Control of mRNA decapping by positive and negative regulatory elements in the Dcp2 C-terminal domain

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ABSTRACT

Decapping commits an mRNA to complete degradation and promotes general 5' to 3' decay, nonsense-mediated decay (NMD), and transcript-specific degradation. In *Saccharomyces cerevisiae*, a single decapping enzyme composed of a regulatory subunit (Dcp1) and a catalytic subunit (Dcp2) targets thousands of distinct substrate mRNAs. However, the mechanisms controlling this enzyme's *in vivo* activity and substrate specificity remain elusive. Here, using a genetic approach, we show that the large C-terminal domain of Dcp2 includes a set of conserved negative and positive regulatory elements. A single negative element inhibits enzymatic activity and controls the downstream functions of several positive elements. The positive elements recruit the specific decapping activators Edc3, Pat1, and Upf1 to form distinct decapping complexes and control the enzyme's substrate specificity and final activation. Our results reveal unforeseen regulatory mechanisms that control decapping enzyme activity and function *in vivo*, and define roles for several decapping activators in the regulation of mRNA decapping.

Keywords: mRNA decapping; decapping enzyme; decapping activators; positive and negative regulation

INTRODUCTION

Decapping plays an important role in multiple aspects of eukaryotic cytoplasmic mRNA turnover (Song et al. 2010; Parker 2012) and is critical to numerous biological processes, including development (Xu et al. 2006; Ma et al. 2013), DNA replication (Mullen and Marzluff 2008; Schmidt et al. 2011), stress response (Hilgers et al. 2006; Xu and Chua 2012), synapse plasticity (Hillebrand et al. 2010), retrotransposition (Dutko et al. 2010), and viral replication (Hopkins et al. 2013). In yeast, mRNA decapping is carried out by a single enzyme composed of a regulatory subunit (Dcp1) and a catalytic subunit (Dcp2), but also requires the functions of specific regulators commonly designated as “decapping activators” (Parker 2012). Decapping of general mRNAs requires the functions of the decapping activators Pat1, Dhh1, Edc3, and the Lsm1-7 complex, and decapping of nonsense-containing mRNAs requires the NMD-specific regulators Upf1, Upf2, and Upf3. All these decapping factors are conserved from yeast to humans, but their precise functions and modes of regulation, and that of the decapping enzyme itself, are largely unknown. There are, however, mechanistic models for decapping enzyme regulation and these models are largely based on the results of *in vitro* enzymatic assays (Steiger et al. 2003; Harigaya et al. 2010; Borja et al. 2011), structural anal-

yses (She et al. 2006, 2008; Deshmukh et al. 2008; Floor et al. 2010), and protein–protein interaction studies (Decker et al. 2007; Nissan et al. 2010). These experiments have led to the notion that the catalytic step is limiting for mRNA decapping and that decapping activators function mainly to enhance decapping chemistry, possibly by promoting a conformational change in Dcp2 required for the decapping reaction (Floor et al. 2010; Borja et al. 2011). However, essentially all of the studies on which the mechanistic models are based have two significant shortcomings. First, they used Dcp2 fragments lacking approximately 700 C-terminal amino acids of that protein's 970 residues. Although the large C-terminal truncation of Dcp2 yielded an apparently functional decapping enzyme (Dunkley and Parker 1999), the resulting Dcp2 fragment could lack important regulatory activities. And, second, the *in vitro* decapping experiments used generic mRNAs as substrates, and thus missed the opportunity to determine how the decapping enzyme controls its target specificity critical for *in vivo* regulation.

Here, we investigate the molecular mechanisms involved in regulating the yeast decapping enzyme *in vivo*. Using deletions, point mutations, and two-hybrid analysis to dissect protein:protein interactions, and quantitative Northern analyses of multiple mRNAs to assess mRNA decay activity, we

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identified the decapping activators that bind to Dcp2 and revealed that the yeast decapping enzyme is subject to both negative and positive regulation. Surprisingly, the key regulatory elements for both negative and positive regulation all mapped to the C-terminal domain of Dcp2 that had been neglected in almost all previous *in vitro* decapping experiments.

RESULTS

A small subset of mRNA decapping activators interacts with the Dcp2 catalytic subunit of the yeast decapping enzyme

To delineate the molecular mechanisms involved in decapping enzyme regulation, we first identified the factors that interact with Dcp1 and Dcp2. Using the yeast two-hybrid assay (Fields and Song 1989), we tested for potential interactions between Dcp1 or Dcp2 and the general decapping activators Edc3, Dhh1, Pat1, and Lsm1, or the NMD-specific regulators Upf1, Upf2, and Upf3. In parallel, and as a positive control, we also analyzed interactions between Dcp1 and Dcp2. In this initial set of experiments, we analyzed full-length Dcp1 and Dcp2, as well as a Dcp2 C-terminal fragment lacking the protein's conserved N-terminal 244-amino acid catalytic domain. We included the Dcp2 C-terminal domain in our initial experiments because Dcp2 may exhibit conformation-dependent interactions with its interacting partners. The interactions of Dcp1 or Dcp2 with the decapping activators were tested in both Gal4-DB and Gal4-AD orientations and success in either orientation was deemed evidence for interaction. As shown in Supplemental Figure S1A, Dcp1 and Dcp2 interacted with each other as expected. However, the interactions of Dcp1 and Dcp2 with the decapping activators were more complex. When tested against the full-length proteins, Edc3 interacted with both Dcp1 and Dcp2, Dhh1 and Upf1 interacted with Dcp1 but not Dcp2, and Pat1, Lsm1, Upf2, and Upf3 failed to interact with either Dcp1 or Dcp2. However, both Pat1 and Upf1 interacted strongly with the C-terminal Dcp2-ND244 fragment lacking the protein's catalytic domain. The lack of positive two-hybrid interactions of Lsm1, Upf2, and Upf3 with either subunit of the decapping enzyme was not likely to be due to insufficient expression or misfolding of the respective Gal4-DB or AD fusion proteins in yeast cells as each of these fusion proteins interacted with their respective positive controls (Supplemental Fig. S2A and data not shown).

Since protein:protein interaction results can reflect "bridging" by interacting partners, we generated tester strains harboring complete deletions of *EDC3*, *PAT1*, *DHH1*, and *UPF1*, or a C-terminal deletion of *DCP2* (*N245* allele) (He et al. 2014), and assayed each binary interaction in these strains. The *N245* allele lacks Dcp2's C-terminal 725 amino acids, including its Edc3-binding site (Fig. 1A; Harigaya et al. 2010), and was utilized because *DCP1* and *DCP2* were essential and could not be deleted completely in our tester

strain. As shown in Supplemental Figure S1B, the interactions between Dcp2 and Dcp1, Edc3, Pat1, or Upf1 still occurred in each deletion/truncation strain. However, interactions between Dcp1 and Edc3 or Upf1 were largely eliminated in the *dcp2-N245* strain, as were interactions between Dcp1 and Dhh1 in both the *dcp2-N245* and *edc3Δ* strains. Dhh1 interacted with Edc3 in all the tester strains (Supplemental Fig. S2B), suggesting direct interactions between these two factors, as recently demonstrated by *in vitro* experiments (Sharif et al. 2013). Together, these results indicate that the decapping enzyme interacted directly with only three decapping activators, Edc3, Pat1, and Upf1, and that all three interactions are mediated by Dcp2. Further, the use of tester strains harboring specific deletions or truncations allowed us to conclude that the observed interactions between Dcp1 and Edc3 or Upf1 were bridged through Dcp2 and that between Dcp1 and Dhh1 was bridged through both Dcp2 and Edc3.

These two-hybrid data validate previously observed *in vitro* interactions between Dcp2 and Edc3 (Harigaya et al. 2010), but challenge other results derived from *in vitro* pull-down experiments, including those suggesting direct interactions between Dcp1 and Pat1 (Nissan et al. 2010) or between Dcp2 and Dhh1 (Decker et al. 2007). Previous *in vitro* pull-down experiments also revealed a direct interaction between Dcp2 and Pat1 (Nissan et al. 2010), but this Dcp2:Pat1 interaction differed from that shown by our two-hybrid assays. The *in vitro* Dcp2:Pat1 interaction was mediated by the N-terminal 300-amino acid fragment of Dcp2 whereas our two-hybrid Dcp2:Pat1 interaction was mediated by a nonoverlapping C-terminal fragment of Dcp2 (see below).

Discrete and generally nonoverlapping Dcp1-, Edc3-, Pat1-, and Upf1-interacting domains within Dcp2

To elucidate the potential roles of Dcp1, Edc3, Pat1, and Upf1 in regulating Dcp2 activity, we used the two-hybrid assay and systematic *DCP2* deletions to map the binding sites of each factor on Dcp2 (Fig. 1A,B). Consistent with previous biochemical analyses (She et al. 2008; Harigaya et al. 2010), Dcp1 bound to an N-terminal region encompassing the first 100 amino acids of Dcp2, and Edc3 bound to an internal region (amino acids 246–300) immediately downstream from the Dcp2 catalytic Nudix domain. Pat1 and Upf1 interacted with overlapping or neighboring Dcp2 C-terminal domain fragments. However, the actual Dcp2 binding sites for these two factors appeared to be distinct: Pat1 bound to five small segments from three different regions (amino acids 416–525, 721–880, and 881–970) (Fig. 1A) and Upf1 bound strongly to two small segments (amino acids 416–475 and 661–720), but failed to interact with the 300-amino acid N-terminal Dcp2 segment observed in a previous two-hybrid analysis (Fig. 1B; Swisher and Parker 2011). The Pat1-interacting segments do not exhibit significant overall sequence similarity, but each shares at least one short 5-amino acid leucine-rich

FIGURE 1. Identification of Dcp1, Edc3, Pat1, and Upf1-interacting domains of Dcp2. (A) Mapping of Dcp1, Edc3, and Pat1-interacting domains of Dcp2. (B) Mapping of Upf1-interacting domains of Dcp2. Dcp2 fragments fused to Gal4(DB) or Gal4(AD) were assayed for interactions with Gal4(AD)-Dcp1, Edc3, and Pat1 (A) or Gal4(DB)-Upf1 (B), respectively. A and B include schematic representations of the Dcp2 domain structure and the Dcp2 fragments used in the experiment. Shaded bars indicate interacting fragments, whereas white bars indicate fragments that failed to interact. The minimal binding sites for Dcp1, Edc3, Pat1, and Upf1 are bracketed by dashed lines. In A and B, blue colony color indicates an interaction and white colony color indicates no interaction. Constructs highlighted with a black diamond indicate Dcp2 segments that interact with Upf1, but not Pat1, or vice versa.

motif, suggesting that these motifs are directly involved in Pat1 binding. A total of eight such motifs were identified in these Pat1-interacting segments (Fig. 2A). Multiple leucine-rich motifs were also identified in the C-terminal domain of *S. pombe* Dcp2 (Fromm et al. 2012). In contrast to our two-hybrid data, previous in vitro pull-down and NMR analyses indicated that some of these motifs bind Edc3 or Scd6 (Fromm et al. 2012). The two Upf1-interacting segments exhibit significant similarity over their entire 60-amino acid regions (data not shown), but also share extremely high local identity over an 11 amino acid stretch (eight out of 11) (Fig. 2A). The Dcp1, Edc3, Pat1, and Upf1-interacting domains or motifs identified here likely play important regulatory roles, as each of these domains or motifs is conserved during eukaryotic evolution and exists in the vast majority of fungal Dcp2 orthologs (Fig. 2A; Supplemental Fig. S3). Short linear interacting motifs have also been identified in many other decapping factors from yeast to humans and appear to be localized primarily to the disordered regions of these proteins (Jonas and Izaurralde 2013), suggesting that protein–protein interactions mediated by short linear motifs may be a general feature of eukaryotic decapping regulators.

Mapping of Dcp2-interacting domains of Edc3, Pat1, and Upf1

Using similar assays, we also identified the domains of Edc3, Pat1, and Upf1 involved in Dcp2 binding. Since both Edc3 and Upf1 can self-associate (Decker et al. 2007; He et al. 2013), we utilized two-hybrid tester strains with and without complete deletions of *EDC3* or *UPF1*. Full-length Edc3 interacted strongly with Dcp2 in *edc3Δ* cells and this interaction was eliminated by deletion of Edc3's Lsm domain and substantially diminished by deletion of the YjeF-N domain (Supplemental Fig. S4A). These data suggest that Dcp2 binding by the Edc3 Lsm domain is weak and requires enhancement by the YjeF-N dimerization domain. Consistent with this interpretation, Edc3 fragments containing the YjeF-N domain but lacking the Lsm domain showed no interaction with Dcp2 in *edc3Δ* cells, but exhibited fairly strong interaction with Dcp2 in wild-type cells (Supplemental Fig. S4A, construct F2–C1). Dcp2 interacted strongly with the C-terminal domain of Pat1 (amino acids 422 to 796) (Supplemental Fig. S4B, construct F3–C1), a region that forms repeat helical structures and is also involved in binding the Lsm1-7

FIGURE 2. Identification of the conserved Edc3, Pat1, and Upf1-binding Motifs in the C-terminal domain of Dcp2 and a summary of the interacting domains involving Dcp2 and Dcp1, Edc3, Pat1, and Upf1. (A) Multiple sequence alignments of Dcp2's Edc3-, Upf1-, and Pat1-binding motifs with the respective conserved sequences from other fungal Dcp2 orthologs. Amino acids are colored based on physicochemical properties ([h] hydrophobic, [l] aliphatic, [p] polar, [s] small, [t] turn-like, [u] tiny). (B) Protein domains involved in interactions between Dcp1 and Dcp2, and between Dcp2 and Edc3, Pat1, or Upf1. Schematic representations of the domain structures for each factor are shown. Interactions between factors are indicated by red lines with arrowheads, and domains implicated in each interaction are marked by straight blue lines. For Dcp2, E-BD indicates Edc3-binding domain, U-BD indicates Upf1-binding domain, and P-BD indicates Pat1-binding domain.

complex (Sharif and Conti 2013; Wu et al. 2014). Binding of Dcp2 to Upf1 was found to be mediated by amino acids 62–181 of the Upf1 CH-domain (Supplemental Fig. S4C, construct 2–16). In contrast to a previous study (Swisher and Parker 2011), we found that deleting *UPF1*, *EDC3*, or *PAT1* from the tester strain did not affect Dcp2 binding to this Upf1 region (Supplemental Fig. S4C), indicating that the observed Dcp2:Upf1 interaction is not bridged by any of these factors. This region of Upf1 is also involved in Upf2 binding (He et al. 1997) and self-association (He et al. 2013), suggesting that it may play a role in sequential molecular interactions during activation of NMD.

Dcp2 C-terminal domain contains an inhibitory element that subjects the enzyme to negative regulation

Even though our two-hybrid analyses identified specific Dcp2 C-terminal domain binding sites for Edc3, Pat1, and Upf1 (Fig. 2B), it was important to determine whether these sites had functional significance. Accordingly, we evaluated their possible regulatory roles by generating incremental Dcp2 C-terminal deletions and analyzing their consequences on the steady-state levels of *YRA1* pre-mRNA in the presence or absence of Edc3, i.e., in *dcp2Δ* or *edc3Δdcp2Δ* cells. *YRA1* pre-mRNA is a substrate of the Edc3-mediated decay pathway and we have previously shown that this transcript is degraded in the cytoplasm by a translation-independent decapping mechanism (Dong et al. 2010). *DCP2* wild-type and deletion mutant alleles on centromere-based plasmids were individually introduced into *dcp2Δ* and *edc3Δdcp2Δ* cells and *YRA1* pre-mRNA levels in the resulting cells were analyzed by Northern blotting. Cells harboring the empty vector served as negative controls. In the presence of Edc3 (i.e., in *dcp2Δ* cells), Dcp2 C-terminal deletions up to amino acid 245, including the Edc3-binding site, had no apparent consequence on *YRA1* pre-mRNA decay. Almost all the mutant alleles behaved like the wild-type allele and promoted efficient *YRA1* pre-mRNA degradation (Fig. 3A). Cells harboring the *dcp2-N300* and *N245* alleles had subtle differences from the other alleles, consistently accumulating slightly lower or higher levels of *YRA1* pre-mRNA, respectively (Fig. 3A). The *dcp2-N210* allele, whose large deletion encompasses box B sequences of the Nudix domain, appeared to eliminate Dcp2 function for *YRA1* pre-mRNA. Cells harboring the *dcp2-N210* allele were also defective in NMD and general mRNA decay (data not shown), indicating that this allele encodes a nonfunctional decapping enzyme. In the absence of Edc3 (i.e., in *edc3Δdcp2Δ* cells), the *dcp2* alleles that were apparently functional in *dcp2Δ* cells yielded two distinct decay phenotypes. Alleles harboring deletions up to amino acid 475 behaved like wild-type *DCP2*, i.e., *edc3Δdcp2Δ* cells harboring these alleles accumulated high levels of *YRA1* pre-mRNA (Fig. 3A, lanes 2–5), indicating a failure to promote *YRA1* pre-mRNA decapping in the absence of

Edc3 activation. In contrast, *edc3Δdcp2Δ* cells with *dcp2* alleles harboring deletions up to amino acids 300 or 245 accumulated significantly lower levels of *YRA1* pre-mRNA (Fig. 3A, lanes 6,7), indicating an ability to promote *YRA1* pre-mRNA decapping even in the absence of Edc3. These results indicate that the Dcp2 C-terminal domain contains a *cis*-inhibitory element between amino acids 300–475, and that this element inhibits *YRA1* pre-mRNA decay in the absence of Edc3.

To determine whether the decreased steady-state accumulation of *YRA1* pre-mRNA in *edc3Δdcp2Δ* cells harboring the *dcp2-N300* or *N245* allele is directly attributable to increased decapping activity, we generated *dcp2-N245* alleles harboring mutations in catalytically important residues, including those functioning as general acid (K135A) or general base (E153Q), or involved in metal-binding (E198Q) (Aglietti et al. 2013), and analyzed the effects of these mutations in *YRA1* pre-mRNA decay in *dcp2Δ* and *edc3Δdcp2Δ* cells. In contrast to the unmodified *dcp2-N245* allele, the *dcp2-N245* alleles harboring each of these amino acid substitutions failed to promote *YRA1* pre-mRNA decay and resulted in the increased accumulation of *YRA1* pre-mRNA in both *dcp2Δ* and *edc3Δdcp2Δ* cells (Fig. 3B). These results indicate that the decreased accumulation of *YRA1* pre-mRNA in *edc3Δdcp2Δ* cells harboring the *dcp2-N245* allele is indeed caused by increased activity of the decapping enzyme and suggests that the inhibitory element mainly targets the functions of the Dcp2 catalytic domain. In addition to *YRA1* pre-mRNA, the *dcp2-N245* allele also targets the *ade2-1* mRNA, an NMD substrate. As shown in Figure 3B, *dcp2Δ* and *edc3Δdcp2Δ* cells harboring the *dcp2-N245* allele accumulated low levels of *ade2-1* mRNA, but cells harboring the *dcp2-N245-K135A*, *E153Q*, or *E198Q* alleles all accumulated high levels of *ade2-1* mRNA. Together, these results indicate that elimination of the Dcp2 inhibitory element results in constitutive activation of the decapping enzyme and that the activated enzyme appears to target substrates of both the Edc3 and NMD pathways in yeast cells.

Since deleting the putative *cis*-inhibitory element resulted in constitutively active Dcp2 activity for *YRA1* pre-mRNA, we determined whether the constitutively active *dcp2* alleles depended on additional decapping regulators. We generated *edc3Δdcp2Δ* cells harboring deletions of *DCP1*, *UPF1*, *DHH1*, *PAT1*, or *LSM1* and analyzed the activity of the different *dcp2* alleles in the resulting cells. Deleting *DCP1* eliminated the constitutive decay activity of the *dcp2-N300* and *N245* alleles for *YRA1* pre-mRNA (Fig. 3A), whereas deleting *UPF1*, *DHH1*, *PAT1*, or *LSM1* had no significant effects (Supplemental Fig. S5A). These results indicate that the constitutively active *dcp2* alleles require Dcp1, but are independent of other decapping activators for decapping activity. Consistent with the requirement for Dcp1, the constitutively active *dcp2* alleles also require their N-terminal Dcp1-interacting region for mRNA decay activity (Supplemental Fig. S5B).

FIGURE 3. The Dcp2 C-terminal domain harbors an inhibitory element that targets the Dcp2 catalytic domain and subjects the decapping enzyme to negative regulation. (A) Northern analysis of the consequences of incremental deletions from the C-terminus of Dcp2 on *YRA1* pre-mRNA decay in *dcp2*Δ and *edc3*Δ*dcp2*Δ cells. (B) Northern analysis of the effects of amino acid substitutions in the catalytically important residues on *dcp2*-N245 decay activity in *dcp2*Δ and *edc3*Δ*dcp2*Δ cells. In A and B, schematic representations of Dcp2 domain structure and *dcp2* alleles used in the experiment are shown on top and the positions of the Edc3, Upf1, and Pat1-binding sites are indicated by dashed lines. Black dots in the Nudix domains shown in B demark positions of amino acid substitutions. Representative Northern blots were hybridized with random-primed probes specific for *YRA1* pre-mRNA, *ade2-1* mRNA, and the *SCR1* transcript, with the last serving as a loading control. The relative levels of *YRA1* pre-mRNA or *ade2-1* mRNA derived from the Northern blots are depicted as bar graphs at the bottom of each panel. The data were the average of at least three independent experiments and were normalized to the respective cells harboring the wild-type *DCP2* allele (labeled 1 in red). The red error bars indicate standard deviations. The red stars indicate the *P*-values from paired two-tailed Student's *t*-tests ([*] *P* < 0.05, [**] *P* < 0.01).

Activity of the Dcp2 *cis*-acting inhibitory element requires amino acids 350–375

To map the Dcp2 *cis*-inhibitory element more precisely, we generated additional deletions between amino acids 300 and 475 and analyzed their effects on *YRA1* pre-mRNA levels in *edc3Δdcp2Δ* cells (Fig. 4A). C-terminal deletions up to amino acid 375, including the overlapping Upf1/Pat1-binding fragment, failed to activate Dcp2 decay activity for *YRA1* pre-mRNA, but additional deletion of amino acids 350–375 was sufficient for activation. Further deletions up to amino acid 245, including the Edc3-binding site, did not enhance *YRA1* pre-mRNA decay. Internal deletion of amino

acids 300–350 of Dcp2 was inconsequential, but internal deletion of amino acids 300–400 promoted the *YRA1* pre-mRNA decay activity (Fig. 4B). Together, these results indicate that Dcp2's *cis*-inhibitory activity requires amino acids 350–375, a segment located between but distinct from the Edc3- and Upf1/Pat1-binding sites (Fig. 4A). This inhibitory element is rich in both proline and phenylalanine, and is conserved in Dcp2 orthologs from yeast species closely or remotely related to *Saccharomyces cerevisiae* (Fig. 4C).

To test whether the increased *YRA1* pre-mRNA decay activity observed with constitutively active *dcp2* alleles results mainly from loss of the inhibitory element, and not from increased protein expression, we generated HA-tagged versions

FIGURE 4. Mapping of the *cis*-inhibitory element in the C-terminal domain of Dcp2, and analyzing the effects of loss of this element on Dcp2 protein accumulation and mRNA decay function. (A) Northern analysis of the effects of small incremental deletions between amino acids 300–475 of Dcp2 on *YRA1* pre-mRNA decay in *edc3Δdcp2Δ* cells. (B) Northern analysis of the effects of small internal deletions between amino acids 300–400 of Dcp2 on *YRA1* pre-mRNA decay in *edc3Δdcp2Δ* cells. (C) Sequence alignment of the Dcp2 inhibitory element with conserved sequences from Dcp2 orthologs of several other yeast species. (D) Western analysis of the effects of C-terminal and internal deletions on Dcp2 protein expression in *edc3Δdcp2Δ* cells using triple-HA tagged *dcp2* alleles. (E) Northern analysis of the consequences of the Dcp2 C-terminal N245 deletion on *EDC1* mRNA and *POR1* mRNA levels in control cells or cells harboring deletions of the *EDC3*, *UPF1*, *PAT1*, *LSM1*, or *DHH1* genes. In A and B, schematic representations of Dcp2 domain structure and *dcp2* alleles used in the experiment are shown on top and the positions of Edc3, Upf1, and Pat1-binding sites are indicated by dashed lines. The Dcp2 amino acid sequence required for *cis*-inhibitory activity is highlighted by a red bar. In A, B, and E, representative Northern blots were hybridized with random-primed probes specific for *YRA1* pre-mRNA, *EDC1* mRNA, *POR1* mRNA, or the *SCR1* transcript. The relative levels of *YRA1* pre-mRNA, *EDC1* mRNA, and *POR1* mRNA derived from Northern blots are depicted as bar graphs of the corresponding panels. The data comprise the average of at least three independent experiments and were normalized to the respective cells harboring the wild-type *DCP2* allele (labeled 1 in red). The red error bars indicate standard deviations. The red stars indicate the *P*-values from paired two-tailed Student's *t*-tests ([*] $P < 0.05$, [**] $P < 0.01$). In D, Western blots were probed with monoclonal antibodies against the HA epitope or Pgk1, with the latter serving as a loading control.

of each *dcp2* allele analyzed above (Supplemental Fig. S6A) and examined their levels of expression and extent of activity in *YRA1* pre-mRNA decay in *dcp2Δ* and *edc3Δdcp2Δ* cells (Fig. 4D; Supplemental Fig. S6B,C). These experiments led to several important observations. First, each of the HA-tagged *dcp2* alleles behaved the same as its untagged counterpart in *YRA1* pre-mRNA decay activity (compare the untagged *dcp2* alleles in Fig. 3A to the HA-tagged counterparts in Supplemental Fig. S6B, and data not shown), indicating that HA-tagging does not alter the function of the *dcp2* alleles. Second, different HA-tagged *dcp2* alleles exhibited different levels of protein. However, each HA-tagged *dcp2* allele had almost identical protein levels in *dcp2Δ* and *edc3Δdcp2Δ* cells (Fig. 4D; Supplemental Fig. S6C), indicating that the presence or absence of Edc3 does not affect the expression of these *dcp2* alleles. Third, HA-tagged *dcp2* alleles harboring larger C-terminal deletions generally resulted in increased Dcp2 levels (Fig. 4D; Supplemental Fig. S6C), but these increased Dcp2 levels did not correlate with increased *YRA1* pre-mRNA decay activity. For example, *HA-dcp2-N430* and *HA-dcp2-N300* had similar levels of Dcp2 protein (Fig. 4D; Supplemental Fig. S6C), but exhibited distinct *YRA1* pre-mRNA decay activity. The former was inert and the latter was constitutively active (Fig. 4A). Collectively, these observations indicate that the increased *YRA1* pre-mRNA decay activity of the constitutively active *dcp2* alleles is due to loss of the inhibitory element, not to changes in the levels of protein expression. C-terminal truncations have also been shown to increase accumulation of human Dcp2 (Erickson et al. 2015). These observations indicate that in vivo levels of yeast and human Dcp2 are both subject to regulation by protein degradation. However, whether yeast Dcp2 is also controlled by proteasome-mediated degradation is currently unknown.

Since loss of the Dcp2 *cis*-inhibitory activity leads to Edc3 independent decay of *YRA1* pre-mRNA, this indicates that negative regulation plays a role in determining the decapping enzyme's substrate specificity. Consistent with this idea, we also found that eliminating the Dcp2 C-terminus also resulted in loss of Dhh1 control of its two substrates, *EDC1* mRNA (Muhlrad and Parker 2005) and *POR1* mRNA (Fig. 4E, cf. lanes 1 and 6 and lanes 7 to 12; Chang and Lee 2012).

Roles of the decapping activators and their interactions with Dcp2 in decapping regulation

In addition to harboring an inhibitory element, the Dcp2 C-terminal domain also contains a set of conserved binding sites for the decapping activators Edc3, Pat1, and Upf1 (see above). We hypothesized that interactions of these conserved binding sites with their corresponding decapping activators could control the decapping enzyme's substrate specificity, targeting, and final activation. In the following experiments, we exploited the fact that the product of the *dcp2-N245* allele is a constitutively active catalytic subunit that no longer binds to Edc3, Pat1, or Upf1 to dissect the roles of the decapping ac-

tivators and their interactions with Dcp2 in decapping regulation. We focused on Edc3 and Upf1. To assess the roles of Edc3:Dcp2 and Upf1:Dcp2 interactions and to determine whether Edc3 and Upf1 may have additional functions independent of their Dcp2 interactions in decapping regulation, we constructed a set of yeast strains harboring genomic wild-type *DCP2*, *dcp2-N245*, or *dcp2-N245* tagged with a basic zipper R34 (Bashor et al. 2008) in *EDC3*, *UPF1*, *edc3Δ*, *upf1Δ*, and *edc3Δupf1Δ* backgrounds and analyzed mRNA decay phenotypes for several representative transcripts of the Edc3, NMD, and general 5' to 3' decay pathways. As shown in Figure 5A and Supplemental Figure S7, eliminating the C-terminal domain of Dcp2 compromised both Edc3-mediated decay and NMD, causing complete stabilization of *RPS28B* mRNA and partial stabilization of *YRA1* pre-mRNA, two substrates of Edc3-mediated decay (Badis et al. 2004; Dong et al. 2007), as well as modest (about 1.5-fold) but consistent stabilization of *ade2-1* mRNA and *CYH2* pre-mRNA, two NMD substrates (He et al. 1993; Maderazo et al. 2003). These observations show that the constitutively active Dcp2-N245 decapping enzyme is at least partially deficient in degrading the substrates of the Edc3 and NMD pathways, probably due to loss of positive Edc3:Dcp2 and Upf1:Dcp2 interactions. One implication of these observations is that decapping of Edc3 and NMD substrates is dependent on positive regulatory mechanisms. In cells harboring either *dcp2-N245* allele, eliminating Edc3 caused further stabilization of *YRA1* pre-mRNA (approximately twofold) and eliminating Upf1 caused significant stabilization of *ade2-1* mRNA and *CYH2* pre-mRNA (>10-fold in each case) (Fig. 5A). Since *Dcp2-N245* binds neither Edc3 nor Upf1, the observed effect of deleting *EDC3* or *UPF1* on mRNA stability in *dcp2-N245* cells most likely reflects the existence of Edc3 and Upf1 functions that are independent of Dcp2 interactions. Furthermore, regardless of the status of *EDC3* or *UPF1*, eliminating the C-terminal domain of Dcp2 promoted the decay of *PGK1* and *TCM1* mRNAs, two general mRNA decay substrates (Decker and Parker 1993; He and Jacobson 2001). This result indicates that decapping of these two general mRNAs is mostly controlled through inhibitory mechanisms that likely include the direct inhibition of the decapping enzyme by the Dcp2 inhibitory element and sequestration of the decapping enzyme by competitive Edc3:Dcp2 and Upf1:Dcp2 interactions. Consistent with this interpretation, we found that deletion of *UPF1* in a wild-type *DCP2* background routinely led to decreased accumulation of general mRNAs (data not shown). These results suggest that Edc3 and Upf1 probably compete with Pat1 for binding to the decapping enzyme and thus limit the available pool of decapping enzyme for general mRNAs. Together, these results indicate that Edc3:Dcp2 and Upf1:Dcp2 interactions have positive roles in targeting the decapping enzyme to specific mRNAs, but can also have negative effects on the decapping of unrelated general mRNAs. In addition, Edc3 and Upf1 both appear to have additional functions that are independent of their interactions with Dcp2.

FIGURE 5. The Dcp2 C-terminal domain plays an important role in both transcript-specific and general mRNA decapping. (A) Northern analysis of the consequences of the Dcp2 C-terminal N245 deletion on Edc3-mediated mRNA decay, NMD, and general mRNA decay in yeast cells harboring either deletions of the *EDC3* or *UPF1* genes or the respective wild-type genes. Schematic representations of the Dcp2 domain structure and the genomic R34-tagged or untagged *dcp2-N245* allele used in the experiments are shown on top. Overexposed blots for *CYH2* pre-mRNA and *ade2-1* mRNA are indicated by an asterisk. (B) Northern analysis of the effect of restoring Edc3 interaction with *dcp2-N245* on *YRA1* pre-mRNA decay. Schematic representations of the Edc3 domain structure and *EDC3* alleles tagged with either a basic or acidic leucine zipper used in the experiments are shown on top. V indicates the empty vector control. In A and B, representative Northern blots were hybridized to random-primed probes specific for the indicated transcripts with the *SCR1* transcript serving as a loading control. The relative levels of each transcript derived from Northern blots are depicted in bar graphs on the right side of each panel. The data are the average of at least three independent experiments and are normalized to the cells harboring the wild-type *DCP2* allele (A) or the empty vector (B) (labeled 1 or V in red, respectively). The red error bars indicate standard deviations. The red stars indicate the *P*-values from paired two-tailed Student's *t*-tests ([*] $P < 0.05$, [**] $P < 0.01$).

One potential role of Edc3:Dcp2 and Upf1:Dcp2 interactions is the formation of distinct decapping enzyme complexes that can target specific mRNAs. To test this possibility, we examined whether restoring interaction between Edc3 and Dcp2-N245 through artificial leucine-zippers capable of heterodimerization (Bashor et al. 2008) could promote *YRA1*

pre-mRNA decay. A set of *EDC3* alleles with C-terminal tags of acidic E34-I, E34-V, or E34-N zippers was generated on centromere-based plasmids. These tagged *EDC3* alleles were individually introduced into an *edc3Δdcp2-N245-R34* strain (expressing *dcp2-N245* tagged with a basic zipper) and the resulting cells were analyzed for *YRA1* pre-mRNA

levels. As controls, we also created strains harboring wild-type or R34-tagged *EDC3* alleles, or the empty vector, and also introduced each *EDC3* allele into an *edc3Δdcp2-N245* strain (expressing *dcp2-N245* with no zipper). The *edc3Δdcp2-N245-R34* and *edc3Δdcp2-N245* strains harboring the empty vector behaved identically and both showed high levels of *YRA1* pre-mRNA accumulation (Fig. 5B, both lanes designated V), indicating that the R34 tag does not alter the function of Dcp2-N245. When wild-type *EDC3* and each of its tagged alleles were introduced into the control *edc3Δdcp2-N245* strain, they behaved similarly and each allele enhanced *YRA1* pre-mRNA decay slightly relative to the empty vector control (Fig. 5B, left panel, cf. lanes 1–5 to V). This decay-enhancing effect most likely reflects an Edc3 function independent of its interaction with the decapping enzyme (see above). When wild-type *EDC3* and each of its tagged alleles were introduced into the *edc3Δdcp2-N245-R34* strain, they yielded distinct *YRA1* pre-mRNA decay phenotypes. The wild-type and R34-tagged *EDC3* alleles again enhanced *YRA1* pre-mRNA decay slightly whereas the E34-I, E34-V, and E34-N-tagged alleles promoted more efficient decay (Fig. 5B, right panel, lanes 3–5). These results show that coexpression of Dcp2-N245 and Edc3 tagged with pairs of leucine zippers capable of heterodimerization promotes *YRA1* pre-mRNA decay. Although E34-I, E34-V, or E34-N bind R34 with different affinities in vitro ($K_d = 6.1, 421, \text{ and } 810 \text{ nM}$, respectively) (Bashor et al. 2008), *EDC3* alleles tagged with E34-I, E34-V, or E34-N reduced *YRA1* pre-mRNA levels to similar extents, indicating that Edc3 interaction with Dcp2 readily targets the decapping enzyme to *YRA1* pre-mRNA.

In addition to targeting specific transcripts, our genetic experiment in Figure 3A revealed that Edc3:Dcp2 interaction also plays an important role in decapping enzyme activation. We observed that *dcp2* alleles harboring the inhibitory element were completely deficient in *YRA1* pre-mRNA decay in the absence of Edc3. However, each of these alleles promoted efficient *YRA1* pre-mRNA degradation in the presence of Edc3. Significantly, *dcp2* alleles lacking the inhibitory element promoted *YRA1* pre-mRNA decay even in the absence of Edc3 (Fig. 3A). These results indicate that the presence of the inhibitory element in Dcp2 imposes a strict functional requirement for Edc3 and that elimination of the inhibitory element in Dcp2 can bypass the Edc3 function for *YRA1* pre-mRNA decapping. These observations argue that Edc3:Dcp2 interaction functions at least in part to counteract the inhibitory activity imposed on Dcp2, a step required for decapping activation.

DISCUSSION

The yeast decapping enzyme is subject to both negative and positive regulation

In contrast to current models that are largely based on in vitro experimentation (Floor et al. 2010; Nissan et al. 2010; Borja

et al. 2011), the in vivo studies reported here reveal that the yeast decapping enzyme is subject to both negative and positive regulation. Using a combination of two-hybrid assays, deletion alleles, point mutants, and quantitative assessments of mRNA decay activity, we found that the long-neglected large C-terminal domain of Dcp2 contains two key types of regulatory elements: (i) a negative regulatory element that inhibits decapping enzyme activity and (ii) a set of positive regulatory elements that recruit the specific decapping activators Edc3, Pat1, and Upf1. Although the Dcp2 sites through which they appear to act are novel, positive regulation of the decapping enzyme by specific decapping activators is largely expected (Parker 2012). However, negative regulation of the decapping enzyme by a *cis*-inhibitory element in Dcp2 is surprising, and undoubtedly significant, as elimination of the inhibitory element results in constitutive activation of the decapping enzyme and loss of the enzyme's specificity for substrate mRNAs. Negative regulation of the decapping enzyme by an inhibitory element could serve two important purposes for eukaryotic cells. One would be the avoidance of accidental or unregulated mRNA decapping and the other would enable temporal control of decapping enzyme activation.

Positive regulation of the decapping enzyme appears to be mediated by the binding of specific decapping activators to the Dcp2 C-terminal domain and the likely formation of distinct complexes that control the decapping enzyme's substrate specificity and final activation (see below). Positive regulation of the yeast decapping enzyme by several decapping activators has been documented in many previous in vitro studies (Harigaya et al. 2010; Nissan et al. 2010; Fromm et al. 2012), but those results differ substantially from the in vivo experiments reported here. These differences are not surprising. The in vitro experiments, including structural, enzymatic, and protein-binding analyses (Steiger et al. 2003; Decker et al. 2007; Deshmukh et al. 2008; She et al. 2008; Floor et al. 2010; Harigaya et al. 2010; Nissan et al. 2010; Borja et al. 2011) used either of two N-terminal Dcp2 fragments (Dcp2-N245 or Dcp2-N300), both of which we have now shown to lack the inhibitory element and most or all of the binding sites for Edc3, Pat1, and Upf1, and to be constitutively active in mRNA decapping in vivo (Fig. 3A). Further, the activity of these two constitutively active forms of Dcp2 is independent of the functions of almost all characterized decapping activators, including Edc3, Pat1, Dhh1, Lsm1, and Upf1 (Supplemental Fig. S5).

Regulatory functions of yeast decapping activators

Our experiments reveal that the decapping activators Edc3, Pat1, and Upf1 bind to a set of distinct conserved regulatory elements in the C-terminal domain of Dcp2 and suggest at least two key functions for these interactions: control of the decapping enzyme's substrate specificity and triggering of enzyme activation. The latter point is best illustrated by Edc3-

mediated decay of *YRA1* pre-mRNA. Figures 3A and 4A demonstrate that when the inhibitory element is present in Dcp2 the decay of *YRA1* pre-mRNA has a strict requirement for Edc3. In contrast, when the inhibitory element is deleted, *YRA1* pre-mRNA decay can bypass its requirement for Edc3. This implies that the binding of Edc3 to Dcp2 alleviates the inhibitory activity otherwise imposed on Dcp2, thus promoting decapping of the Edc3 substrate, *YRA1* pre-mRNA.

Edc3, Pat1, and Upf1 each appear to bind directly to their respective substrate mRNAs (Chowdhury et al. 2007; Johansson et al. 2007; He et al. 2014). These results, and our observations that the same proteins also bind to distinct sites in Dcp2, strongly suggest that these decapping activators directly control the targeting or substrate specificity of the decapping enzyme. Further, the binding of Edc3, Pat1, and Upf1 to the Dcp2 C-terminal domain most likely leads to the formation of distinct decapping complexes instead of a single large complex encompassing most activators, as suggested earlier (Nissan et al. 2010; Swisher and Parker 2011). Support for this conclusion follows from three observations. First, the Edc3, Pat1, and Upf1-binding sites in the Dcp2 C-terminal domain are close to each other (Fig. 1A,B), suggesting that the binding of these factors to Dcp2 may well be mutually exclusive. Second, the effects of deleting *EDC3*, *PAT1*, or *UPF1* are highly specific and each of these gene deletions selectively inhibits the decay of mRNAs of a specific decay pathway, but not the unrelated decay pathways (Fig. 5A). Third, although deletion of *EDC3*, *PAT1*, or *UPF1* each inhibits the activity of a specific decay pathway, we observed that deletion of *UPF1* also has indirect effects on apparently unrelated mRNA decay pathways and consistently promotes the degradation of the substrates of the Edc3 or general decay pathways (Fig. 5A; Supplemental Fig. S7), i.e., elimination of the factor controlling one pathway may make additional Dcp2 available for another pathway. In turn, this implies that in addition to providing the decapping enzyme with unique substrate or targeting specificity, the formation of distinct decapping enzyme complexes by Edc3, Pat1, and Upf1 may also allow dynamic regulation between different decapping-dependent decay pathways in vivo in response to changes in development, growth, or stress signals.

In addition to roles in targeting and activating the decapping enzyme, Edc3 and Upf1 both appear to have additional functions in mRNA decapping that are independent of their interactions with Dcp2. A key observation is that in yeast cells harboring the *dcp2-N245* allele, deletion of *EDC3* or *UPF1* resulted in significant stabilization (two- to 10-fold) of the respective substrate mRNAs (Fig. 5A). Since the *dcp2-N245* allele encodes a constitutively active decapping enzyme that no longer binds to Edc3 or Upf1, these effects of *EDC3* or *UPF1* deletion in *dcp2-N245* cells must result from loss of Edc3 or Upf1 functions that occur independently of Dcp2 interactions. One possibility is that Edc3 and Upf1 both have functions in mRNA decapping that are upstream of the formation of a decapping complex or activation of the decapping

enzyme, such as repressing mRNA translation, or tagging or remodeling specific mRNPs (Coller and Parker 2005; Franks et al. 2010; Ghosh et al. 2010). The occurrence of functions independent of Dcp2 interaction, and the ability of these factors to bind directly to their substrate mRNAs (Chowdhury et al. 2007; Johansson et al. 2007; He et al. 2014), suggests that the assembly of the active decapping complexes most likely occurs on mRNAs and that Edc3 and Upf1 function in recruiting the decapping enzyme to their targeted mRNAs. Since both Edc3 and Upf1 self-associate and the self-association of these factors appears to be critical for their respective decay activities (He et al. 2013, 2014), it is possible that the presumptive upstream functions for Edc3 and Upf1 are carried out by distinct Edc3 or Upf1 molecules other than those in complexes with the decapping enzyme. In this scenario, the decapping enzyme would be recruited to the respective decay substrates by either Edc3 or Upf1 dimerization.

Roles of negative and positive regulation in mRNA decapping in vivo

Although deleting the entire C-terminal domain of Dcp2 would in principle eliminate both the negative and positive regulation proposed here, the actual consequences of that deletion did not have a uniform effect on the decapping of different transcripts, including those degraded by the same decay pathway (Fig. 5A). For example, deletion of the entire Dcp2 C-terminal domain led to complete stabilization of one Edc3 substrate, *RPS28B* mRNA, but only partial stabilization of another Edc3 substrate, *YRA1* pre-mRNA (Fig. 5A). Concurrently, the same deletion yielded only marginal stabilization of two NMD substrates, *CYH2* pre-mRNA and *ade2-1* mRNA. Since elimination of the entire Dcp2 C-terminal domain resulted in constitutively active decapping enzyme, the different consequences of this deletion on decay of the Edc3 and NMD substrates may reflect distinct requirements or sensitivities of these transcripts for positive regulation. Decapping of *RPS28B* mRNA may require Edc3:Dcp2 interaction for both enzyme recruitment and activation whereas decapping of *YRA1* pre-mRNA may require Edc3:Dcp2 interaction mostly for enzyme activation, but be less critical for the enzyme's recruitment because *YRA1* pre-mRNA is translationally repressed (Dong et al. 2010). In support of this argument, restoring the interactions between constitutively active *dcp2-N245* and Edc3 only had a modest effect on *YRA1* pre-mRNA decay, accelerating the decay of this transcript about twofold (Fig. 5B). The same may be true for decapping of nonsense-containing mRNAs. Such NMD substrates may be principally dependent on Upf1:Dcp2 interaction for enzyme activation, but less so for enzyme recruitment as recognition of NMD substrates also leads to translational repression (Muhlrad and Parker 1999). In addition, since deletion of *UPF1* in *dcp2-N245* cells still resulted in substantial stabilization of NMD substrates (Fig. 5A), Upf1-

mediated decapping enzyme activation and recruitment probably do not control a rate-limiting step for NMD, i.e., Upf1's major function in NMD may occur earlier and involve remodeling of terminating mRNPs. Complicating the interpretation of the consequences of Dcp2 C-terminal deletion on the decay of individual transcripts is the fact that the levels of any mRNA reflect the net outcome of eliminating both the negative and positive regulation. Because loss of negative regulation results in decapping enzyme activation, any loss of positive regulation is likely to be offset to some extent by a gain of function from the loss of negative regulation. Thus, for example, the partial effect of Dcp2 C-terminal domain deletion on *YRA1* pre-mRNA levels and the marginal effect of Dcp2 C-terminal domain deletion on *CYH2* pre-mRNA and *ade2-1* mRNA levels are likely to be underestimates of the role of positive regulation in the decapping of these transcripts. Future analyses of small deletions or point mutations in each of these positive elements that specifically disrupt Dcp2 interactions with Edc3, Upf1, and Pat1 (or combinations of such mutations) are required to further define the roles of the positive elements in decapping regulation of different mRNAs.

Constitutive activation of the decapping enzyme caused by eliminating the Dcp2 C-terminal domain also promoted the degradation of two general transcripts, the *PGK1* and *TCM1* mRNAs (Fig. 5A). This result indicates that constitutively active Dcp2 also targets general mRNAs and that, in contrast to the Edc3 and NMD substrates, decapping of general mRNAs is in large part controlled through negative regulatory mechanisms. These may include direct inhibition of decapping enzyme activity by the Dcp2 inhibitory element and/or sequestration of the decapping enzyme through the formation of distinct and competing decapping complexes. Consistent with the latter possibility, we observed that, in wild-type *DCP2* cells, deletion of *UPF1* consistently caused decreased accumulation of general mRNAs (Supplemental Fig. S7 and data not shown). We also found that constitutively active Dcp2-N245 also targets two decapping substrate mRNAs regulated by decapping activator Dhh1 (Fig. 4E). In short, our results indicate that in vivo the decapping enzyme is controlled through both negative and positive regulatory elements.

A new model for decapping enzyme regulation

Collectively, our data reveal a pivotal role for the Dcp2 C-terminal domain in mRNA decapping and lead to a new model for decapping enzyme regulation in vivo (Fig. 6). In this model, the Dcp2 C-terminal domain contains both negative

FIGURE 6. Model for in vivo regulation of the yeast mRNA decapping enzyme. Details are described in the main text. In the Dcp2 images, *N* indicates the negative regulatory element. *P1*, *P2*, *P3*, and *P4* indicate different positive regulatory elements, including the Edc3, Pat1, and Upf1-binding sites.

and positive regulatory elements. The negative element is thought to inhibit the enzymatic or substrate-binding activity of the decapping enzyme and promote downstream functions of the positive elements. The positive elements promote the binding of specific decapping activators (such as Edc3, Pat1, and Upf1) to Dcp2. In turn, binding of the decapping activators to the positive elements is thought to alleviate the inhibitory activity imposed on Dcp2 by the negative element and promote the formation of distinct decapping complexes. These decapping complexes possess unique substrate specificities and target specific mRNAs. Decapping activation is thus coupled to both the assembly of decapping complexes and substrate recognition by these complexes, a regulatory mechanism reminiscent of protein phosphatase regulation (Shi 2009). Much like protein phosphatases, a single decapping enzyme targets a variety of substrate mRNAs and uses stepwise assembly of decapping complexes to control both substrate specificity and enzymatic activation.

MATERIALS AND METHODS

General methods

Preparation of standard yeast media and methods of cell culture were as described previously (Guthrie and Fink 1991). Transformation of yeast was done by the high-efficiency method (Schieftel and Gietz 1989), and DNA manipulations were performed by standard techniques (Sambrook et al. 1989). All PCR amplifications were performed with Taq DNA polymerase and confirmed by DNA sequencing.

Yeast strains

All strains used in this study are listed in Supplemental Table S1. Strains containing complete deletions of *DCP1*, *DCP2*, *EDC3*, *PAT1*, *LSM1*, *DHH1*, and *UPF1* were constructed by gene

replacement (Guthrie and Fink 1991), using DNA fragments harboring the corresponding null alleles. All null alleles were generated from plasmid DNA by restriction enzyme digestion except for the *edc3::KanMX6* allele. The *edc3::KanMX6* allele was generated by PCR using the oligonucleotide pair *EDC3-DSK-F* and *EDC3-DSK-R* (Supplemental Table S3). Strains containing a 725-amino acid C-terminal deletion of *DCP2* were also constructed by gene replacement, using a DNA fragment harboring either the *dcp2-N245::KanMX6* or the *dcp2-N245-R34::KanMX6* allele. Each genomic DNA deletion or integration was confirmed by PCR analysis. Deletion of *DCP1* or *DCP2* resulted in lethality in the GGY1::171 background, but slow growth in the HFY114 background. Accordingly, all genetic experiments in this study used strains derived from HFY114.

Plasmids

All plasmids used in this study are listed in Supplemental Table S2. Plasmids harboring the *upf1::HIS3*, *dcp2::HIS3*, *dcp2-N245::KanMX6*, and *edc3::URA3* alleles, or containing *UPF1* fragments fused to either the *GAL4* DNA-binding domain (DB) or activation domain (AD), or containing *EDC3* fragments fused to *GAL4-AD* were described previously (He et al. 1997, 2013, 2014). Details of plasmid construction are described in Supplemental Information.

Oligonucleotides

The oligonucleotides used in this study were obtained from Eurofins MWG/Operon, Inc., and are listed in Supplemental Table S3.

Yeast two-hybrid interaction assay

Two-hybrid assays utilized previously described procedures (Fields and Song 1989). All the two-hybrid interactions were initially assayed in the GGY1::171 tester strain. In each case, a *GAL4(DB)* fusion construct was cotransformed with a *GAL4(AD)* construct into the tester strain. Transformants were incubated for 3–5 d at 30°C until colonies were fairly large and then replicated on X-Gal-containing plates. The color phenotypes of two independent transformants from each interaction assay were presented in the figures. To assess the potential for self-activation, each of the *GAL4(DB)* fusions was also cotransformed with a *GAL4(AD)* empty vector into the tester strain and assayed in parallel with constructs testing for interaction (Supplemental Fig. S2C). In experiments where interactions were tested in both *DB* and *AD* orientations (see Supplemental Fig. S1A), success in either orientation was deemed evidence for an interaction. To assess the potential for molecular interactions that were bridged, we also carried out most two-hybrid analyses in tester strains harboring complete deletions of *EDC3*, *PAT1*, *DHH1*, or *UPF1*, or a 725-amino acid C-terminal truncation of *DCP2*. In our two-hybrid analyses, we noticed that *Pat1* and *Upf1* interacted with N-terminally truncated *Dcp2* fragments but failed to interact with full-length *Dcp2* (Supplemental Fig. S1A). One possible explanation for this observation is that in vivo full-length *Dcp2* may only interact with *Pat1* and *Upf1* when it is in a specific conformation and most likely in a complex with *Dcp1* and *Dcp1* is a limiting factor for this complex formation. This explanation is supported by observations that *Upf1* interacted with *Dcp1* and *Upf1::Dcp1* interaction is

bridged though *Dcp2* (Supplemental Fig. S1A,B). Coexpression of several combinations of *GAL4-DB* and *GAL4-AD* fusions resulted in lethality in the *dhh1Δ* background (Supplemental Fig. S1B). The underlying mechanism for the lethality is currently unclear, but is probably related to *Dhh1* function in mRNA decapping.

RNA analysis

Cells were grown in either YEPD (Figs. 4E, 5A; Supplemental Fig. S7), or synthetic complete (SC) medium lacking leucine (Figs. 3A, B, 4A,B, 5B; Supplemental Figs. S5, S6B,C). In each case, cells (15 mL) were grown at 30°C to an OD₆₀₀ of 0.7 and harvested by centrifugation. Cell pellets were frozen on dry ice and then stored at –80°C until RNA isolation. The procedures for RNA isolation and Northern blotting were as previously described (He and Jacobson 1995). Transcript-specific signals on Northern blots were determined with a FUJI BAS-2500 analyzer. Each experiment was repeated independently at least three times and data are presented as mean ± SD. Statistical significance of the data was assessed by paired two-tailed Student's *t*-tests.

Analysis of protein levels

Preparation of whole-cell extracts and Western blotting procedures were as described previously (Dong et al. 2007). The Western blots in Figure 4D and Supplemental Figure S6C were probed with monoclonal antibodies against the HA epitope (H6908, Sigma) or against *Pgk1* (22C5-D8, Molecular Probes), with the latter polypeptide serving as a loading control. Proteins were detected using ECL Western blotting detection reagents and Kodak BioMax film.

Protein motif analysis

Amino acid sequences of fungal *Dcp2* orthologs were downloaded from the *Saccharomyces* Genome Database (SGD) (<http://www.yeastgenome.org>). Sequence similarity between different *Upf1* or *Pat1*-interacting segments and sequence conservation of the *Dcp1*, *Edc3*, *Pat1*, and *Upf1*-binding domains or motifs and the *Dcp2* *cis*-inhibitory element among the *Dcp2* orthologs were carried out with the multiple sequence alignment software Clustal Omega hosted on a web server (<http://www.ebi.ac.uk/Tools/msa/>).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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