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Decapping Activators in *Saccharomyces cerevisiae* Act by Multiple Mechanisms

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SUMMARY

Eukaryotic mRNA degradation often occurs in a process whereby translation initiation is inhibited and the mRNA is targeted for decapping. In yeast cells, Pat1, Scd6, Edc3, and Dhh1 all function to promote decapping by an unknown mechanism(s). We demonstrate that purified Scd6 and a region of Pat1 directly repress translation in vitro by limiting the formation of a stable 48S preinitiation complex. Moreover, while Pat1, Edc3, Dhh1, and Scd6 all bind the decapping enzyme, only Pat1 and Edc3 enhance its activity. We also identify numerous direct interactions between Pat1, Dcp1, Dcp2, Dhh1, Scd6, Edc3, Xrn1, and the Lsm1-7 complex. These observations identify three classes of decapping activators that function to directly repress translation initiation and/or stimulate Dcp1/2. Moreover, Pat1 is identified as critical in mRNA decay by first inhibiting translation initiation, then serving as a scaffold to recruit components of the decapping complex, and finally activating Dcp2.

INTRODUCTION

The process of mRNA degradation is a key step in the regulation of gene expression. One major pathway of mRNA decay occurs by deadenylation leading to decapping, which effectively ends the life of the mRNA by allowing rapid 5′ to 3′ degradation (Parker and Song, 2004; Franks and Lykke Andersen, 2008). The decapping rate is inversely related to translation initiation rate, suggesting that these two processes are in competition (reviewed in Coller and Parker, 2004). Moreover, the Dhh1 and Pat1 proteins, which activate decapping (Tharun et al., 2000; Bonnerot et al., 2000; Bouveret et al., 2000; Coller et al., 2001), can also affect translation repression in vivo (Coller and Parker, 2005; Holmes et al., 2004; Pilkington and Parker, 2008). Thus, key to understanding decapping, as well as translation repression, will be to understand the process by which mRNAs cease translation initiation and become targeted either for decapping and/or translation repression. In yeast, decapping is catalyzed by a Dcp1/Dcp2 holoenzyme and is accelerated in vivo by Pat1, Dhh1, the Lsm1-7 complex, Edc3, and Scd6, all of which are conserved proteins (Tharun et al., 2000; Bonnerot et al., 2000; Bouveret et al., 2000; Coller et al., 2001; Fischer and Weis, 2002; Tharun et al., 2000; Decourty et al., 2008).

A key protein in promoting decapping is Pat1. Except for the dcp1Δ or dcp2Δ strains lacking the decapping enzyme, pat1Δ strains show the strongest defect in mRNA decapping of any known mutant (Tharun et al., 2000; Bonnerot et al., 2000; Bouveret et al., 2000). Pat1 also functions in the formation of P bodies, which are cytoplasmic mRNP granules containing the decapping machinery and translationally repressed mRNAs (Parker and Sheth, 2007; Teixeira and Parker, 2007; Pilkington and Parker, 2008). Pat1 is conserved in eukaryotes, and orthologs are found in P body-like RNP granules in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *C. elegans*, and mammalian cells (Sheth and Parker, 2003; Eulalio et al., 2007; Boag et al., 2008; Scheller et al., 2007). In addition, Pat1 knockdown in *Drosophila* S2 cells affects the degradation of certain miRNA targets (Eulalio et al., 2007).

A key question in understanding decapping is determining the direct effects of the decapping activators on translation and the decapping enzyme. Previous results demonstrate Dhh1 can directly inhibit translation, but whether it can also directly affect the decapping, or how the Scd6, Edc3, and Pat1 proteins function, has not been determined. In this work, we use recombinant proteins to demonstrate that Scd6 and a region of Pat1 directly repress translation in vitro by limiting the formation of a stable 48S preinitiation complex. Moreover, while Pat1, Edc3, Dhh1, and Scd6 all bind the decapping enzyme, only Pat1 and Edc3 enhance its activity. We also identify numerous direct interactions between Pat1, Dcp1, Dcp2, Dhh1, Scd6, Edc3, Xrn1, and the Lsm1-7 complex. Taken together, these observations indicate that activators of decapping function to directly repress translation initiation and/or stimulate the decapping enzyme. Moreover, we identify Pat1 as playing a key role in mRNA decay by first inhibiting translation initiation, then serving as a scaffold to recruit components of the decapping complex, and finally activating the enzymatic activity of Dcp2.

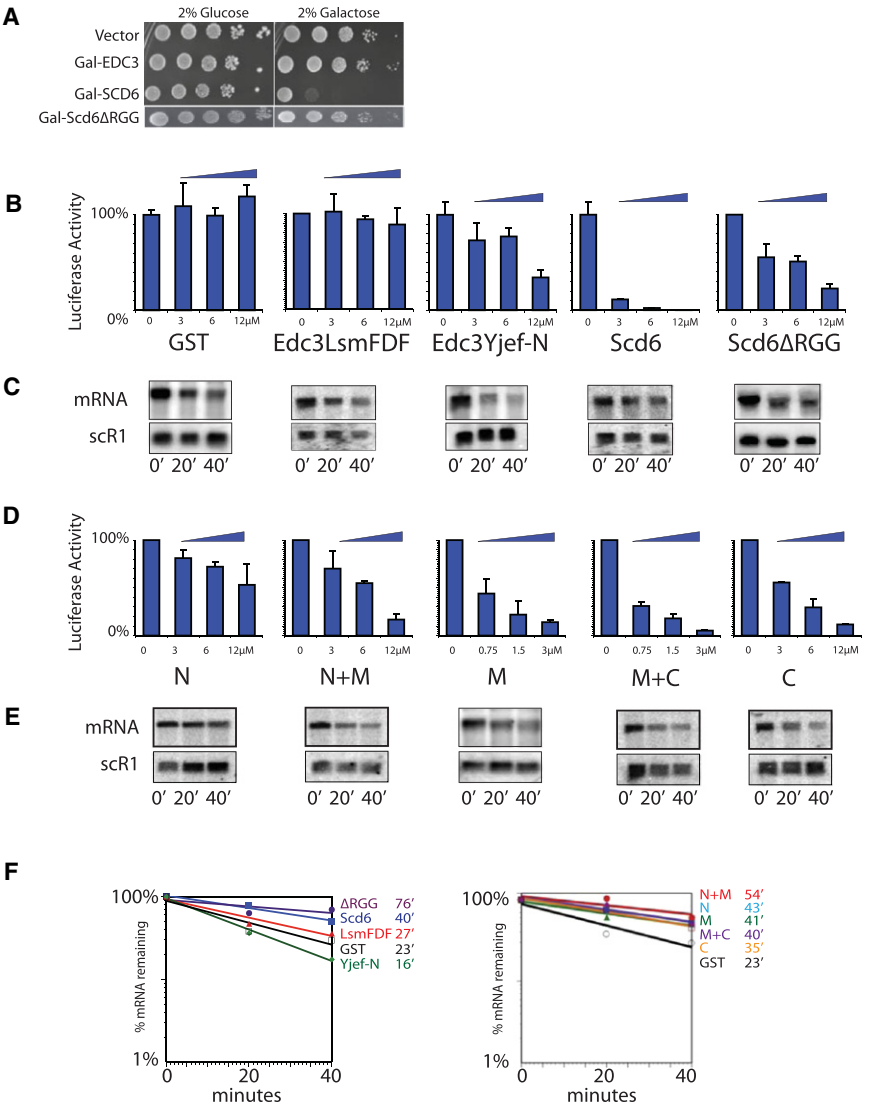

Figure 1. Pat1 and Scd6, but Not Edc3, Directly Repress Translation Initiation

(A) Growth assay for wild-type cells transformed either with empty vector or a vector overexpressing wild-type Edc3, wild-type Scd6, or the Scd6ΔRGG mutant under the GAL promoter. See also Figures S1 and S4.

(B) Effect of Scd6, the domains of Edc3, or GST alone on translation of luciferase mRNA. The graphs depict the luciferase activity at indicated protein concentrations. Error bars represent standard deviations from a minimum of three independent experiments.

(C) mRNA stability of luciferase translated with the proteins listed above. The amount of the luciferase mRNA present in translation extracts is shown at 0′, 20′, and 40′ with an scR1 RNA loading control. Protein used to test RNA stability was at the highest concentration used in the respective translation assays.

(D) Repression of translation of luciferase mRNA by the domains of Pat1 as above. Error bars represent standard deviations from a minimum of three independent experiments.

(E) mRNA stability of luciferase translated with the domains of Pat1 as above.

(F) The percentage mRNA remaining during the reaction at 0, 20, and 40 min normalized to scR1 levels. The amount of mRNA remaining over time is plotted using a logarithmic scale. The calculated half-lives are listed to the right of each graph.

RESULTS

Pat1 and Scd6, but Not Edc3, Directly Repress Translation Initiation

Given the inverse relationship between translation initiation and decapping, these decapping activators could act by directly inhibiting translation. Moreover, overexpression of Pat1, or its C-terminal regions, in yeast directly or indirectly leads to inhibition of translation (Coller and Parker, 2005; Pilkington and Parker, 2008). Similarly, we observe that overexpression of Scd6 inhibits growth of yeast in a manner dependent on its C-terminal RGG box (Figure 1A), despite this variant being well expressed (see Figure S1 available online). In contrast, overexpression of Edc3 does not inhibit growth (Figure 1A). These results imply that Scd6 and Pat1 might directly repress translation.

To examine if any of these decapping activators directly affected translation, we examined the affect of recombinant Scd6, and different domains of Edc3 and Pat1, on translation in vitro. Purification of sufficient full-length Edc3 and Pat1 protein for measuring their effects on translation was not possible due to solubility issues.

We observed that Scd6 (Figure 1B), but not Edc3 domains, repressed protein production in vitro (Figure 1B). The ability of Scd6 to repress protein production in vitro is related to Scd6 function in vivo, since deletion of the RGG box of Scd6 also reduces its ability to repress protein synthesis in vitro.

For Pat1, we observed that the N-terminal region had a two-fold effect on protein synthesis by itself but when fused to the middle domain served as an effective inhibitor of protein synthesis (Figure 1D). We also observed that the C-terminal domain inhibited protein synthesis in vitro (Figure 1D), which is consistent with this domain having a strong effect on translation in vivo when overexpressed (Pilkington and Parker, 2008). The middle domain of Pat1 was also effective at translation repression (Figure 1D), although this Pat1 domain purified with contaminants from *E. coli*, limiting the interpretation of this result. More importantly, the combination of the middle and C-terminal portions of Pat1 was the most effective at reducing protein production (Figure 1D), which is the most relevant construct, as it more accurately represents the full-length protein.

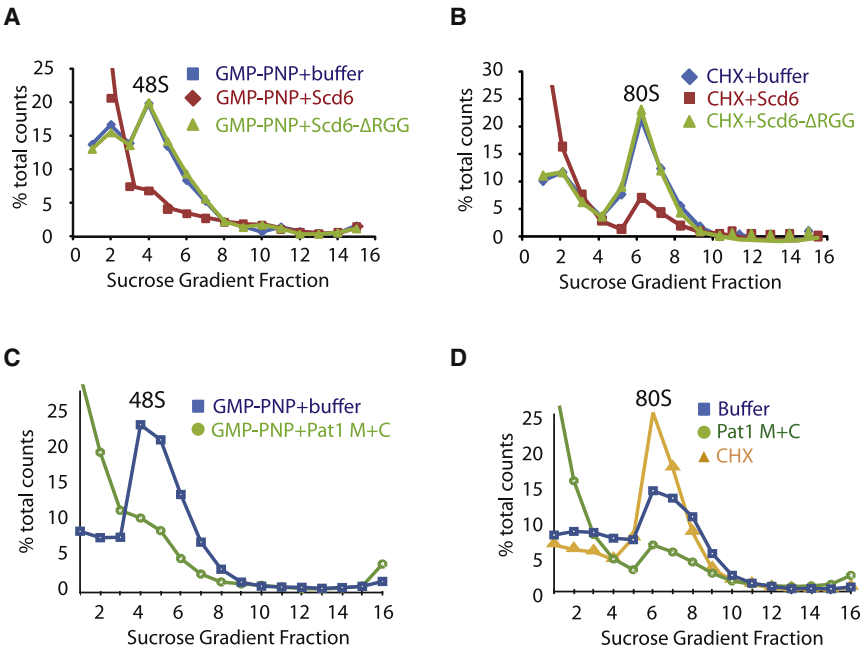

Figure 2. Scd6 and Pat1 Affect Translation at or Prior to the Initiation Step

Localization of radiolabeled capped MFA2 mRNA in translation reactions, which are assembled as described in methods with (A) GMP-PNP either alone, with Scd6 or Scd6ΔRGG mutant; (B) cycloheximide with buffer alone, Scd6, or Scd6ΔRGG mutant; (C) GMP-PNP either with buffer alone or with Pat1 M+C; or (D) no additional components (buffer), cycloheximide, or Pat1 M+C. After assembly and translation, the mRNA is separated by sucrose gradient centrifugation. The localization of the 48S and 80S ribosomal peaks is indicated in the figures. See also Figure S2.

The reduction in protein production due to the addition of Scd6 or Pat1 polypeptides could be due to reduced translation, or increased degradation of the mRNA in the extracts. We examined the rates of mRNA degradation in these extracts by northern blots and observed that none of these proteins increased the rates of mRNA degradation (Figures 1C, 1E, and 1F). The only exception was the Yjef-N domain of Edc3, which increased the decay rate of the reporter mRNA and led to a corresponding decrease in protein production, presumably due to an indirect effect on mRNA stability (Figures 1B and 1F). More importantly, these results indicate that Scd6 and Pat1 can directly repress translation in vitro, and the activity of Pat1 is most efficient in the presence of the middle and C-terminal domains of the protein.

We observed that both Scd6 and Pat1 repressed translation whether the mRNA was capped or not (data not shown), indicating that their function in translation repression is independent of the cap structure.

Pat1 and Scd6 Reduce the Accumulation of 48S Translation Initiation Intermediates

To determine how Scd6 and Pat1 repress translation, we examined how each protein affected the accumulation of intermediates in the process of translation initiation. We utilized the strategy of using GMP-PNP or cycloheximide to block translation at the 48S or 80S complex, respectively (Gray and Hentze, 1994; Coller and Parker, 2005). The 48S complex represents an mRNP complexed with translation initiation factors, the 40S subunit and the initiator tRNA, while the 80S complex has progressed to joining of the 60S subunit. We then examined the effect of exogenous Scd6 or the Pat1 M+C domains (which had the strongest effect on translation) on the accumulation of the 48S and 80S intermediates using the yeast MFA2 mRNA as a substrate.

We observed that both Scd6 and the M+C domain of Pat1 reduced the accumulation of the 80S complex, indicating that these proteins inhibit translation upstream of the formation of the 80S complex. Moreover, we observed that Scd6 and the M+C domains of Pat1 reduced the accumulation of the 48S complex in the presence of GMP-PNP (Figures 2A–2D). This indicates that Scd6 and the M+C region of Pat1 inhibit the formation of 48S complexes or function to dissociate them from mRNAs, thus limiting the formation of a stable 48S preinitiation complex. Consistent with this interpretation, we observed that an spb2Δ strain, which has an excess of 40S subunits (Sachs and Davis, 1990), is resistant to Pat1 overexpression in vivo (Figure S2A). Moreover, we observed that 43S complexes, wherein the initiator tRNA and initiation factors interact with the ribosomal 40S subunit, still formed effectively in the presence of the Pat1 M+C domain (Figure S2C), suggesting that Pat1 limits the interaction of the 43S complex with the mRNA.

Numerous Direct Contacts within the Decapping Machinery: Pat1 Serves as a Scaffold for mRNA Decapping Factors

Pat1 has been shown to copurify with the Lsm1-7 complex (Tharun et al., 2000; Bonnerot et al., 2000; Bouveret et al., 2000), demonstrates two-hybrid and protein fragment complementation assay (PCA) interactions with components of the decapping machinery (Fromont-Racine et al., 2000; Pilkington and Parker, 2008; Tarassov et al., 2008), and accumulate in P bodies (Sheth and Parker, 2003). These observations suggest that Pat1 has one or more direct interactions with the decapping machinery. Moreover, because Pat1 can accumulate in P bodies independent of Lsm1 (Teixeira and Parker, 2007), Pat1 must have additional direct contacts in these complexes. To determine the direct interactions of Pat1, we utilized the purified subdomains of Pat1 in pull-down experiments with purified Dcp1, Dcp2, Dhh1, Scd6, Lsm1-7 complex, and Xrn1. We also examined the interactions between Scd6, Edc3, and other components of the decapping machinery. All proteins were purified from *E. coli*, with the exception of the Lsm1-7 complex,

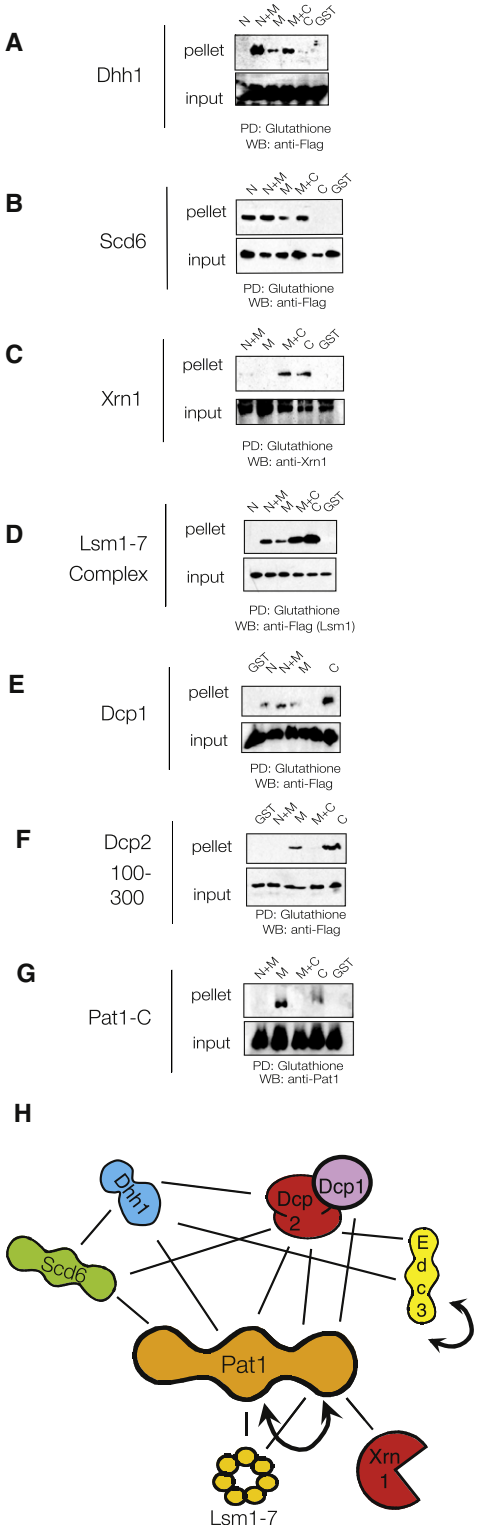

Figure 3. Interactions of Pat1 with Factors Involved in mRNA Decay and Translational Repression

See also Figure S3. (A) Dhh1, (B) Scd6, (C) Xrn1, (D) Lsm1-7 complex, (E) Dcp1, (F) Dcp2, and (G) Pat1 C domain with the GST moiety cleaved with PreScission protease. The protease and cleaved GST were removed by binding to GST resin. (H) Diagram showing the determined interactions.

which was purified from pat1Δ yeast. These experiments revealed the following observations:

First, we observed that Dhh1 interacts with any Pat1 polypeptide containing the middle segment of Pat1 (Figure 3A). This indicates there is a direct interaction between Pat1 and Dhh1, and is consistent with our observation that Dhh1 copurifies with Pat1 from yeast cells in TAP purifications (Figure S3).

Second, we observed that Scd6 interacts with any Pat1 polypeptide containing the N-terminal domain, and to a lesser extent the M domain, but does not interact with the C domain (Figure 3B). This indicates there are direct interactions between Scd6 and two regions of Pat1.

Third, we observed that purified Xrn1, the primary cytoplasmic 5′-3′ exonuclease, directly interacts with the C-terminal region of Pat1 (Figure 3C). Xrn1 did not interact with the M or N+M regions. A direct interaction of Pat1 with Xrn1 is consistent with Xrn1 copurifying with Pat1 in numerous experiments (Figure S3; and Bouveret et al., 2000; Gavin et al., 2006; Krogan et al., 2006).

Fourth, we observed that the Lsm1-7 complex strongly interacts with any peptide containing the C-terminal region, and to a lesser extent with the middle section of Pat1 (Figure 3D). This indicates that the Lsm1-7 complex interacts with two regions of Pat1. The interaction of the Lsm1-7 complex with the C-terminal domain of Pat1 is consistent with prior observations that the C-terminal domain is required for Lsm1 recruitment to P bodies (Pilkington and Parker, 2008).

Fifth, Pat1 directly bound both subunits of the decapping enzyme. Specifically, we observed interactions between the C-terminal region of Pat1 and Dcp1, the noncatalytic component of the decapping enzyme (Figure 3E). Moreover, we observed that both the middle and the C-terminal regions of Pat1 were able to interact with Dcp2 (Figure 3F). This indicates that Pat1 can interact with Dcp1 and contains two regions that can directly interact with Dcp2.

Surprisingly, we observed no interaction of Dcp2 with a Pat1 construct containing the M+C region, even though Dcp2 was clearly pulled down with either the M- or the C-terminal domain alone (Figure 3F). One possible explanation for this set of interactions would be that the M and C domains of Pat1 interact with each other, in a manner that blocks Dcp2 binding to either portion. To test this possibility, we examined the ability of the various regions of Pat1 to pull down the Pat1 C-terminal region. Strikingly, we observed that the middle domain, and to a lesser extent the C-terminal region, was able to interact with an independent Pat1 C-terminal region (Figure 3G). This suggests that Pat1 contains self-interaction domains that might be important in the modulation of Pat1 function (see the Discussion).

We also observed that the Edc3LsmFDF domain, or the Edc3Lsm domain alone, binds directly to Dcp2 (Figures 4A and 4C, left panels). We did not observe any interaction between the Lsm domain or LsmFDF double domain with Dcp1 (Figure 4A, right panel and data not shown). In addition, we observed Scd6 bound to Dcp2 and not Dcp1 (Figure 4B). Both an N-terminal portion of Scd6 containing the Lsm domain and an C-terminal portion of Scd6 containing the FDF domain interacted with Dcp2, suggesting there are two regions of Scd6 that interact with Dcp2 (Figure 3C, right panel). We also observed that Dhh1 bound Dcp2 as reported earlier (Figure 4D and Decker

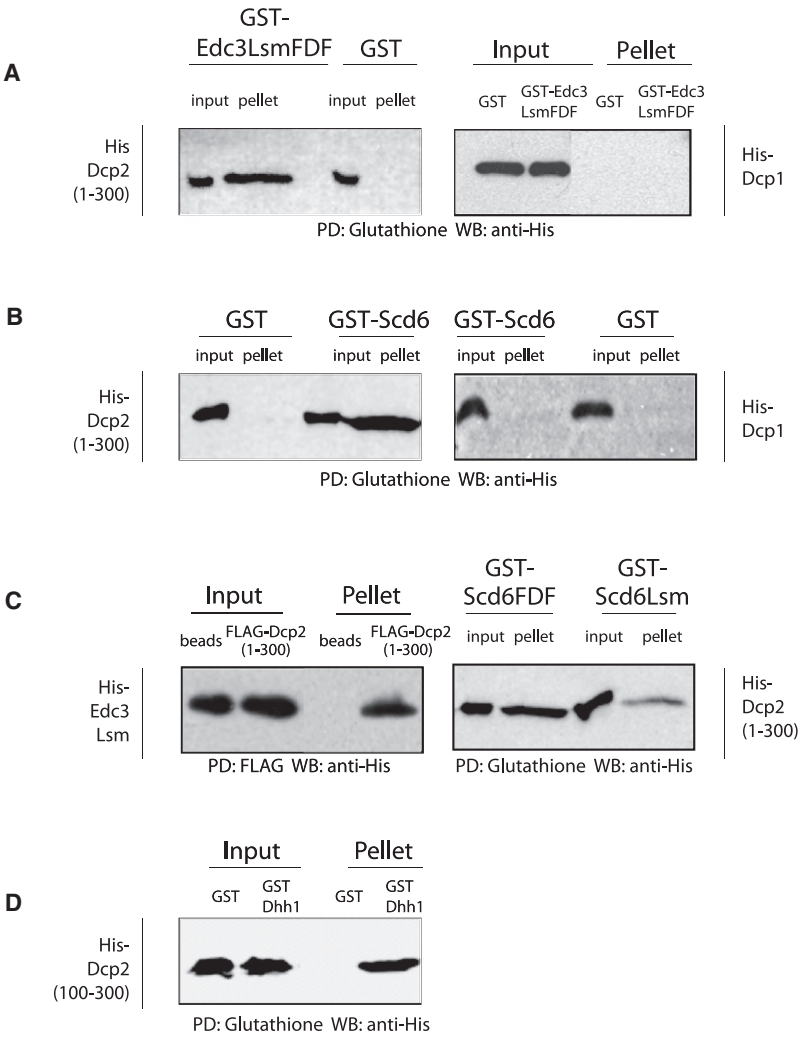

Figure 4. Edc3, Scd6, and Dhh1 Bind to Dcp2

The pull-downs "PD" were performed by the resin indicated, and the resulting western blot was probed for the antibody indicated by "WB."

(A) Purified Dcp2 (1–300) (left panel) or Dcp1 (right panel) was incubated with GST-Edc3LsmFDF and GST (control).

(B) Purified Dcp2(1–300) (left panel) or Dcp1 (right panel) was incubated with either GST-Scd6 or GST protein.

(C) (Left panel) Purified His-Edc3Lsm was incubated with FLAG-Dcp2(1–300) or anti-FLAG M2 agarose beads (Sigma). (Right panel) Purified GST-tagged Scd6 domains were incubated with Dcp2(1–300).

(D) Purified Dcp2(100–300) was incubated with GST-Dhh1 or GST protein.

et al., 2007). Thus, Edc3, Dhh1, and Scd6 directly interact with Dcp2.

These results document numerous direct interactions between components of the decapping machinery, with Pat1 playing a central scaffolding role for assembly of the decapping complex (summarized in Figure 3H). The number of direct interactions with Pat1 suggests this protein plays a critical role in the nucleation of a decapping complex, which is consistent with pat1Δ strains having a strong defect in mRNA decapping and being defective in P body assembly (Teixeira and Parker, 2007). In addition, the interaction of Edc3, Scd6, and Pat1 with Dcp2 raises the possibility that these proteins might directly influence the activity of the decapping enzyme.

Edc3 and Pat1 Directly Stimulate the Dcp1/Dcp2 Decapping Enzyme

To test if Scd6, Edc3, Dhh1, or Pat1 could directly stimulate decapping, we examined the effect of the purified proteins on recombinant Dcp1/Dcp2 decapping enzyme (Steiger et al., 2003).

We observed that Pat1 and Edc3 both stimulated the decapping enzyme, while Dhh1 and Scd6 had little or no effect on Dcp1/Dcp2 in vitro (Figures 5A, 5D, 5E, and 5I).

Purification of subdomains of the Edc3 protein demonstrated that the Lsm domain was sufficient to stimulate the decapping activity (Figure 5B, left TLC panel, and Figure 5F). In contrast, the FDF and Yjef-N individual domains did not stimulate decapping (Figure 5B, right TLC panel). Interestingly, the full-length Edc3 led to enhanced stimulation as compared with the Lsm-FDF protein lacking the Yjef-N domain (Figure 5B, middle TLC panel, and Figure 5G). These observations argue that the Yjef-N domain can enhance the function of Edc3, possibly by contributing to dimer formation or RNA binding (Ling et al., 2008). This identifies the Lsm domain as the key domain in Edc3 for enhancing Dcp1/Dcp2 activity in vitro. Moreover, the interaction of Edc3Lsm with Dcp2 explains its effect on decapping, since the Lsm domain of Edc3 is sufficient to enhance the activity of Dcp2 in the absence of Dcp1 (Figures 5C and 5H). This is also consistent with recent observations that mutations in Dcp2 that prevent Edc3 interaction prevent the ability of Edc3 to promote decapping in vitro and in vivo (Harigaya et al., 2010).

For Pat1, the C-terminal domain of Pat1 stimulated the initial decapping rate nearly 8-fold (Figures 5D and 5I). In contrast, the N and the M domains of Pat1 did not stimulate Dcp2 activity (Figures 5D and 5I). This demonstrates that the C-terminal domain of Pat1, which directly binds Dcp1 and Dcp2 (Figures 3E and 3F), stimulates the activity of the Dcp1/Dcp2 enzyme.

Interestingly, and consistent with binding to Dcp1 and Dcp2 being important for decapping, we also observed that the M+C region of Pat1, which fails to bind Dcp2 (Figure 3F), reduces the ability of the C region to stimulate decapping (Figures 5D and 5I). The failure of M+C to bind to Dcp2 and stimulate decapping suggests that Pat1 may undergo conformational rearrangements during the assembly and activation of decapping (see the Discussion).

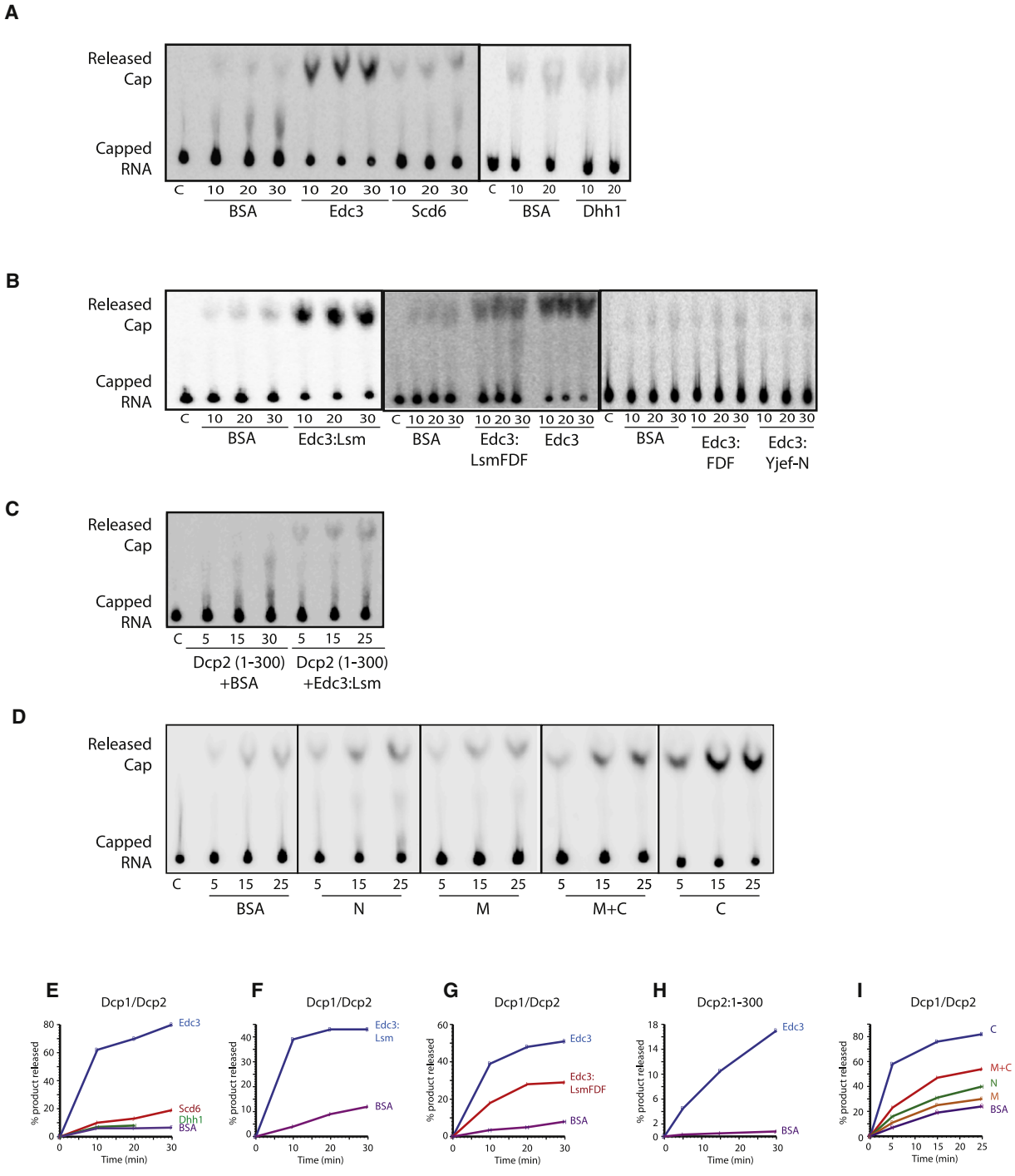

Figure 5. Pat1 and Edc3 Directly Stimulate Decapping

TLC analyses of decapping assay.

(A) Decapping complex (Dcp1/Dcp2) incubated with equimolar amounts of BSA, Edc3, Scd6, or Dhh1.

(B) Decapping reaction of the Dcp1/Dcp2 complex with BSA, Edc3, Edc3Lsm, Edc3LsmFDF, Edc3FDF, or Edc3YjeF-N as indicated below each panel.

(C) Dcp2(1–300) incubated with either BSA or Edc3Lsm.

(D) Decapping complex reaction with BSA or the indicated Pat1 domain. The reconstituted complex was then incubated with cap-labeled MFA2pG at 30° C for the time points indicated.

(E–I) Graphs of the quantified panels from above. Each panel indicates if either the entire decapping complex (Dcp1/Dcp2) or Dcp2(1–300) alone was used.

DISCUSSION

Individual Decapping Factors Act in Different Manners

In this work, we identify three different classes of proteins that activate decapping (summarized in Table 1). The first class consists of proteins that function by directly activating the decapping enzyme. For example, several observations indicate that Edc3 directly binds and stimulates the decapping activity of Dcp2. First, purified Edc3 binds directly to Dcp2 (Figure 4A), which is consistent with earlier work with less-purified proteins (Decker et al., 2007). Second, we observe that Edc3 stimulates the Dcp1-Dcp2 decapping enzyme or just Dcp2 alone (Figures 5A, 5C, 5E, and 5H). We did not observe any affect of Edc3 inhibiting translation either in vitro or when overexpressed in vivo (Figures 1A and 1B), suggesting its sole role in decapping is to activate the Dcp1/Dcp2 decapping enzyme. The exact biochemical mechanism by which Edc3 enhances Dcp2 activity remains to be determined. Edc3 activation of Dcp2 may include both conformational changes in Dcp2, since the Lsm domain of Edc3 is sufficient to enhance decapping but does not bind RNA, and RNA binding, since the full-length protein can dimerize and bind RNA (Ling et al., 2008) and shows increased stimulatory activity as compared to the LsmFDF domain alone. Similarly, the Edc1 and Edc2 proteins directly stimulate the decapping enzyme (Schwartz et al., 2003; Steiger et al., 2003) and are unlikely to repress translation, as their overexpression is not lethal to yeast (Sopko et al., 2006).

A second class of decapping activators, such as Dhh1 and Scd6, promote decapping by primarily repressing translation initiation, which then indirectly increases the rate of decapping by altering the competition between translation initiation and decapping. This was first suggested for Dhh1, which was shown to inhibit translation in cell extracts and to only affect the decapping of mRNAs that complete translation initiation (Coller and Parker, 2005). Recombinant Scd6 is also a potent inhibitor of translation in vitro (Figure 1B). In addition, overexpression of Scd6 in vivo leads to a decrease in growth and the accumulation of P bodies and stress granules, suggesting Scd6 can also repress translation in vivo (Figure 1A and Figure S4). In contrast, neither purified Dhh1 nor Scd6 significantly stimulates the recombinant decapping enzyme in vitro (Figures 5A and 5E), supporting the model that Dhh1 and Scd6 largely promote decapping by inhibiting translation initiation. It should be noted that previous results have suggested Dhh1 can stimulate the activity of a decapping enzyme immunopurified from yeast (Fischer and Weis, 2002), which might indicate that Dhh1 can enhance decapping in the presence of additional factors.

Pat1 Directly Reduces Translation Initiation and Stimulates Decapping by Dcp1/2

We identify Pat1 as the sole member to date of a third class of protein that affects decapping both by directly stimulating the decapping enzyme and inhibiting translation initiation. The key observations that Pat1 directly activates the decapping enzyme are that Pat1 binds both subunits of the decapping enzyme (Figures 3E and 3F), and that the purified C-terminal domain of Pat1 stimulates the activity of Dcp1/Dcp2 in vitro (Figures 5D and 5I). Moreover, two observations also indicate that Pat1 directly represses translation initiation. First, the addition of recombinant M+C, or C domains to in vitro translation, extracts inhibits translation and 48S complex formation (Figures 1D and 2C and data not shown). Similarly, overexpression of Pat1 or the C-terminal regions of Pat1 in vivo leads to translation repression and mRNAs accumulating in P bodies (Coller and Parker, 2005; Pilkington and Parker, 2008). Thus, Pat1 is the only protein identified to date that both represses translation and stimulates the decapping enzyme, which is consistent with the strong decapping defect seen in pat1Δ strains. These results indicate Pat1 is a critical protein that spans both translation repression and decapping of the mRNA.

Decapping Activators Affect Early Steps in Translation Initiation

Several observations argue that Pat1 and Scd6 reduce translation initiation by limiting the formation of a 48S preinitiation complex. First, the addition of the translation repression region of Pat1 (M+C) or Scd6 reduces the accumulation of 48S complexes trapped by the addition of GMP-PNP (Figures 2A and 2C). Second, spb2Δ strains having an overabundance of 40S subunits are resistant to the growth inhibitory effects of Pat1 overexpression (Figures S2A and S2B). Third, Pat1 does not affect the formation of a 43S complex, suggesting that Pat1 acts to limit the interaction of the 43S complex with the mRNA (Figure S2C). Strikingly, Dhh1 also reduces 48S complex formation (Coller and Parker, 2005). Taken together, these results demonstrate that decapping activators typically repress translation upstream of 48S complex formation, which could provide a mechanism for cap exposure to the decapping enzyme.

We also observed that Pat1 associates with ribosomes through its N-terminal domain and sediments on sucrose gradients in a position consistent with interactions with the 48S complexes (Figure S3). However, since the Pat1 M+C domains can repress translation both in vivo and in vitro, and does not interact with ribosomes (Figure S3), the Pat1-ribosome interaction is not essential for Pat1 function. It remains possible that the efficiency of Pat1 in repressing translation and/or promoting decapping is enhanced by interactions with ribosomes, since the N terminal domain can function redundantly with the C-terminal domain of Pat1 to promote decapping (Pilkington and Parker, 2008).

Pat1 Serves as a Scaffold to Connect Inhibition of Translation and mRNA Decapping

Several observations suggest that Pat1 functions as a scaffold for physical interactions that promote translation repression and decapping. First, Pat1 associates with ribosomes through its N-terminal domain (Figure S3). In addition, Pat1 is likely to have interactions with other translation factors, since it can repress translation initiation through its M+C domains, which do not interact with ribosomes, and because it immunoprecipitates with eIF4G, eIF4E, and Pab1 (Tharun and Parker, 2001; Gavin et al., 2006). Second, Pat1 also has direct interactions with Scd6, Dhh1, and the Lsm1-7 complex (Figures 3A, 3B, and 3D) that can also directly repress translation (Figure 1D), which may allow for the assembly of a robust translation

Table 1. Biochemical Activities and Phenotypes of Decapping Activators

Class/Mode of Action	Protein	Inhibits Growth In Vivo	Represses Translation In Vitro	Activates Decapping In Vivo	Stimulates Decapping Enzyme	Binds RNA
I: activates Dcp1/Dcp2	Edc1	–[f]	ND	+[g]	+++[h,i]	+++[h]
I: activates Dcp1/Dcp2	Edc2	–[f]	ND	+[g]	+++[h,i]	+++[h]
I: activates Dcp1/Dcp2	Edc3	–[a,b]	–[b]	+[j,k]	+++[b]	+++[l]
II: represses initiation and activates Dcp1/Dcp2	Pat1	+++[a,q]	+++[b]	+++[a,m,n,o,q]	++[b]	+++[q]
III: represses initiation	Scd6	+++[b]	+++[b]	+[k]	–[b]	+++[r,s]
III: represses initiation	Dhh1	+++[a]	+++[b]	+++[a,c,d]	–[b]	+++[e]
ND	Lsm1-7	+ (but indirect effect)[t]	ND	+++[m,n,o]	– (data not shown)	+++[p]

[a] Coller and Parker, 2005.
[b] This work.
[c] Coller et al., 2001.
[d] Fischer and Weis, 2002.
[e] Cheng et al., 2005.
[f] Sopko et al., 2006.
[g] Dunckley et al., 2001.
[h] Schwartz et al., 2003.
[i] Steiger et al., 2003.
[j] Kshirsagar and Parker, 2004.
[k] Decourty et al., 2008.
[l] Ling et al., 2008 (human).
[m] Bonnerot et al., 2000.
[n] Bouveret et al., 2000.
[o] Tharun et al., 2000.
[p] Chowdhury et al., 2007.
[q] Pilkington and Parker, 2008.
[r] Audhya et al., 2005 (*C. elegans*).
[s] Tanaka et al., 2006 (*Xenopus*).
[t] Luhtala and Parker, 2009.

repression complex. Finally, Pat1 also directly interacts with the decapping enzyme and Xrn1, triggering decapping and 5′ to 3′ degradation. These interactions occur in both the M and the C domains of Pat1, both of which affect Pat1 function in vivo (Pilkington and Parker, 2008). Thus, Pat1 has a myriad of interactions that orchestrate the inhibition of translation and target an mRNA for decapping. The interactions of Pat1 are also integrated into a large meshwork of direct protein-protein interactions of components of the decapping machinery that facilitate the processes of translation repression and mRNA degradation (Figure 3H).

Three lines of evidence argue that Pat1 does not interact with all of its binding partners at the same time, and therefore goes through a series of transitions in its associated factors. First, Pat1 associates with ribosomal subunits based on gel filtration, sucrose gradients, and coimmunpurification, yet the Lsm1-7 complex does not share these same properties (Figure S3). This argues that Pat1's interaction with ribosomes occurs at a different time than its interactions with the Lsm1-7 complex. Similarly, Pat1 immunoprecipitates with eIF4E, eIF4G, and Pab1, but Lsm1 does not (Tharun and Parker, 2001). Finally, the fact that Dcp2 separately binds the M and C domains of Pat1 but not M+C together (Figure 3) suggests that additional interactions with Pat1, either with RNA or protein, alter the self-interactions between domains M and C to allow interaction with the decapping enzyme. An important goal in future work will be to determine the precise nature of different Pat1-containing complexes and how they are related to each other in assembly and function.

An Integrated Model for mRNA Decapping

The results in this work, and additional data from the literature, allow us to develop an integrated model for the process of mRNA decapping (Figure 6). In this model, the first key step in targeting an mRNA for decapping is inhibition of translation initiation, which occurs due to competition between key steps in translation initiation and function of decapping activators that can inhibit initiation such as Dhh1, Scd6, and Pat1. The precise interactions that repress initiation for each of these factors remain to be determined. Since the M and C domains of Pat1 can bind RNA (Pilkington and Parker, 2008), one possibility is that Pat1 represses initiation by direct interactions with the mRNA, thereby explaining why the combination of the M+C domains is a more effective translation repressor in vitro.

The competition between decapping and initiation is likely to be affected by stochastic events as well as mRNA-specific features that limit substeps in translation initiation that are targeted by decapping activators, the recruitment of decapping activators to individual mRNAs by sequence specific mRNA-binding factors, and the regulation of translation initiation factors

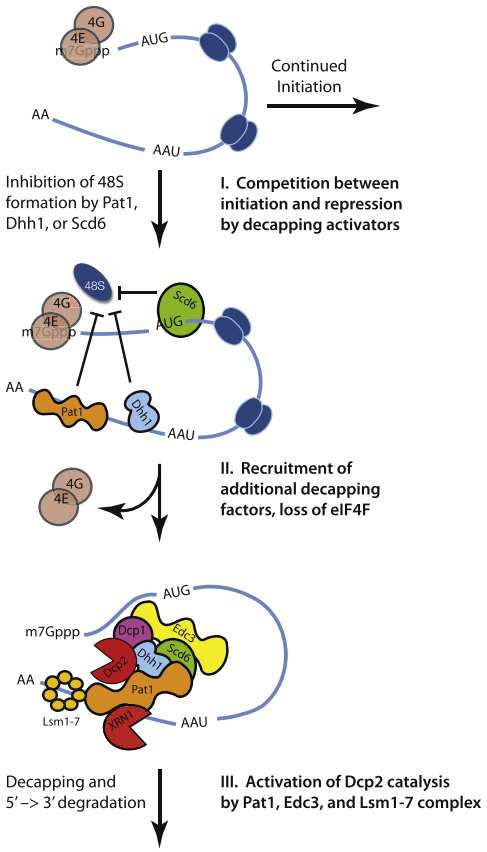

Figure 6. Model for Transitions Mediated by Activators of Decapping

Three-step mechanism for effect of decapping activators on translation and enhancement of mRNA decapping.

and decapping factors in response to environmental cues. The presence of multiple decapping activators that can inhibit translation explains why loss of no single decapping activator completely blocks decapping and why double mutants lacking Dhh1 and Pat1 show a very strong defect in decapping (Coller and Parker, 2005).

Following an initial inhibition of translation initiation, a second step is the recruitment of additional decapping activators and the decapping enzyme. Since Scd6, Dhh1, and Pat1 all show direct interactions, the initial interaction of any one of these translational repressors can then lead to the subsequent recruitment of two or more translation repressors on an individual mRNA creating a robust block to translation initiation. One key step during this transition in mRNP organization is the loss of the cap-binding complex, which is an effective inhibitor of decapping both in vivo and in vitro (Schwartz and Parker, 2000). An unresolved issue is whether the cap-binding complex passively dissociates or is actively removed from the mRNA. A second key step is recruitment of the decapping enzyme to the mRNA, which occurs by redundant mechanisms given the multitude of direct interactions between the decapping activators and the decapping enzyme (Figure 3H). In addition, the Xrn1 exonuclease and Lsm1-7 complex appear to be recruited to the complex by the Pat1 protein (Teixeira and Parker, 2007; Pilkington and Parker, 2008; Figures 3C and 3D).

The final steps in decay require cleavage of the cap linkage and 5′ to 3′ degradation by Xrn1, which is likely to require additional interactions in the decapping complex involving the Lsm1-7 complex since lsm1Δ mutants accumulate P bodies and show defects in the decapping of mRNAs that never enter translation initiation (Coller and Parker, 2005; Teixeira and Parker, 2007). Interestingly, removal of the M domain of Pat1 also leads to a strong defect in decapping in vivo and the accumulation of P bodies similar to an lsm1Δ strain (Pilkington and Parker, 2008). This suggests that a late step in efficient decapping is specific interactions with the M domain of Pat1, perhaps with the Lsm1-7 complex, that are required for a final activation of the Dcp1/Dcp2 enzyme in vivo. The requirement for additional steps that activate decapping also extend to nonsense-mediated decay in yeast, where Upf1 is sufficient to recruit Dcp1/Dcp2 to mRNAs but requires Upf2 and Upf3 for actual decapping to occur (Sheth and Parker, 2006). Important issues in future work will be to understand the dynamics of the decapping complex and at what stage the decapping enzyme becomes capable of catalysis and how cells regulate this transition to modulate translation repression and mRNA degradation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids

The genotypes of strains used in this study are listed in Table S1. Strains were grown on either standard yeast extract/peptone medium (YP) or synthetic medium (SC) supplemented with the appropriate amino acids and 2% glucose. Strains were grown at 28°C unless otherwise stated. All plasmids used in this study are listed in Table S2. Mutations in Scd6, addition of stop codons, C-terminal 6xHis, and FLAG tags to proteins were performed using QuikChange mutagenesis according to the manufacturer's instructions (Stratagene).

RNA Analysis

RNA was purified after translation with MEGAclear (Ambion), followed by northern blotting of agarose gels and probed for luciferase mRNA by oRP1435 (CAA TTT GGA CTT TCC GCC CTT). Quantification of blots was performed using a Phosphorimager. Loading corrections were done using oRP100, an oligonucleotide directed against scR1 RNA, a stable RNA polymerase III transcript (Cao and Parker, 2001).

Polysome Analysis

Polysome analysis was performed by growing cells in YP media containing 2% dextrose. Cells were harvested by centrifugation at 4000 rpm for 1 min at RT, washed in ice-cold lysis buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 30 mM MgCl2, 1% Igepal CA-630), and frozen at −80°C. Cells were lysed in 400 μl lysis buffer containing 0.5 mg/ml heparin and 1 mM DTT, and 200 μl volume of glass beads was added and vortexed. After cell lysis, a clarifying centrifugation was done for 2 min at 4000 rpm at 4°C. Approximately ten A260 units were loaded on a 15%–50% sucrose gradient with 80% cushion and sedimented in a SW41 rotor at 4°C for 2.5 hr at 39,000 rpm or 15 hr at 27,000 rpm.

Protein Purification, In Vitro Protein-Protein Interaction Assays, and Western Blotting

TAP affinity purification of Pat1 complexes from yeast was from 2–6 L of mid-log phase yeast culture cultured at 25°C in YPD medium. Cells were harvested and lysed using a French press. Two-column purification was performed as previously described (Puig et al., 2001). Briefly, cleared cell lysate was purified using IgG resin (GE Healthcare), eluted with TEV protease (Invitrogen), purified using calmodulin resin (GE), and eluted with EGTA. Purified proteins were

concentrated by TCA precipitation and separated on 4%–12% NuPAGE gels. The TEV eluate from each TAP-tagged protein was analyzed on a TSK Gel G4000SW$_{XL}$ column (Tosoh Bioscience) in 50 mM TRIS-HCl (pH 7.5), 100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, using the ProStar HPLC System (Varian).

Proteins were purified from *E. coli* according to standard protocols using glutathione Sepharose beads (GE), Talon IMAC resin (Clontech), or Ni-NTA agarose (QIAGEN) according to standard protocols. Purified protein was concentrated and dialyzed into 150 mM NaCl, 10 mM HEPES (pH 7.4), 2 mM DTT with 50% glycerol and stored at −20°C. In the case of Scd6 and Edc3, purified proteins/domains were dialyzed in 20 mM Tris, 100 mM NaCl, and 10% glycerol. Purified yeast Xrn1 was purchased from NEB. Purification of the Lsm1-7 complex was performed using Flag purification (Sigma) from yeast cells grown to mid-log phase in minimal media (6–8 L) using the pat1Δ lsm1Δ strain with Lsm5-His6 (yRP2758) harboring a plasmid expressing Flag-Lsm1 (pRP1911).

Binding reactions were performed at 4°C in binding buffer (50 mM HEPES [pH 7.4], 100 mM NaCl, 2 mM DTT, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 1% Igepal CA-630 [USB], 10% glycerol, and 10 mg/ml BSA) containing 25 ng/μl of the target protein (without GST tag) and 25 ng/μl of the GST-tagged bait protein. In the case of the assay with cleaved Pat1 C-terminal region (422–796), the NaCl concentration was increased to 350 mM.

Western analysis was performed using anti-GST (Abgent), purified anti-Flag M2 (Sigma), anti-His (Abcam), anti-Rpl3 (Jonathan Warner), anti-Xrn1 (Arlen Johnson) antibodies or a polyclonal anti-C Pat1 raised in rabbits to the purified protein (Cocalico Biologicals).

In Vitro Translation Assays

Yeast extracts were prepared according to a modified protocol based on Iizuka and Sarnow (1997) and Wu et al. (2007). Briefly, yRP930 cells were grown to high OD overnight, lysed using a Retsch PM200 mill with cells frozen in liquid nitrogen, and lysed twice at 300 rpm in canisters cooled with liquid nitrogen for 3 min. After thawing, gel filtration and extract preparation were similar to the published protocols.

Translation repression assays were conducted using 200 ng uncapped poly $(A)^+$ luciferase mRNA (Promega) on nuclease-treated extracts, which was used to start the reaction. Reactions were assembled and incubated 50′ at RT. Translation was monitored using a luciferase enzymatic assay (Promega). Prior to the experiment, the protein was dialyzed into 50 mM KOAc, 1 mM DTT, 10 mM HEPES (pH 7.4) and diluted in this buffer. In Figure 2, translation was performed on capped radiolabeled MFA2 p(G) poly$(A)^+$ RNA transcribed from XbaI linearized pRP803 with MAXIScript (Ambion) and preincubated with or without purified M+C Pat1 for 20′ at RT before translation. These reactions were supplemented with 5 mM GMPPNP and 7.5 mM MgOAc or 0.5 g/L cycloheximide as appropriate. The extract was centrifuged as above and radioactivity assayed by Cherenkov counting.

Decapping Assay

Uncapped MFA2 mRNAs lacking poly(A) tails were transcribed as above. The resulting uncapped transcript was run on urea 8% polyacrylamide gel and the correct size band excised. RNA was eluted from gel by incubating overnight with 0.3 M sodium acetate, 0.1% SDS, and 1 mM EDTA, followed by phenol chloroform extraction. The m7G cap was added posttranscriptionally with Scriptcap (Epicenter) using [α^{32}P]-GTP.

Decapping reactions with yeast Dcp1/Dcp2 purified from *E. coli* were assayed at 30°C. The reaction mixtures generally contained 5 fmol of $m^7G[^{32}P]$ pppMFA2 mRNA, 5 pmol of Dcp1/Dcp2, 5 pmol of either BSA or protein being tested, 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM DTT, and 1 μl of RNasin in a volume of 15 μL. Reactions were stopped with EDTA followed by storage on ice. The products of the reaction were separated by PEI-cellulose TLC developed in 0.75 M LiCl and detected with a PhosphorImager.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.molcel.2010.08.025.

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