Analysis of P-Body Assembly in *Saccharomyces cerevisiae*

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Recent experiments have defined cytoplasmic foci, referred to as processing bodies (P-bodies), that contain untranslating mRNAs in conjunction with proteins involved in translation repression and mRNA decapping and degradation. However, the order of protein assembly into P-bodies and the interactions that promote P-body assembly are unknown. To gain insight into how yeast P-bodies assemble, we examined the P-body accumulation of Dcp1p, Dcp2p, Edc3p, Dhh1p, Pat1p, Lsm1p, Xrn1p, Ccr4p, and Pop2p in deletion mutants lacking one or more P-body component. These experiments revealed that Dcp2p and Pat1p are required for recruitment of Dcp1p and of the Lsm1-7p complex to P-bodies, respectively. We also demonstrate that P-body assembly is redundant and no single known component of P-bodies is required for P-body assembly, although both Dcp2p and Pat1p contribute to P-body assembly. In addition, our results indicate that Pat1p can be a nuclear-cytoplasmic shuttling protein and acts early in P-body assembly. In contrast, the Lsm1-7p complex appears to primarily function in a rate limiting step after P-body assembly in triggering decapping. Taken together, these results provide insight both into the function of individual proteins involved in mRNA degradation and the mechanisms by which yeast P-bodies assemble.

INTRODUCTION

The regulation of mRNA translation and degradation is an important aspect of the control of eukaryotic gene expression. In eukaryotes, two major decay pathways initiate with deadenylation of the 3' polyadenosine [poly(A)] tail, with the predominant cytoplasmic deadenylase being the Ccr4p/Pop2p/Not1-5p complex (reviewed in Meyer *et al.*, 2004; Parker and Song, 2004). Deadenylation can lead to 3' to 5' degradation, but primarily is followed by removal of the 5' end cap structure by the Dcp1p/Dcp2p decapping enzyme and 5' to 3' degradation of the body of the transcript by the exonuclease Xrn1p. Decapping is a key step of this process, because it precedes and permits the degradation of the body of the mRNA and represents the site of multiple control inputs.

The process of mRNA decapping and translation are mechanistically intertwined and appear to compete with each other, at least in yeast (reviewed in Collier and Parker, 2004). For example, decreasing translation initiation by a variety of means increases the rate of mRNA decapping (LaGrandeur and Parker, 1999; Muhlrud and Parker, 1999; Schwartz and Parker, 1999, 2000). Conversely, inhibition of translation elongation leads to a significant decrease in the rate of mRNA decapping (Beelman and Parker, 1994). Moreover, coimmunoprecipitation experiments suggested that before decapping, the mRNA must exit translation and assemble into a translationally repressed messenger ribonucleoprotein (mRNP) complex capable of decapping (Tharun and Parker, 2001). These results indicate that before decap-

ping mRNAs cease translation and assemble an mRNP containing the decapping machinery.

Additional evidence for a discrete population of nontranslating mRNPs has been that nontranslating mRNAs and the decapping machinery accumulate in discrete cytoplasmic foci called P-bodies (also referred as GW182 or Dcp bodies) (Bashkurov *et al.*, 1997; Ingelfinger *et al.*, 2002; Lykke-Andersen, 2002; van Dijk *et al.*, 2002; Sheth and Parker, 2003; Cougot *et al.*, 2004). P-bodies have now been observed in yeast, insect cells, nematodes, and mammalian cells and contain various proteins implicated in mRNA degradation, including the decapping enzyme (Dcp1p/Dcp2p), activators of decapping Dhh1p, Pat1p, Lsm1-7p, Edc3p, and the exonuclease Xrn1p (reviewed in Anderson and Kedersha, 2006; Eulalio *et al.*, 2007; Sheth and Parker, 2007). Consistent with the reciprocal relationship between mRNA translation and degradation, the assembly of P-bodies is in a dynamic competition with translation (Teixeira *et al.*, 2005; Sheth and Parker, 2007). Moreover, the mRNPs that accumulate in P-bodies have been suggested to be functionally involved in mRNA decapping (Sheth and Parker, 2003; Cougot *et al.*, 2004), mRNA storage (Brenques *et al.*, 2005; Bhattacharyya *et al.*, 2006), general translation repression (Holmes *et al.*, 2004; Collier and Parker, 2005), miRNA-mediated repression (Jakymiw *et al.*, 2005; Liu *et al.*, 2005; Pillai *et al.*, 2005), nonsense-mediated decay (Unterholzner and Izaurralde, 2004; Sheth and Parker, 2006), and possibly viral packaging (Beliakova-Bethell *et al.*, 2006). The existence of P-bodies as a discrete cytoplasmic compartment containing nontranslating mRNAs that can be either degraded or stored suggests that understanding the nature of the protein-protein and protein-RNA interactions that allow the assembly of both mRNPs containing the decapping machinery, as well as larger P-bodies visible by light microscopy, will be important in understanding the control of mRNA translation and degradation.

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Two aspects of the assembly of P-bodies have emerged. First, it has been shown that P-bodies require mRNA for their formation and integrity (Teixeira *et al.*, 2005). Second, coimmunoprecipitation and two-hybrid experiments have revealed a dense network of interactions between the components of the mRNA decapping and degradation machinery found in P-bodies (e.g., Hata *et al.*, 1998; Collier *et al.*, 2001; Ho *et al.*, 2002; Fenger-Gron *et al.*, 2005; Gavin *et al.*, 2006; Krogan *et al.*, 2006). However, the specific interactions that mediate the assembly of the translation repression and decapping machinery on mRNAs as well as the aggregation of individual mRNPs into larger P-bodies are largely unknown.

Several proteins have been described as affecting P-body assembly in yeast and mammalian cells. However, the competition between translation and P-body formation suggests that lack of a specific protein can affect P-body formation by either reducing the pool of nontranslating mRNAs, or by decreasing the aggregation of the nontranslating "P-body" mRNPs. For example, in mammalian cells, P-bodies are greatly reduced by knockdown of GW182, RCK/p54, RAP55, miRNA biogenesis in general, LSM4, Hedls/Ge-1, or 4E-T (Andrei *et al.*, 2005; Ferraiuolo *et al.*, 2005; Jakymiw *et al.*, 2005; Pauley *et al.*, 2006). However, for at least LSM4 knockdowns, P-bodies are restored when translation initiation is inhibited by arsenite (Kedersha *et al.*, 2005). Additionally, depletion of GW182 relieves translational repression by miRNAs (Jakymiw *et al.*, 2005; Liu *et al.*, 2005a; Meister *et al.*, 2005; Rehwinkel *et al.*, 2005; Behm-Ansmant *et al.*, 2006). Moreover, 4-ET and RCK/p54 are known to function in translational repression (Andrei *et al.*, 2005; Ferraiuolo *et al.*, 2005). These observations argue that LSM4p, and possibly these other factors as well, are not required for P-body assembly per se, but instead contribute to P-body formation in mammalian cells by increasing the pool of translationally repressed mRNAs. Similarly, in yeast, Dhh1p and Pat1p are required for global translation repression of mRNAs, targeting mRNAs for decapping, and promoting their assembly into P-bodies (Collier and Parker, 2005). Strains lacking Dhh1p and Pat1p are defective in translation repression in response to glucose deprivation and amino acid starvation, mRNA decapping, and consequently in P-body formation (Holmes *et al.*, 2004; Collier and Parker, 2005). In contrast, overexpression of Dhh1p or Pat1p results in inhibition of translation and induces P-body formation (Collier and Parker, 2005).

Given the limited understanding of how P-bodies assemble our goal in this work was to determine the requirement for numerous yeast proteins in P-body assembly and organization. To this end, we examined P-body formation and composition in strains defective in proteins known to accumulate in P-bodies. These experiments revealed specific dependencies in P-body assembly for individual proteins. In addition, this work argues that P-body assembly is redundant and no single known component of P-bodies is absolutely required for P-body assembly although Dcp2p and Pat1p can affect P-body assembly. Taken together, these results provide insight both into the function of individual proteins involved in mRNA degradation and the mechanisms by which yeast P-bodies assemble.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

The genotypes of all strains used in this study are listed in Table 1. All strains have *GAL1* upstream activating sequence-regulated *PGK1pG* and *MFA2pG* genes, as well as the *LEU2* gene, collectively termed *LEU2 pm*, integrated at

the *CUP1* locus (Hatfield *et al.*, 1996). Proteins were C terminal tagged with green fluorescent protein (GFP) following the PCR-based gene modification method described by Longtine *et al.* (1998). These fusion proteins include the full-length protein and are all at least partially functional (Sheth and Parker, 2003). Yeast crosses were carried out using standard laboratory procedures. Strains were grown on standard yeast extract/peptone medium (YP) supplemented with 2% Dextrose (Glu) as carbon source. Strains were grown at 30°C.

Preparation of Cells for Confocal Microscopy

Cells were grown to an OD₆₀₀ of 0.3–0.35 in YPD. For observation, cells were washed once and resuspended in synthetic medium (SC) supplemented with amino acids and Glu and immediately observed. For glucose depletion, cells were washed in YP without Glu and resuspended in the same medium for 10 more minutes before being collected. Cells were then washed with SC plus amino acids without Glu, resuspended in the same medium, and examined. For observation at high cell density, cells were grown in SC to an OD₆₀₀ of 1.0. Cells were then washed and resuspended in SC plus amino acids without Glu before observation. For *in vivo* 4',6-diamidino-2-phenylindole (DAPI, Sigma) staining, 1 ml of cells was harvested by centrifugation and resuspended in 150 μ l of medium containing 1.5 μ l of DAPI (1 mg/ml). After shaking for 30 min at 30°C, cells were again pelleted, resuspended in 150 μ l of media alone, and observed. Observations were made using Nikon PCM 2000 confocal microscope (Melville, NY) using a 100 $\times$ objective with a 3 $\times$ zoom using Compix software (Sewickley, PA). All images are a z-series compilation of 6–10 images in a stack.

RESULTS

General Strategy

To examine the role of various proteins in P-body assembly, we analyzed the contributions and dependencies to P-body formation of Dcp1p, Dcp2p, Edc3p, Dhh1p, Pat1p, Lsm1p, Xrn1p, Ccr4p, and Pop2p. For each protein a series of strains was constructed lacking one protein and individually containing a GFP fusion version of the other proteins. The GFP fusion proteins are integrated at the relevant genomic locus under the control of the endogenous promoter, include the full length protein C terminal tagged to GFP, and are all at least partially functional (Sheth and Parker, 2003). Each strain was then first examined microscopically for accumulation of each protein in P-bodies in yeast grown in glucose-supplemented rich medium during the midlog growth phase, wherein P-bodies are small in wild-type cells (Teixeira *et al.*, 2005). This provides an ideal condition to identify lesions that increase the size of P-bodies. In some cases, strains were also observed under the stress condition of glucose deprivation, which decreases translation initiation and leads to the accumulation of large P-bodies (Ashe *et al.*, 2000; Teixeira *et al.*, 2005). Because P-bodies are large in wild-type cells undergoing a response to glucose deprivation, this provides an opportunity to identify lesions that reduce or compromise P-body assembly. Finally, where relevant we examined the accumulation of some proteins in strains lacking two proteins. The important results are discussed below.

Defects in the Catalysis of Decapping or 5' to 3' Exonuclease Digestion Lead to the Accumulation of P-Bodies

Several observations demonstrated that blocking the catalytic events of decapping or 5' to 3' exonuclease digestion led to increased P-bodies. For example, strains lacking the 5' to 3' exonuclease Xrn1p, accumulated Dhh1p, Pat1p, Lsm1p, Dcp1p, Dcp2p, and Edc3p in P-bodies even in midlog cultures where yeast P-bodies are generally small (Figure 1B, III–VIII). Similarly, during midlog growth the *dcp1 Δ* strain showed high levels of accumulation of Dhh1p, Pat1p, Lsm1p, Dcp2p, Edc3p, and Xrn1p in P-bodies (Figure 1C, III–IX). These large P-bodies observed in *xrn1 Δ* or *dcp1 Δ* strains did not increase significantly when cells were subjected to glucose deprivation, by shifting the cells for 10 min

Table 1. Yeast strains

Strain	Genotype	Source/reference
yRP 1724	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP (NEO)	Sheth and Parker (2003)
yRP 1726	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP1-GFP (NEO)	Sheth and Parker (2003)
yRP 1727	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP2-GFP (NEO)	Sheth and Parker (2003)
yRP 1728	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG PAT1-GFP (NEO)	Sheth and Parker (2003)
yRP 1729	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG Lsm1-GFP (NEO)	Sheth and Parker (2003)
yRP 1730	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG XRN1-GFP (NEO)	Sheth and Parker (2003)
yRP 1731	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG CCR4-GFP (NEO)	Sheth and Parker (2003)
yRP 1736	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 DHH1-GFP (NEO)	Sheth and Parker (2003)
yRP 1737	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO DHH1-GFP (NEO)	Sheth and Parker (2003)
yRP 1738	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 DHH1-GFP (NEO)	Sheth and Parker (2003)
yRP 1739	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 DHH1-GFP (NEO)	Sheth and Parker (2003)
yRP 1740	MATα leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 DHH1-GFP (NEO)	Sheth and Parker (2003)
yRP 1758	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG EDC3-GFP (NEO)	Kshirsagar and Parker, 2004
yRP 1923	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 DCP2-GFP (NEO)	Teixeira <i>et al.</i> (2005)
yRP 1924	MATa leu2-3,112 trp1 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 Lsm1-GFP (NEO)	Teixeira <i>et al.</i> (2005)
yRP 1936	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 DCP2-GFP (NEO)	Bregues <i>et al.</i> (2005)
yRP 2213	MATα leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG POP2-GFP (NEO)	This study
yRP 2215	MATα leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO DHH1-GFP (NEO)	This study
yRP 2216	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 DHH1-GFP (NEO)	This study
yRP 2187	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO DCP2-GFP (NEO)	This study
yRP 2188	MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO DCP2-GFP (NEO)	This study
yRP 2183	MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 DCP2-GFP (NEO)	This study
yRP 2181	MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 DCP2-GFP (NEO)	This study
yRP 2182	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 DCP2-GFP (NEO)	This study
yRP 2218	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO DCP1-GFP (NEO)	This study
yRP 2219	MAT α leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO DCP1-GFP (NEO)	This study
yRP 2220	MAT α leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 DCP1-GFP (NEO)	This study
yRP 2221	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 DCP1-GFP (NEO)	This study
yRP 2222	MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 DCP1-GFP (NEO)	This study
yRP 2223	MATa leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 DCP1-GFP (NEO)	This study
yRP 2224	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 DCP1-GFP (NEO)	This study
yRP 2226	MATα leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO Lsm1-GFP (NEO)	This study
yRP 2227	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO Lsm1-GFP (NEO)	This study
yRP 2228	MATα leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 Lsm1-GFP (NEO)	This study
yRP 2229	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 LSM1-GFP (NEO)	This study
yRP 2230	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 LSM1-GFP (NEO)	This study
yRP 2231	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 LSM1-GFP (NEO)	This study
yRP 2233	MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO PAT1-GFP (NEO)	This study
yRP 2234	MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO PAT1-GFP (NEO)	This study
yRP 2235	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 PAT1-GFP (NEO)	This study
yRP 2236	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 PAT1-GFP (NEO)	This study
yRP 2237	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 PAT1-GFP (NEO)	This study
yRP 2238	MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 PAT1-GFP (NEO)	This study
yRP 2239	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 PAT1-GFP (NEO)	This study
yRP 2241	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO XRN1-GFP (NEO)	This study
yRP 2242	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO XRN1-GFP (NEO)	This study
yRP 2243	MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 XRN1-GFP (NEO)	This study
yRP 2244	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 XRN1-GFP (NEO)	This study
yRP 2245	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 XRN1-GFP (NEO)	This study
yRP 2246	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 XRN1-GFP (NEO)	This study
yRP 2247	MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 XRN1-GFP (NEO)	This study
yRP 2249	MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO EDC3-GFP (NEO)	This study

Continued

Table 1. *Continued*

Strain	Genotype	Source/reference
yRP 2250	<i>MATa leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO EDC3-GFP (NEO)</i>	This study
yRP 2251	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 EDC3-GFP (NEO)</i>	This study
yRP 2252	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 EDC3-GFP (NEO)</i>	This study
yRP 2253	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 EDC3-GFP (NEO)</i>	This study
yRP 2254	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 EDC3-GFP (NEO)</i>	This study
yRP 2255	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 EDC3-GFP (NEO)</i>	This study
yRP 2256	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 EDC3-GFP (NEO)</i>	This study
yRP 2257	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO CCR4-GFP (NEO)</i>	This study
yRP 2258	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 CCR4-GFP (NEO)</i>	This study
yRP 2259	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 CCR4-GFP (NEO)</i>	This study
yRP 2260	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 CCR4-GFP (NEO)</i>	This study
yRP 2261	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 CCR4-GFP (NEO)</i>	This study
yRP 2262	<i>MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 CCR4-GFP (NEO)</i>	This study
yRP 2263	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 CCR4-GFP (NEO)</i>	This study
yRP 2265	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO POP2-GFP (NEO)</i>	This study
yRP 2266	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 POP2-GFP (NEO)</i>	This study
yRP 2267	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 POP2-GFP (NEO)</i>	This study
yRP 2268	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 POP2-GFP (NEO)</i>	This study
yRP 2269	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 POP2-GFP (NEO)</i>	This study
yRP 2270	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 POP2-GFP (NEO)</i>	This study
yRP 2271	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 POP2-GFP (NEO)</i>	This study
yRP 2273	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 xrn1::URA3 DCP1-GFP (NEO)</i>	This study
yRP 2277	<i>MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 dcp2::TRP1 PAT1-GFP (NEO)</i>	This study
yRP 2279	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 dcp2::TRP1 DHH1-GFP (NEO)</i>	This study
yRP 2280	<i>MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 lsm1::TRP1 PAT1-GFP (NEO)</i>	This study
yRP 2283	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 DCP1-GFP (NEO)</i>	This study
yRP 2284	<i>MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 DCP2-GFP (NEO)</i>	This study
yRP 2286	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 XRN1-GFP (NEO)</i>	This study
yRP 2289	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 EDC3-GFP (NEO)</i>	This study
yRP 2290	<i>MATa leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 DCP1-GFP (NEO)</i>	This study
yRP 2292	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 DCP2-GFP (NEO)</i>	This study
yRP 2294	<i>MAT α leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 XRN1-GFP (NEO)</i>	This study
yRP 2295	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 EDC3-GFP (NEO)</i>	This study
yRP 2297	<i>MAT α leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 xrn1::URA3 CCR4-GFP (NEO)</i>	This study

Continued

Table 1. *Continued*

Strain	Genotype	Source/reference
yRP 2299	<i>MATa leu2-3,112 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 xrn1::URA3 POP2-GFP (NEO)</i>	This study
yRP 2300	<i>MAT α leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 xrn1::URA3 CCR4-GFP (NEO)</i>	This study
yRP 2302	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 xrn1::URA3 POP2-GFP (NEO)</i>	This study
yRP 2303	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 xrn1::URA3 CCR4-GFP (NEO)</i>	This study
yRP 2304	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 xrn1::URA3 POP2-GFP (NEO)</i>	This study
yRP 2382	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 xrn1::URA3 PAT1-GFP (NEO)</i>	This study

to medium lacking glucose (data not shown and Figure 2B), which is consistent with the observation that *xrn1Δ* and *dcp1Δ* strains are unable to repress translation during glucose deprivation (Holmes *et al.*, 2004; Collier and Parker, 2005).

Because the *xrn1Δ* blocks 5' to 3' degradation and the *dcp1Δ* blocks decapping, these observations indicate that defects in the catalytic steps of decapping or 5' to 3' degradation lead to increased P-bodies. Moreover, because all the proteins examined accumulated in P-bodies under these conditions, it argues that no other known component of P-bodies is strictly dependent on Xrn1p or Dcp1p for accumulation in P-bodies. This is consistent and extends earlier work showing an accumulation of Dhh1p in P-bodies in *xrn1Δ* or *dcp1Δ* strains (Sheth and Parker, 2003). Moreover, these observations are consistent with the observation that RNAi-mediated knockdown of the decapping enzyme Dcp2p in mammalian cells leads to an accumulation of P-bodies as assessed by the human ortholog of Dhh1p, RCK/p54, as well as hCcr4p, and hLsm1p (Andrei *et al.*, 2005).

Interestingly, Ccr4p and Pop2p were also clearly observed to accumulate in P-bodies in the *xrn1Δ*, *dcp1Δ*, and *dcp2Δ* mutant strains (Figure 1, B, I and II, C, I and II, and D, I and II). Similarly, we observed that Ccr4p and Pop2p accumulated at low levels in P-bodies during glucose deprivation (see below, Figure 8). This indicates that Ccr4p and Pop2p can accumulate in yeast P-bodies. This is consistent with earlier observations that other components of the Ccr4p/Pop2p/Not complex, the Not1-5 proteins, are detected in P-bodies in *dcp1Δ* strains (Muhlrad and Parker, 2005) and that yeast Ccr4p can be observed in small foci during a water stress response (Sheth and Parker, 2003). In addition, mammalian and *Drosophila* Ccr4p also accumulate in P-bodies (Cougot *et al.*, 2004; Temme *et al.*, 2004; Andrei *et al.*, 2005). These results indicate that the Ccr4p/Pop2p/Not1-5p complex accumulates in P-bodies in yeast as well as other eukaryotes. However, the limited amount of Ccr4p-GFP and Pop2p-GFP seen in P-bodies compared with other factors suggests that these proteins are present at reduced stoichiometry.

Dcp2p Is Required for Dcp1p Accumulation in P-Bodies

Strains lacking Dcp2p also showed increased P-bodies in midlog cultures with accumulations of Ccr4p, Pop2p, Dhh1p, Pat1p, Lsm1p, Edc3p, and Xrn1p in P-bodies (Figure 1D, I–V and VIII–IX), but with some differences from *dcp1Δ* or *xrn1Δ* strains. First, despite the increased P-bodies in

dcp2Δ strains as judged by other protein components, Dcp1p-GFP was distributed throughout the cell and completely absent from P-bodies (Figure 1D, VI). Moreover, Western analysis showed that Dcp1p-GFP was still efficiently expressed in the *dcp2Δ* strain (data not shown). Because *dcp1Δ* and *dcp2Δ* strains have identical and complete blocks to decapping (Beelman *et al.*, 1996; Dunkley and Parker, 1999) and all other proteins accumulate in P-bodies in the *dcp2Δ* strain, this observation argues that Dcp2p is required to recruit Dcp1p to P-bodies.

Two additional observations confirm Dcp2p is required for Dcp1p recruitment to P-bodies. First, even when *dcp2Δ* strains are undergoing a response to glucose deprivation, which increases Dcp1p-GFP accumulation in P-bodies in wild-type strains, Dcp1p-GFP is completely absent from P-bodies in a *dcp2Δ* strain (Figure 2A, IV). In contrast, Dcp2p-GFP accumulates in P-bodies independent of Dcp1p with or without glucose deprivation conditions (Figure 2B, II and IV). Moreover, other P-body markers still accumulate in P-bodies in a *dcp2Δ* with or without glucose deprivation conditions (Figure 1D and data not shown). Second, although Dcp1p-GFP accumulates in P-bodies to high levels in the *xrn1Δ* strain (Figure 2C, I), this accumulation is completely lost in the *xrn1Δ dcp2Δ* double mutant strain (Figure 2C, II). The requirement of Dcp2p for Dcp1p recruitment to P-bodies is consistent with the direct physical interaction between Dcp1p and Dcp2p in yeast (Steiger *et al.*, 2003; She *et al.*, 2004, 2006) and with the observation that specific point mutations in residues of Dcp2p that disrupt the physical interaction between Dcp2p and Dcp1p prevent Dcp1p from accumulating in P-bodies (Decker and Parker, unpublished observation). These results indicate that Dcp1p is recruited to the P-body through interactions with Dcp2p.

Dcp2p Has an Additional Role in P-Body Assembly or Persistence

A second interesting result observed with the *dcp2Δ* strain was that P-bodies were reproducibly smaller than in *dcp1Δ* or *xrn1Δ* strains (Figure 1, B–D). This can be easily seen by comparing the accumulation of Dhh1p, Pat1p, Lsm1p, or Xrn1p in *dcp1Δ* strains compared with *dcp2Δ* strains (compare Figure 1C, III–V and IX with 1D, III–V and IX). Because both *dcp1Δ* and *dcp2Δ* strains show absolute blocks to decapping (Beelman *et al.*, 1996; Dunkley and Parker, 1999), this difference in P-body size must reflect a difference in the function of Dcp1p or Dcp2p in the formation of P-bodies independent of their catalytic role in decapping. Interestingly, the smaller size of P-bodies in the *dcp2Δ* strain relative to the *dcp1Δ* strain was not as pronounced

Figure 1. Blocks in the catalytic steps of decapping or 5' to 3' degradation lead to increased P-bodies. (A) Wild-type (WT), (B) *xrn1Δ*, (C) *dcp1Δ* and (D) *dcp2Δ* cells expressing a GFP-tagged version of Ccr4p (I), Pop2p (II), Dhh1p (III), Pat1p (IV), Lsm1p (V), Dcp1p (VI), Dcp2p (VII), Edc3p (VIII), and Xrn1p (IX) are shown. All images are shown at the same scale and a 3-μm scale bar is shown in the lower right panel.

when Edc3p was examined (Figure 1D, VIII), suggesting some difference in how Edc3p's accumulation in P-bodies is affected compared with other factors.

Two possible models can explain the difference between P-body size in the *dcp1Δ* and *dcp2Δ* strains, which we distinguished by the analysis of a *dcp1Δ dcp2Δ* double mutant. First, it could be that Dcp1p is also an inhibitor of P-body formation and that P-bodies are larger in the *dcp1Δ* strain compared with the *dcp2Δ* strain because of the loss of the

Figure 2. Dcp2p is required for Dcp1p recruitment to P-bodies. (A) Distribution of GFP-tagged version of Dcp1p in WT (I and III) and *dcp2Δ* (II and IV) cells is shown. (B) Distribution of GFP-tagged version of Dcp2p in WT (I and III) and *dcp1Δ* (II and IV) cells is shown. Pictures of cells before glucose deprivation (GLU) and after 10 min of glucose deprivation (10 min NO GLU) treatment are shown. (C) Distribution of a GFP-tagged version of Dcp1p in *xrn1Δ* (I) and *xrn1Δ dcp2Δ* (II) cells is shown. All images are shown at the same scale and a 3-μm scale bar is shown in the lower right panel.

Dcp1p inhibitory role on P-body formation. In this model, a *dcp1Δ dcp2Δ* double mutant is predicted to have large P-bodies similar to the *dcp1Δ* strain. Alternatively, Dcp2p might have an additional role in the assembly or maintenance of P-bodies, possibly through additional protein-protein interactions. In this model, a *dcp1Δ dcp2Δ* strain is predicted to be phenotypically similar to a *dcp2Δ* strain.

To distinguish these models, we examined the accumulation of Dhh1p and Pat1p in *dcp1Δ dcp2Δ* double mutants

Figure 3. Dcp2p plays a role in the formation of P-bodies. Shown are *dcp1Δ* (left panel), *dcp2Δ* (middle panel) and *dcp1Δ dcp2Δ* (right panel) cells expressing a GFP-tagged version of Dhh1p (I, III, and V) and Pat1p (II, IV, and VI). All images are shown at the same scale and a 3-μm scale bar is shown in the lower right panel.

strains as compared with *dcp1Δ* and *dcp2Δ* single mutant strains. Strikingly, we observed that both Dhh1p-GFP and Pat1p-GFP accumulated in P-bodies in the *dcp1Δ dcp2Δ* double mutant to the same extent seen in the *dcp2Δ* single mutant (Figure 3, V and VI). This provides evidence that Dcp2p has some role in the assembly or maintenance of P-bodies. A likely possibility is that the multiple interactions that Dcp2p has with other components of P-bodies contribute to the assembly of the P-body mRNP or to the interactions that allow P-body aggregation into visible structures in the light microscope.

Minor Affects of *Ccr4p* and *Pop2p* on P-Body Assembly

We also examined how *Ccr4p* and *Pop2p* affect the formation and composition of P-bodies in both midlog cultures and in response to glucose deprivation. We observed that *ccr4Δ* led to a small, but clear, decrease in the accumulation of Dcp2p-GFP and Edc3p-GFP in P-bodies in midlog cultures (Figure 4B, V and VI). Furthermore, we observed *pop2Δ* led to a small decrease in the accumulation Dcp1p-GFP and Dcp2p-GFP in P-bodies (Figure 4C, IV and V). This is consistent with the *ccr4Δ* and *pop2Δ* affecting deadenylation (Tucker *et al.*, 2001) and previous work arguing that in midlog growth phase deadenylated mRNAs preferentially associate with P-body components such as Lsm1p (Tharun and Parker, 2001). In midlog cultures, the *ccr4Δ* or *pop2Δ* strains did not show a significant difference in the concentration of Dhh1p-GFP, Pat1p-GFP, Lsm1p-GFP, or Xrn1p-GFP in P-bodies (Figures 4B, I–III and VII, and 4C, I–III and VII). However, because it is difficult to observe these proteins in P-bodies in midlog growth, we are unable to determine if the *ccr4Δ* or *pop2Δ* affects their accumulation in P-bodies under these conditions.

In the *ccr4Δ* strains in response to glucose deprivation stress, where decreases in P-bodies are more easily identified, Pat1p-GFP and Edc3p-GFP accumulated in P-bodies in a manner similar to wild-type cells (Figure 4B, IX and XIII), whereas Dhh1p-GFP, Lsm1p-GFP, Dcp1p-GFP, Dcp2p-GFP, and Xrn1p-GFP accumulated but less pronouncedly compared with wild-type cells (Figure 4B, VIII, X–XII, and XIV). This slightly reduced accumulation of Dhh1p in P-bodies in these mutant may be due to the reduced levels of protein expression in *ccr4Δ* strains, as has been shown to be the case for Dhh1p (Sheth and Parker, 2003). In contrast, after glucose deprivation in a *pop2Δ* strain, Dhh1p, Pat1p, Lsm1p, Dcp1p, Dcp2p, Edc3p, and Xrn1p accumulated in P-bodies in a

manner similar to wild-type cells (Figure 4C, VIII–XIV). Taken generally, these results indicate that during glucose deprivation, neither *Ccr4p* nor *Pop2p* has a large impact on P-body assembly. This is consistent with recent results indicating that poly(A)⁺ mRNAs are translationally repressed during glucose deprivation and accumulate in P-bodies (Brenques and Parker, in press).

Dhh1p, Pat1p, and Lsm1p Have Different Effects on P-Body Assembly and Composition

The proteins Dhh1p, Pat1p, and Lsm1p are all found in P-bodies and function as general decapping activators (reviewed in Collier and Parker, 2004). To determine the roles of these proteins on P-body assembly we examined the accumulation of GFP tagged version of Dcp1p, Dcp2p, Edc3p, Xrn1p, Dhh1p, Pat1p, and Lsm1p in *lsm1Δ*, *pat1Δ* or *dhh1Δ* strains. Because results from mammalian cells indicate that the Lsm1-7p complex must be intact to localize to P-bodies (Ingelfinger *et al.*, 2002), and *lsm1Δ* is sufficient to fully inactivate this complex with respect to decapping (Tharun *et al.*, 2000), the *lsm1Δ* should be sufficient to address the contribution of the entire Lsm1p-7p complex to P-body formation. In addition, we examined both the affect of the deletions on P-bodies during midlog growth, where P-bodies are small and increases are easily seen, and during glucose deprivation, where P-bodies are large, and decreases in P-body assembly are more easily observed. These results are presented in Figure 5 and important observations are discussed below.

One important observation was that *lsm1Δ* strains showed increased accumulation of Dcp1p, Dcp2p, Edc3p, Xrn1p, and Dhh1p in P-bodies even in midlog growth cells (Figure 5B, I–V). Pat1p did not accumulate in P-bodies in midlog cultures in the *lsm1Δ* strain, perhaps because of an effect of the *lsm1Δ* on the nuclear-cytoplasmic distribution of Pat1p (Figure 5B, VI; see below). The accumulation of all P-body components except Pat1p in P-bodies in *lsm1Δ* strain is consistent with the decapping defect seen in *lsm1Δ* strains (Boeck *et al.*, 1998; Bouveret *et al.*, 2000; Tharun *et al.*, 2000). Moreover, this observation argues that the rate-limiting step in decapping in *lsm1Δ* strains is after mRNAs assembly into an mRNP that can accumulate in P-bodies. This identifies an important function of the Lsm1-7p complex in triggering decapping after translation repression and accumulation of the mRNA in the P-body mRNP. Consistent with the partial block to mRNA decapping in the *lsm1Δ* strain, the accumulation of P-bodies is not as strong in the *lsm1Δ* strain compared with the *dcp1Δ* strain, where decapping is absolutely blocked (compare Figure 1C with 5B). Moreover, in *lsm1Δ* strains, P-bodies still increased during glucose deprivation compared with nonstressed *lsm1Δ* cells (Figure 5B, VIII–XIII), which suggests that *lsm1Δ* strains do not have a maximal accumulation of P-bodies.

In contrast to the accumulation of P-bodies in the *lsm1Δ* strain, in general the *pat1Δ* and *dhh1Δ* strains showed either minor or no reduction of P-bodies during midlog growth, respectively, as assessed by Dcp1p, Dcp2p, Edc3p, and Xrn1p (Figure 5, C, I–IV, and D, I–IV). Interestingly, we did observe a minor increase in P-bodies during midlog growth for *dhh1Δ* and *pat1Δ* cells in a subpopulation of the cells (Figure 5). This suggests that Dhh1p and/or Pat1p can affect the rate of mRNA decay within P-bodies or the rate of mRNA exit from P-bodies in at least some cells. However, during glucose deprivation the *pat1Δ* strain and to a more modest effect the *dhh1Δ* strain showed a reduction in the amount of P-bodies formed (compare Figure 5A, VIII–XIV, with 5C, VIII–XIV and 5D, VIII–XIV). This is consistent with earlier results that *dhh1Δ* and *pat1Δ* strains are partially

Figure 4. Defects in the mRNA deadenylases Ccr4p and Pop2p do not significantly affect P-body formation and composition. (A) WT, (B) *ccr4Δ* and (C) *pop2Δ* cells expressing a GFP-tagged version of Dhh1p (I and VIII), Pat1p (II and IX), Lsm1p (III and X), Dcp1p (IV and XI), Dcp2p (V and XII), Edc3p (VI and XIII), and Xrn1p (VII and XIV). Pictures of cells before glucose deprivation (GLU) and after 10 min of glucose deprivation (10 min NO GLU) treatment are shown. All images are shown at the same scale and a 3- μ m scale bar is shown in the lower right panel.

defective in translation repression during glucose deprivation (Holmes *et al.*, 2004; Collier and Parker, 2005). Because both *dhh1Δ* and *pat1Δ* appear to affect translation repression similarly during glucose deprivation (Holmes *et al.*, 2004; Collier and Parker, 2005), this suggests Pat1p may have a more significant role in P-body assembly and aggregation than Dhh1p (see below and *Discussion*).

Pat1p Is Required for Recruitment of Lsm1p to P-Bodies

A second important result from these comparisons was that strains lacking Pat1p failed to accumulate Lsm1p in P-bodies with or without glucose repression (Figure 5C, VII and XIV).

This argued that Pat1p is required for Lsm1-7p to be recruited to P-bodies. However, because the loss of Pat1p can affect the size of P-bodies during glucose repression, we verified this result under conditions where P-bodies were large even in a *pat1Δ* strain. To do this, we examined whether Lsm1p accumulates in P-bodies in a *pat1Δ* strain at high cell density, where P-bodies are large (Teixeira *et al.*, 2005). Consistent with Pat1p being required for Lsm1p to enter into P-bodies, we observed that *pat1Δ* cells did not accumulate Lsm1p in P-bodies at high OD (Figure 6A, II). These observations demonstrate that Pat1p is required to recruit the Lsm1-7p complex to P-bodies.

Figure 5. Dhh1p, Pat1p, and Lsm1p have different affects in P-body assembly and composition. (A) WT, (B) *lsm1Δ*, (C) *pat1Δ*, and (D) *dhh1Δ* cells expressing a GFP-tagged version of Dcp1p (I and VIII), Dcp2p (II and IX), Edc3p (III and X), Xrn1p (IV and XI), Dhh1p (V and XII), Pat1p (VI and XIII), and Lsm1p (VII and XIV). Pictures of cells before glucose deprivation (GLU) and after 10 min of glucose deprivation (10 min NO GLU) treatment are shown. All images are shown at the same scale and a 3- μ m scale bar is shown in the lower right panel.

Lsm1p Affect the Nuclear-Cytoplasmic Distribution of Pat1p

A third observation from these comparisons was that in the *lsm1Δ* strain Pat1p accumulated in a large circular region of the cell that was similar in size the nucleus (Figure 5B, VI). Subsequent DNA staining with DAPI revealed that this circular region of Pat1p concentration colocalized with the nucleus (Figure 6B). This observation indicates that Lsm1p, and presumably the whole Lsm1-7p complex, plays a role in decreasing the concentration of Pat1p in the nucleus and increasing the cytoplasmic concentration of Pat1p. These results suggest that Pat1p is a likely nuclear-cytoplasmic shuttling protein. This conclusion is consistent with earlier work showing that Pat1p shows physical interactions with the mRNA export factor Crm1p (Jensen *et al.*, 2000).

Despite the nuclear concentration of Pat1p in the *lsm1Δ* mutant, three observations suggest that some Pat1p is still in the cytoplasm and is functioning in decapping. First,

even in the *lsm1Δ* strain during midlog growth, some Pat1p-GFP was observed distributed in the cytoplasm (Figure 5B, VI). Second, when *lsm1Δ* cells were glucose deprived some Pat1p was seen to accumulate in P-bodies (Figure 5B, XIII). Third, when P-bodies accumulate because of *dcp1Δ*, Pat1p-GFP is still present in P-bodies, even in the absence of Lsm1p, albeit at a reduced level compared with a *dcp1Δ* alone (Figure 6C, III). These observations indicate that Lsm1p is not absolutely required for Pat1p to enter P-bodies, although it clearly appears to decrease the amount of Pat1p seen in P-bodies, at least in part by affecting the distribution of Pat1p between the nucleus and the cytoplasm. Interestingly, although P-bodies accumulate due to *xrn1Δ*, Pat1p-GFP was no longer observed in P-bodies in the *xrn1Δ lsm1Δ* double mutant (Figure 6C, IV). This further suggests the idea that Pat1p/Lsm1-7p complex/Xrn1p form a functionally important complex, which is consistent with their previous biochemical copurification (Bouveret *et al.*, 2000).

Figure 6. Pat1p is required for recruitment of Lsm1p to P-bodies but Lsm1p is not required for Pat1p targeting to P-bodies. (A) WT (I) and *pat1Δ* (II) cells expressing GFP-tagged version of Lsm1p grown to high cell density of 1.0 OD₆₀₀ unit (OD 1) as indicated at the top of the figure. (B) Distribution of GFP-tagged version of Pat1p in *lsm1Δ* (right panel) cells. The nucleus is visible by DAPI staining of nuclear DNA (left panel). Note that the small foci seen with DAPI staining are due to mitochondrial DNA. (C) Distribution of GFP-tagged version of Pat1p in *dcp1Δ* (I), *dcp1Δ lsm1Δ* (III), *xrn1Δ* (II), and *xrn1Δ lsm1Δ* (IV) cells is shown. All images are shown at the same scale and a 3-μm scale bar is shown in the lower right panel.

Double Mutants Indicate Pat1p Functions Before Lsm1p in mRNA Decapping

The above observation suggest a model for P-body assembly wherein Pat1p acts early in the process and is required to recruit the Lsm1-7p complex, which functions later to enhance decapping rate. A prediction of this model is that a *pat1Δ lsm1Δ* double mutant should show the phenotype of the *pat1Δ* single mutant strain and not the *lsm1Δ* strain. To test this prediction, we examined the accumulation of GFP tagged Dcp1, Dcp2, Edc3p and Xrn1p in *pat1Δ lsm1Δ* strains. For comparison, we also examined the same proteins in a *dhh1Δ lsm1Δ* strain.

A clear and significant observation was that during mid-log growth the *pat1Δ lsm1Δ* strain showed little P-body accumulation of all four proteins similar to a *pat1Δ* strain (Figure 7, E–H) and unlike a *lsm1Δ* strain where P-bodies are increased (Figure 5B). This result provides additional evidence that Pat1p acts upstream of Lsm1p in the process of

Figure 7. Pat1p functions before Lsm1p in P-body assembly. Distribution of GFP-tagged version of Dcp1p (A and E), Dcp2p (B and F), Edc3p (C and G), and Xrn1p (D and H) in *dhh1Δ lsm1Δ* (left panel) and *pat1Δ lsm1Δ* (right panel) cells are shown. All images are shown at the same scale and a 3-μm scale bar is shown in the lower right panel.

P-body assembly and mRNA decapping. In contrast, we observed that *dhh1Δ lsm1Δ* leads to increased concentration of Dcp1p, Dcp2p, Edc3p, and Xrn1p in P-bodies, because P-bodies increased in size and number compared with both wild-type cells and *dhh1Δ* or *lsm1Δ* single-mutant strains (Figure 7, A–D). This suggests that Dhh1p, like Lsm1p, can also play a role in the actual rate of decapping after assembly of the mRNP capable of aggregation in P-bodies.

Dhh1p, Pat1p, and Lsm1p Affect the Recruitment of Ccr4p and Pop2p to P-Bodies

In examining the function of Dhh1p, Pat1p, and Lsm1p in P-body assembly we also examined how they affected the recruitment of Ccr4p and Pop2p, subunits of the major cytoplasmic deadenylase, to P-bodies during glucose deprivation (Figure 8A). We observed that during glucose deprivation the amount of Ccr4p or Pop2p seen in P-bodies declined in *dhh1Δ*, *pat1Δ* or *lsm1Δ* strains compared with a wild-type strain, where small but clear accumulation of Ccr4p and Pop2p in P-bodies could be seen during glucose deprivation (Figure 8A, III, IV, VII, VIII, XI, XII, XV, and XVI). This suggests that the accumulation of Ccr4p and Pop2p in P-bodies requires Dhh1p, Pat1p, and Lsm1p. However, a limitation of this analysis is that the accumulation of Ccr4p and Pop2p in P-bodies in the wild-type strain is relatively low; thus it is difficult to make a robust conclusion from this experiment alone.

Figure 8. Dhh1p, Pat1p, and Lsm1p are required to the recruitment of Ccr4p and Pop2p to P-bodies. (A) Distribution of GFP-tagged version of Ccr4p (top panel) and Pop2p (bottom panel) in WT (I–IV), *dhh1Δ* (V–VIII), *pat1Δ* (IX–XII), and *lsm1Δ* (XIII–XVI) cells. Pictures of cells before glucose deprivation (GLU) and after 10 min of glucose deprivation (10 min NO GLU) treatment are shown. (B) Shown are *xrn1Δ* (I and II), *xrn1Δ dhh1Δ* (III and IV), *xrn1Δ pat1Δ* (V and VI), and *xrn1Δ lsm1Δ* (VII and VIII) cells expressing a GFP-tagged version of Ccr4p (top panel) and Pop2p (bottom panel). All images are shown at the same scale and a 3- μ m scale bar is shown in the bottom right panel.

To further support this observation we asked how *dhh1Δ*, *pat1Δ* or *lsm1Δ* affected the accumulation of Ccr4p or Pop2p in P-bodies in combination with *xrn1Δ* where the accumulation of Ccr4p and Pop2p in P-bodies is easily observed (Figure 1). We observed that the *dhh1Δ*, *pat1Δ* or *lsm1Δ* all reduced the accumulation of Ccr4p and Pop2p in P-bodies compared with *xrn1Δ* single mutant (Figure 8B, III–VIII). This is consistent with the observations during glucose deprivation and argues that the efficient recruitment of Ccr4p and Pop2p to P-bodies requires Dhh1p, Pat1p, and the Lsm1-7p complex.

DISCUSSION

Multiple Proteins Function in Yeast P-Body Assembly

This work begins to reveal the process by which P-bodies assemble and the possible roles of individual proteins within that assembly. One principle that emerges is that P-body assembly appears to be redundant, with no single protein being absolutely required for formation of P-bodies.

One protein that can affect P-body formation was Dcp2p. This was surprising because strains lacking Dcp2p show a complete block to decapping and therefore, like *dcp1Δ* strains, would be expected to accumulate very large P-bodies. However, *dcp2Δ* strains had reproducibly smaller P-bodies than *dcp1Δ* or *xrn1Δ* strains, although *dcp2Δ* strains showed enhanced P-bodies compared with wild-type strains (Figure 1). Moreover, because *dcp2Δ* strains have smaller P-bodies than a *dcp1Δ* strain as assessed by seven different proteins, it strongly argues that the difference is in the actual formation of P-bodies themselves and not simply a change in P-body composition. Finally, because *dcp2Δ dcp1Δ* strains are similar to *dcp2Δ* single mutant strains alone when assessed for P-body formation, it argues that Dcp2p is required for optimal P-body accumulation (Figure 3). Because Dcp2p shows

direct physical interactions with Dcp1p, Dhh1p, Edc3, and possibly Pat1p (Steiger *et al.*, 2003; She *et al.*, 2004; Decker, Pilkington, and Parker, unpublished observation), the simplest possibility is that the multiple protein–protein interactions that Dcp2p participates in either stabilizes the P-body monomer mRNP or helps to provide cross-linking interactions between individual mRNPs, thereby contributing to aggregation of multiple mRNPs into a larger P-body. Thus, Dcp2p is not only the catalytic subunit of the decapping enzyme, but also has a second role in promoting P-body assembly or maintenance.

Several observations argue that Pat1p is likely to affect P-body formation in multiple manners. First, because Pat1p overexpression leads to inhibition of translation (Coller and Parker, 2005), one possible role of Pat1p is likely to be to inhibit translation initiation in some manner, thereby contributing to P-body formation by affecting the size of the pool of nontranslating mRNA, which can then aggregate into P-bodies. Second, because P-bodies are reduced in *pat1Δ* strains, even during glucose deprivation, where translation is markedly repressed even in a *pat1Δ* strain, it suggests that Pat1p also plays a role in assembly of P-bodies (Figure 5). The role of Pat1p in assembly is again likely to be through multiple protein–protein interactions that promote formation of the P-body monomer mRNP, and cross-linking between individual mRNPs. Finally, we suggest that Pat1p has a final role in promoting mRNA decapping after assembly of the P-body mRNP. This is suggested by both the requirement for Lsm1p for efficient decapping after P-body assembly, and the requirement for Pat1p for assembly of Lsm1p into P-bodies.

Specific Dependencies in P-Body Assembly

Our analysis of yeast P-bodies suggests that there are some clear dependencies in the assembly of specific components. For example, the assembly of Dcp1p in P-bodies is depen-

dent on Dcp2p (Figures 1 and 2). This is consistent with the direct physical interaction between these components from yeast (Steiger *et al.*, 2003; She *et al.*, 2004, 2006).

A second clear dependency is that Pat1p is required for the recruitment of Lsm1p to P-bodies (Figures 5 and 6). Because Lsm1p is a component of the Lsm1-7p complex and Lsm1-7p complex formation is required for its recruitment to P-bodies and for the Lsm1-7 complex to function in mRNA decay (Bouveret *et al.*, 2000; Tharun *et al.*, 2000, 2005; Ingelfinger *et al.*, 2002), this suggests that Pat1p is required for the recruitment of the entire Lsm1-7p complex into P-bodies. This is consistent with the physical interactions between Pat1p and the Lsm1-7p complex (Bouveret *et al.*, 2000; Tharun *et al.*, 2000).

Pat1p Acts Early in Translation Repression and mRNA Decapping While Recruiting the Lsm1-7p Complex To Trigger Decapping in a Late Step

Our results also argue that the Lsm1-7p complex has an important role after P-body assembly in the actual triggering of decapping. The key observation is that *lsm1Δ* strains show an accumulation of P-bodies, as judged by the subcellular distribution of Dcp1p, Dcp2p, Edc3p, Xrn1p, and Dhh1p (Figure 5). This is consistent with the observed defect in mRNA decapping in the *lsm1Δ* strains (Boeck *et al.*, 1998; Bouveret *et al.*, 2000; Tharun *et al.*, 2000) and indicates that the decapping mRNP can assemble and aggregate in P-bodies in the absence of the Lsm1p, but is then deficient at actual decapping, and hence a pool of mRNAs accumulates in P-bodies. It should be noted that these results do not rule out a potential earlier role of the Lsm1-7p complex in translation repression or assembly of the monomer unit, they just reveal what becomes the rate-limiting step in the absence of Lsm1p.

An important implication of the function of the Lsm1-7p complex in triggering decapping after P-body formation is that this role of the Lsm1-7p complex could be used to alter the fate of mRNAs within P-bodies. Specifically, individual mRNAs that are destined for storage could simply be packaged into an mRNP that is lacking the Lsm1-7p complex and thereby have a reduced rate of mRNA decapping. Similarly, in certain biological contexts where mRNA storage is important, the Lsm1-7p complex may be lacking from P-bodies and thereby converts the fate of mRNAs within P-bodies into storage.

Our data also indicate that Pat1p is required for Lsm1p to assemble into P-bodies. The key observation is that P-bodies formed in *pat1Δ* strains lack Lsm1p, even under glucose deprivation (Figure 5) or high OD (Figure 6). This is also consistent with earlier work showing that the coimmunoprecipitation of Lsm1p with Dcp2p is greatly reduced in a *pat1Δ* strain (Tharun and Parker, 2001). The simplest model is that Pat1p directly interacts with Lsm1-7p complex and recruits it to P-bodies, although one anticipates that Pat1p might also facilitate the interaction of the Lsm1-7p complex with other components of P-bodies.

The phenotypes of *lsm1Δ* and *pat1Δ* strains suggest a model for these proteins function wherein Pat1p participates early in translation repression and the movement of mRNAs into P-bodies and the Lsm1-7p complex has a rate limiting role at a later stage to trigger decapping. The early role of Pat1p is suggested by the observations that overexpression of Pat1p inhibits translation (Coller and Parker, 2005) and that *pat1Δ* strains show reduced P-bodies even under glucose deprivation conditions (Figure 5). The late role of Lsm1p is indicated by the accumulation of P-bodies in the *lsm1Δ* strain. Moreover, epitasis analysis is consistent with

Pat1p acting before Lsm1-7p because *pat1Δ lsm1Δ* double mutant strains show reduced P-bodies similar to *pat1Δ* strains (Figure 7).

Pat1p Is a Nuclear-Cytoplasmic Shuttling Protein and Its Distribution Is Affected by Lsm1p

Our results indicate that Pat1p is likely to be a nuclear-cytoplasmic shuttling protein and its distribution between the nucleus and the cytoplasm is affected by the Lsm1-7p complex. The key observation is that in *lsm1Δ* strains, Pat1p accumulates in the nucleus (Figures 5 and 6). Consistent with Pat1p being a nuclear-cytoplasmic shuttling protein, previous work has identified a two-hybrid interaction between Pat1p and Crm1p, which is involved in mRNA export (Jensen *et al.*, 2000). In addition, Pat1p was identified in a proteomic analysis of the components of the penta-snRNP, which is a large nuclear complex involved in pre-mRNA splicing (Stevens *et al.*, 2002). Interestingly, this nuclear localization is not unique to Pat1p in *lsm1Δ* strains, because we also detect a clear accumulation of Dhh1p in the nucleus in *lsm1Δ* strains under glucose deprivation conditions (Figure 5).

In principle, the accumulation of Pat1p in the nucleus in *lsm1Δ* strain could be explained in two manners. First, it could be that interaction of the Lsm1-7p complex with Pat1p in the nucleus is required for efficient export of Pat1p from the nucleus, perhaps in association with mRNAs. This model would imply that an mRNP structure similar to a P-body mRNP may form on some, or all, mRNAs in the nucleus and be transported with them to the cytoplasm. An alternative, and potentially overlapping, model is that interaction of Pat1p with the Lsm1-7p complex in the cytoplasm limits import of Pat1p into the nucleus. Future experiments should be able to distinguish between these models.

Pat1p might function in the nucleus in one of two manners. First, it could be that Pat1p plays a role in a nuclear process such as splicing or degradation of aberrant mRNAs in the nucleus. Alternatively, Pat1p may enter the nucleus to become assembled into nascent mRNPs before export to the cytoplasm, which might lead to those mRNAs entering the cytoplasm in a translationally repressed mRNP that is directly targeted to P-bodies. Such potential targeting of nascent mRNPs might occur on all mRNAs or might be more specific to subclasses of mRNAs, or biological contexts, where translation repression of nascent transcripts is particularly important. Interestingly, one example of such a context is in the biogenesis of maternal mRNAs, which are exported to the cytoplasm and then stored in maternal germ granules, which are related to P-bodies (Anderson and Kedersha, 2006; Sheth and Parker, 2007). Here it is worth noting that the *Xenopus* ortholog of Dhh1p is a nuclear-cytoplasmic shuttling protein (Smillie and Sommerville, 2002) and may play a role in packaging nascent mRNAs for direct targeting to storage particles. An interesting area of future work will be to understand the functional significance of P-body components shuttling between the nucleus and the cytoplasm.

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