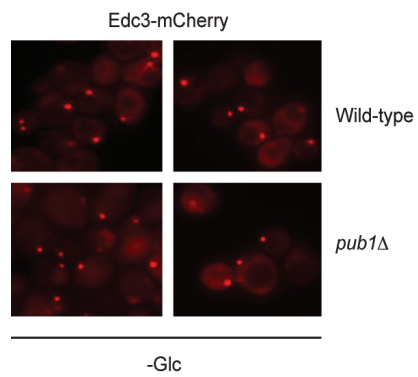


Figure S7 Similar numbers of P body foci were detected in wild-type and *pub1*Δ cells that had been exposed to a 15 min glucose starvation. Two representative images are shown for each strain background.

Table S1 Yeast strains used in this study

PHY1736 (GF260-2)	<i>MATa leu2 trp1 ura3 lys2</i>	(Valay <i>et al.</i> 1993))
PHY 1954	<i>MATa leu2 trp1 ura3 lys2 ras2::URA3</i>	This study
PHY3193 (XPY310a)	<i>MATa/α pep4::HIS3/pep4::HIS3 prb1_1.6R/prb1_1.6R can1/can1 ura3-52/ura3-52 his3_200/his3_200 GAL1-GST-TPK1/TPK1</i>	(Pan and Heitman 2002)
PHY3194 (XPY311a)	<i>MATa/α pep4::HIS3/pep4::HIS3 prb1_1.6R/prb1_1.6R can1/can1 ura3-52/ura3-52 his3_200/his3_200 GAL1-GST-TPK2/TPK2</i>	(Pan and Heitman 2002))
PHY3362 (W303-1A)	<i>MATa ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1 TPK1 TPK2 TPK3</i>	(Mazon <i>et al.</i> 1993)
PHY3364 (MB23)	<i>PHY3362 TPK1 tpk2: :HIS3 tpk3: :URA3</i>	(Mazon <i>et al.</i> 1993)
PHY3367 (MB13)	<i>PHY3362 tpk1: :LEU2 TPK2 tpk3: :URA3</i>	(Mazon <i>et al.</i> 1993)
PHY3369 (MB12)	<i>PHY 3362 tpk1: :LEU2 tpk2: :HIS3 TPK3</i>	(Mazon <i>et al.</i> 1993)
PHY4231 (BY4741)	<i>MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open Biosystems
PHY 4311	<i>MATα his3 leu2 ura3</i>	(Coller and Parker 2005)
PHY4313 (yRP2067)	<i>MATα his3 leu2 ura3 pat1Δ::NEO</i>	(Coller and Parker 2005)
PHY4697 (Y3175)	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 tpk2::KAN tpk3::TRP1 tpk1(M164G)</i>	Dr. James Broach
PHY4773	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 DCP2-GFP</i>	Invitrogen
PHY4774	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 XRN1-GFP</i>	Invitrogen
PHY4827 (yRP2162)	<i>MATa his4-539 leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG Dcp2-GFP(NEO)</i>	(Decker <i>et al.</i> 2007)
PHY 4833 (yRP 2183)	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 DCP2-GFP (NEO)</i>	(Teixeira and Parker 2007)
PHY 4834 (yRP1724)	<i>MATa2 his4-539 leu2-3,112 trp1 ura3-5 cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP (NEO)</i>	(Decker <i>et al.</i> 2007)
PHY 4898	<i>MATa leu2 trp1 ura3 lys2 ras2::URA3 pat1::KAN</i>	This study

PHY 4899	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PAB1-GFP</i>	Invitrogen
PHY 4900	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PBP1-GFP</i>	Invitrogen
PHY 4927	<i>MATα his3 leu2 ura3 pat1Δ:: NEO GFP-[PAT1-SS]</i>	This study
PHY 4928	<i>MATα his3 leu2 ura3 pat1Δ:: NEO GFP-[PAT1-AA]</i>	This study
PHY 4929	<i>MATα his3 leu2 ura3 pat1Δ:: NEO GFP-[PAT1-EE]</i>	This study
PHY 4939	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PBP4-GFP</i>	Invitrogen
PHY 4940	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PUB1-GFP</i>	Invitrogen
PHY 4941	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LSM12-GFP</i>	Invitrogen
PHY 4942	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PAT1-GFP</i>	Invitrogen
PHY 4859	<i>MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 pbp4::KAN</i>	Open Biosystems
PHY 4860	<i>MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 lsm12::KAN</i>	Open Biosystems
PHY 4869	<i>MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 pub1::KAN</i>	Open Biosystems
PHY 4870	<i>MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0 pbp1::KAN</i>	Open Biosystems

Table S2 Plasmids used in this study

Plasmid	Description	Reference
pPHY795	<i>MET3_{pro}-RAS2^{Val19}</i> in pRS415 (<i>CEN, LEU2</i>)	(Ramachandran <i>et al.</i> 2011)
pPHY796	<i>MET3_{pro}-RAS2^{Val19}</i> in pRS416 (<i>CEN, URA3</i>)	(Ramachandran <i>et al.</i> 2011)
pPHY921	<i>RAS2^{Val19}</i> in pRS316	(Ramachandran <i>et al.</i> 2011)
pPHY2203	<i>CUP1_{pro}-TPK1-HA₃</i> (<i>URA3</i>)	(Deminoff <i>et al.</i> 2006)
pPHY2659	DHH1-GFP (<i>CEN, URA3</i>)	Dr. Tien-Hsien Chang
pDHH1018		
pPHY2915	<i>CUP1_{pro}-TPK2-HA₃</i> (<i>URA3</i>)	(Deminoff <i>et al.</i> 2006)
pPHY2916	<i>CUP1_{pro}-TPK3-HA₃</i> (<i>URA3</i>)	(Deminoff <i>et al.</i> 2006)
pPHY3360	<i>PAT1_{pro}-Myc-PAT1-AA</i> (<i>CEN, HIS3</i>)	(Ramachandran <i>et al.</i> 2011)
pPHY3362	<i>PAT1_{pro}-Myc-PAT1-SS</i> (<i>CEN, HIS3</i>)	(Ramachandran <i>et al.</i> 2011)
pPHY3506	<i>PAT1_{pro}-GFP-PAT1-SS</i> (<i>CEN, HIS3</i>)	(Ramachandran <i>et al.</i> 2011)
pPHY3507	<i>PAT1_{pro}-GFP-PAT1-AA</i> (<i>CEN, HIS3</i>)	(Ramachandran <i>et al.</i> 2011)
pPHY3509	<i>PAT1_{pro}-GFP-PAT1-EE</i> (<i>CEN, HIS3</i>)	(Ramachandran <i>et al.</i> 2011)
pPHY3660	<i>EDC3-mCherry</i> (<i>CEN, URA3</i>)	(Buchan <i>et al.</i> 2011)
(pRP1574)		
pPHY3661	<i>PAB1-GFP</i> (<i>URA3</i>)	(Buchan <i>et al.</i> 2008)
(pRP1362)		
pPHY3665	<i>PAB1-GFP, EDC3-mCherry</i> (<i>CEN, URA3</i>)	(Buchan <i>et al.</i> 2011)
(pRP1657)		
pPHY3667	<i>PAB1-GFP, DCP2-mCherry</i> (<i>CEN, TRP1</i>)	(Buchan <i>et al.</i> 2011)
(pRP1660)		
pPHY3671	<i>EDC3-mCherry; PBP1-GFP</i> (<i>CEN, TRP1</i>)	(Swisher and Parker 2010)
(pRP1944)		
pPHY3685	<i>PAT1_{pro}-GFP-Pat1-SS</i> (<i>HIS3</i>)	This study
pPHY3691	<i>PAT1_{pro}-GFP-Pat1-AA</i> (<i>HIS3</i>)	This study
pPHY3693	<i>PAT1_{pro}-GFP-Pat1-EE</i> (<i>HIS3</i>)	This study
pPHY3702	<i>ADH2_{pro}-H2B-mCherry</i> (<i>CEN, LEU2</i>)	Dr. James Hopper
pOE79		

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