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Yeast Edc3 Targets *RPS28B* mRNA for Decapping by Binding to a 3′ Untranslated Region Decay-Inducing Regulatory Element

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mRNA decapping commits a transcript to complete turnover in eukaryotic cells. In yeast, general mRNA decapping requires the Dcp1/Dcp2 decapping enzyme and a set of decapping activators, including Pat1, Dhh1, Edc3, and the Lsm1-7 complex. The exact function and mode of action of each of these decapping activators in mRNA decapping largely remain elusive. Here, we analyzed the role of Edc3 in the decay of yeast *RPS28B* mRNA, a pathway triggered by a negative-feedback autoregulatory mechanism. We show that Edc3-mediated *RPS28B* mRNA decay requires either of two orthologous proteins, Rps28a and Rps28b, expressed from the *RPS28A* and *RPS28B* genes, respectively. Contrary to a generally accepted model, we found that Rps28b does not bind to the 3′-untranslated region (UTR) regulatory element in *RPS28B* mRNA. Instead, Edc3 is directly involved in binding the element, and Rps28b binds Edc3 and regulates its activity. Decay of *RPS28B* mRNA requires the Lsm and YjeF-N domains of Edc3, but surprisingly, decay of *YRA1* pre-mRNA, the only other known substrate of Edc3, requires only the Lsm domain. Collectively, our experiments reveal a new role for Edc3 in mRNA substrate recognition and suggest that this activity is subject to intricate regulation by additional factors, including the Rps28 ribosomal protein.

Decapping of mRNA is an important and usually irreversible step that commits a transcript to complete turnover in the pathways for general 5′-to-3′ decay (1, 2), nonsense-mediated decay (NMD) (2–4), adenine/uridine-rich element (ARE)-mediated decay (5), microRNA-mediated gene silencing (6), and transcript-specific degradation (7, 8). In the yeast *Saccharomyces cerevisiae*, general mRNA decapping occurs after extensive poly(A) shortening and requires the functions of the Dcp1/Dcp2 decapping enzyme and a set of regulatory factors, including Pat1, Dhh1, Edc3, and the Lsm1-7 complex (2, 9). *In vivo*, the regulatory factors do not catalyze decapping *per se* but appear to stimulate the activity of the Dcp1/Dcp2 decapping enzyme and are dubbed general activators of mRNA decapping (2, 9). Based on genetic and biochemical studies, decapping activators are currently thought to promote mRNA decapping by performing two general functions in the cell. One function is to repress mRNA translation, and another is to activate the Dcp1/Dcp2 decapping enzyme (10, 11). The exact function and mode of action for each of these decapping activators in mRNA decapping, however, remain largely elusive.

Edc3 is an Lsm-like protein with a long C-terminal extension (12). The protein is conserved in eukaryotes and exhibits a modular structure, containing an Lsm domain at its N terminus, an FDF domain in the middle, and a YjeF-N domain at its C terminus (12–14). Yeast and human Edc3 proteins copurify with the Dcp1/Dcp2 decapping enzyme (5, 15, 16) and also physically interact with Dcp1, Dcp2, and Dhh1 (17, 18). Further biochemical and two-hybrid analyses show that the Lsm domain of Edc3 binds to Dcp1 and Dcp2 (10, 19–21), the FDF domain binds to Dhh1 (20, 22), and the YjeF-N domain self-associates (20, 23). Remarkably, although Edc3 is considered a general decapping activator (2, 10), deletion of the *EDC3* gene upregulates only two transcripts in yeast and has no effect on the decay of other mRNAs (7, 8). One of the upregulated transcripts is the intron-containing *YRA1* pre-mRNA (7), and the other is the *RPS28B* mRNA (8). The spliced *YRA1* pre-mRNA encodes an hnRNP-like protein (Yra1) involved in an early stage of mRNA export, and the *RPS28B* mRNA encodes a ribosomal protein (Rps28b) of the 40S subunit.

We recently elucidated the mechanism of Edc3-mediated *YRA1* pre-mRNA decay (7, 24), defining specific steps that occur in the cytoplasm, are independent of translation, and are controlled through five functionally distinct regulatory elements in the *YRA1* intron. Two elements (EREs [*E*dc3 *r*esponse *e*lements]) control Edc3 substrate specificity, and the other three (TREs [*t*ranslational *r*epression *e*lements]) repress *YRA1* pre-mRNA translation. Translational repression of *YRA1* pre-mRNA inhibits the transcript's susceptibility to NMD and ensures the transcript's decay by Edc3. Furthermore, we also showed that Edc3-mediated *YRA1* pre-mRNA decay is an integral part of a negative-feedback autoregulatory loop functionally linked to *YRA1* pre-mRNA splicing and nuclear export (7).

Edc3-mediated *RPS28B* mRNA decay is also triggered by a negative-feedback mechanism. This decay pathway serves to autoregulate *RPS28B* expression and is controlled by a single regulatory element located in the *RPS28B* 3′ untranslated region (UTR) (8). Based on observations that Rps28b exhibits two-hybrid interactions with Dcp1 and Edc3 (17) and manifests binding to the *RPS28B* 3′ UTR decay element in a three-hybrid assay, a model for Edc3-mediated *RPS28B* mRNA decay was proposed (8). In this model, excess Rps28b is thought to bind directly to the *RPS28B* 3′ UTR element and, through its interaction with Dcp1 and Edc3, to recruit the decapping enzyme to trigger decapping of *RPS28B* mRNA.

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Here, we tested this model and sought commonalities in the events promoting the decay of the only two apparent direct substrates of Edc3. We analyzed the molecular interactions between Rps28b and Edc3, examined the effects of disrupting these interactions on *RPS28B* mRNA decay, reevaluated the identity of the protein involved in binding the *RPS28B* regulatory element and compared the Edc3 domain requirements for decay of the *RPS28B* and *YRA1* transcripts. Consistent with a recent finding (25), we found that Rps28b binds directly to an internal fragment of Edc3. However, contrary to the current model (8), our experiments revealed that Edc3, but not Rps28b, binds to the 3′ UTR element in *RPS28B* mRNA and is thus directly involved in identifying the substrate mRNA for decapping. Surprisingly, Edc3 accomplishes this task with domains that are distinctly different from those required for promoting decay of the *YRA1* pre-mRNA.

MATERIALS AND METHODS

General methods. Preparation of standard yeast media and methods of cell culture were as described previously (26). Transformation of yeast was done by the high-efficiency method (27), and DNA manipulations were performed by standard techniques (28). All PCR amplifications were performed with *Taq* DNA polymerase and confirmed, when appropriate, by DNA sequencing.

Yeast strains. All the strains used in this study are listed in Table S1 in the supplemental material. Strains containing deletions of *RPS28A*, *RPS28B*, *EDC3*, *UPF1*, *DCP1*, and *DCP2* were constructed by gene replacement (26), using DNA fragments harboring the corresponding null alleles. All null alleles were generated from plasmid DNA by restriction enzyme digestion, except for the *rps28a*::*LEU2* and *edc3*::*KanMX6* alleles. These two alleles were generated by PCR using the oligonucleotide pair RPS28A-5-2 and RPS28A-3-3-r or EDC3-DSK-F and EDC3-DSK-R (see Table S3 in the supplemental material), respectively. Strains containing a 726-amino-acid C-terminal deletion of Dcp2 were also constructed by gene replacement, using a DNA fragment harboring the *dcp2-N245*::*KanMX6* allele. Each genomic DNA deletion or integration was confirmed by PCR analysis.

Plasmids. All the plasmids used in this study are listed in Table S2 in the supplemental material. Plasmids harboring the *edc3*::*URA3*, *YRA1*, *YRA-DE2*, and *upf1*::*LEU2* alleles were described previously (7, 29). Plasmids harboring the wild-type or mutant *RPS28B* 3′ UTR Edc3 response element fused to two tandem MS2 coat protein binding sites were received as a gift from Gwenael Badis-Breard and Alain Jacquier (8).

Plasmids harboring wild-type, full-length *EDC3*, *RPS28A*, and *RPS28B* alleles were generated by PCR and molecular cloning. In each case, a pair of oligonucleotides that contain the NotI (5′ primer) and SalI (3′ primer) sites were used to amplify the corresponding genomic locus, and the resulting DNA fragment was then inserted into a plasmid previously digested by NotI and SalI. The plasmid harboring the *RPS28B-MS2* allele was constructed in two steps. First, an *RPS28B* allele that contains a deletion in the 3′ UTR Edc3 reponse element region from nucleotides (nt) 474 to 625 and an insertion of a STYI site immediately downstream of the deleted sequences was generated. Second, two oligonucleotides containing tandem MS2 coat protein binding sites were annealed, and the resulting DNA fragment was then inserted into the EcoRI and STYI sites flanking the deleted region. The plasmid harboring the *SL-RPS28B* allele was constructed by PCR and a three-way ligation reaction. This allele is identical to the wild-type gene but contains an insertion of a 26-nt stem-loop structure (GGATCTCGCGGTTCGCCGCGAGATCC) in the *RPS28B* 5′ UTR 6 nucleotides upstream of the translation initiation codon. Plasmids harboring *RPS28B* or *YRA1-DE2* PTC alleles were generated using a QuikChange site-directed mutagenesis kit from Stratagene.

The plasmids used in the yeast two-hybrid or three-hybrid analyses were all constructed by PCR and molecular cloning. In each case, a pair of oligonucleotides that contain sites for EcoRI or BamHI (5′ primer) and SalI (3′ primer) were used for amplification, and the resulting DNA fragments were then inserted into plasmids previously digested by EcoRI and SalI or BamHI and SalI.

Plasmids expressing the MS2 coat protein fusions with *EDC3* or *RPS28A* were generated by PCR and molecular cloning. In each case, a DNA fragment harboring the corresponding coding and 3′ UTR sequences of *EDC3* or *RSP28A* was amplified using a pair of oligonucleotides that contain the NheI (5′ primer) and SalI (3′ primer) sites. The resulting DNA fragment was then inserted between the NheI and SalI sites of a plasmid that contains the *ADH1* promoter and the MS2 coding sequence. Each of these fusion proteins was expressed *in vivo* under the control of the *ADH1* promoter.

Plasmids harboring the *EDC3* N- or C-terminal deletion alleles (*ND-70*, *ND260*, *CD261*, *CD341*, *CD401*, and *CD481*), *RPS28B-YRA1* 3′ UTR chimeric alleles (*B-Y-R1* and *B-Y-R2*), *YRA1-RPS28B* intron chimeric alleles (*Y-B1-Y* and *Y-B2-Y*), and *RPS28A-RPS28B* or *PGK1-RPS28B* coding region chimeric alleles (*B-A-B* and *B-P-B*) were all constructed through *in vivo* recombination in yeast cells as described previously (30). In each case, a PCR product and a linearized plasmid cut in the gene to be recombined (*EDC3* by HindIII or BglII, *YRA1* by EcoRI, and *RPS28B* by ClaI) were introduced into yeast cells, and the recombinant allele was screened by PCR analysis and restriction enzyme digestion. A plasmid harboring the *EDC3* Δ*FDF* allele was generated by replacing the 1,242-bp wild-type NotI-PvUII fragment from the 5′ end of the *EDC3* gene with a PCR-generated 684-bp DNA fragment, resulting in an internal deletion of coding sequences from codon 71 to 260. Plasmids harboring the *YRA1*- or *RPS28B-RC-RPS28B* coding region chimeric alleles were constructed in two steps. First, a modified *RPS28B* allele containing an NcoI site at the translation initiation codon and a BglII site downstream of the translation termination codon was generated. Second, a DNA fragment containing sequences coding for the first 67 amino acids of Yra1 or complementary to the *RPS28B* coding region from nt 3 to 203 in a reverse orientation was amplified using a pair of oligonucleotides that contain the NcoI (5′ primer) and BglII (3′ primer) sites. The resulting DNA fragment was then used to replace the coding region of the modified *RPS28B* allele. A plasmid harboring the *HA-CUP1-RPS28B* reporter was constructed by PCR. This reporter allele contains 322 bp from the promoter region of *RPS28B*, 99 bp of triple hemagglutinin (HA), 186 bp of the *CUP1* coding region, and 1,102 bp from the 3′ UTR of *RPS28B*.

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

RNA analysis. Cells were grown in either yeast extract-peptone-dextrose (YEPD) (see Fig. 3C and 4A and B) or synthetic complete (SC) medium lacking leucine (see Fig. 1), tryptophan (see Fig. 2, 3B and D, and 4C; see Fig. S2 in the supplemental material), or leucine and tryptophan (see Fig. 7). For normal cell cultures, cells (15 ml) were grown at 30°C to an optical density at 600 nm (OD_{600}) of 0.7 and harvested by centrifugation. For cultures involving cycloheximide or thiolutin treatment, cells (100 ml) were grown at 30°C to an OD_{600} of 0.7, harvested by centrifugation, and resuspended in 20 ml of the same medium. Cycloheximide or thiolutin was added to the cell cultures at final concentrations of 100 μg/ml and 20 μg/ml, respectively, and 2 ml of cell cultures was harvested at different time points. In each case, the 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 (31). Transcript-specific signals on Northern blots were determined with a Fuji BAS-2500 analyzer.

Protein analysis. Preparation of whole-cell extracts and Western blotting procedures were as described previously (7). One Western blot (Fig. 3D) was 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 (GE Healthcare) and Kodak BioMax film.

Polyribosome analysis. Cells were grown at 30°C in either YEPD medium (Fig. 3A) or SC medium lacking tryptophan (see Fig. S1 in the supplemental material) to an OD_{600} of 0.7. Cell extracts were prepared and fractionated on 7 to 47% sucrose gradients as described previously (24, 32).

Yeast two- and three-hybrid interaction assays. Two- and three-hybrid assays utilized previously described procedures (33, 34). All the two-hybrid interactions were initially assayed in the GGY1::171 tester strain (33). In each case, a *GAL4*(*DB*) fusion construct was cotransformed with a *GAL4*(*AD*) construct into the tester strain. Interactions between Edc3 or Rps28b and the Edc3-responsive RNA element located in the 3′ UTR of *RPS28B* mRNA were assayed in the YBZ1 tester strain (35). In each case, a *GAL4*(*AD*) fusion construct was cotransformed with a hybrid RNA construct. In all these assays, transformants were incubated for 3 to 5 days at 30°C until colonies were fairly large and then replicated on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing plates. The color phenotypes of two independent transformants from each interaction assay are presented in the figures. To assess the potential for molecular interactions that were bridged, we also carried out most two- and three-hybrid analyses in the respective tester strains harboring a complete deletion of *EDC3* or a 726-amino-acid C-terminal truncation of Dcp2.

RESULTS

Degradation of both YRA1 pre-mRNA and RPS28B mRNA is Edc3 mediated, but with different requirements for Edc3. *YRA1* pre-mRNA and *RPS28B* mRNA are both subject to Edc3-mediated decay (7, 8), but the *cis*-regulatory elements that control the Edc3 substrate specificities for the two transcripts appear to be different. The decay of the *YRA1* pre-mRNA is controlled by a set of functionally redundant composite elements in the *YRA1* intron (24). In contrast, *RPS28B* mRNA decay is controlled through a single simple element in its 3′ UTR (8). Although decay of both *YRA1* pre-mRNA and *RPS28B* mRNA is regulated by Edc3, these observations raised the possibility that the mechanisms of degradation for these two transcripts are still different. To test this notion, we generated deletions of different Edc3 domains and assessed the functional consequences of these deletions for Edc3-mediated *YRA1* pre-mRNA and *RPS28B* mRNA decay. In this experiment, *edc3* alleles harboring different domain deletions were individually introduced into an *edc3*Δ strain, and the steady-state levels of *YRA1* pre-mRNA and *RPS28B* mRNA in the resulting cells were determined by Northern blotting. This analysis led to several important observations (Fig. 1). First, deleting the Lsm domain (amino acids 1 to 70; allele *ND70*) eliminated Edc3-mediated decay of both *YRA1* pre-mRNA and *RPS28B* mRNA (compare the *ND70* allele in Fig. 1, lane 3, to the null and *EDC3* alleles in lanes 1 and 2). Second, deleting the YjeF-N domain (amino acids 261 to 551; allele *CD261*) eliminated Edc3-mediated *RPS28B* mRNA decay but did not have a significant effect on Edc3-mediated *YRA1* pre-mRNA decay (compare the *CD261* allele in Fig. 1, lane 5, to the null and *EDC3* alleles in lanes 1 and 2). Third, deleting the FDF domain (amino acids 71 to 260; allele Δ*FDF*) affected neither Edc3-mediated *YRA1* pre-mRNA decay nor Edc3-mediated *RPS28B* mRNA decay (compare the Δ*FDF* allele in Fig. 1, lane 9, to the null and *EDC3* alleles in lanes 1 and 2). Finally, deleting both the FDF domain and the YjeF-N domain (amino acids 71 to 551; allele *CD481*) also failed to have a notable effect on Edc3-mediated *YRA1* pre-mRNA decay, and in fact, the resulting 70-amino-acid Lsm domain of Edc3 was sufficient to promote efficient *YRA1* pre-mRNA decay (compare the *CD481* allele in Fig. 1, lane 8, to the null and *EDC3* alleles in lanes 1 and 2). Together, these results indicate that

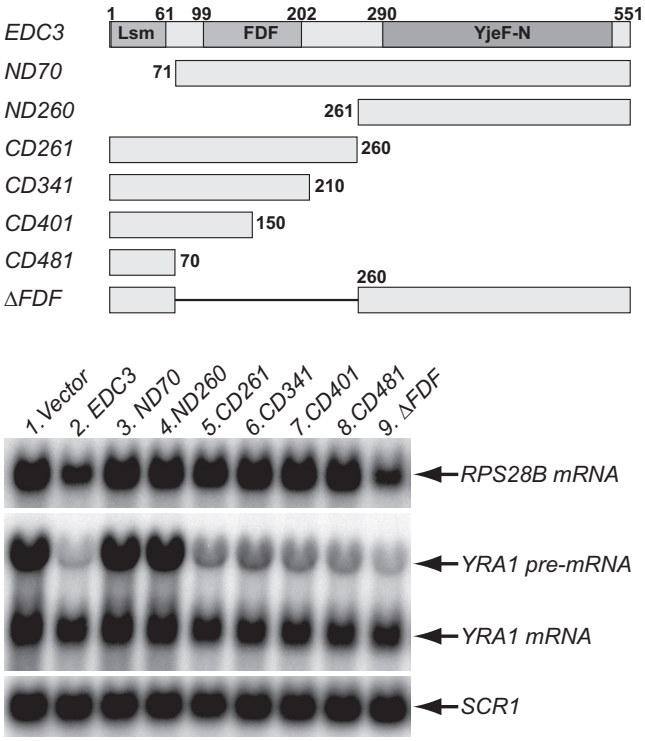

FIG 1 Edc3-mediated decay of *YRA1* pre-mRNA and that of *RPS28B* mRNA differ in their respective requirements for Edc3. A set of *edc3* alleles encoding Edc3 with N-terminal, C-terminal, or internal deletions was constructed, and each of the alleles was individually introduced into the *edc3*Δ strain (CFY25). Total RNA was isolated from each of the resulting strains, and the steady-state levels of the *RPS28B* mRNA and *YRA1* pre-mRNA in each strain were analyzed by Northern blotting, using random-primed probes specific for *RPS28B*, *YRA1* pre-mRNA, or *SCR1* transcripts, with the last serving as a loading control. Schematic representations of the Edc3 domain structure and *edc3* alleles used in this experiment are shown at the top. The numbers refer to amino acid positions.

Edc3-mediated *YRA1* pre-mRNA decay and *RPS28B* mRNA decay have different requirements for Edc3. *YRA1* pre-mRNA decay requires the function of only the Lsm domain, whereas Edc3-mediated *RPS28B* mRNA decay requires the functions of both the Lsm and the YjeF-N domains.

***cis*-regulatory elements involved in Edc3-mediated decay of YRA1 pre-mRNA and RPS28B mRNA are not functionally equivalent.** Our observation that degradation of *YRA1* pre-mRNA and *RPS28B* mRNA requires different functional domains of Edc3 suggests that the decay of these two transcripts likely involves different mechanisms. To substantiate this conclusion, we tested whether the *cis*-regulatory elements involved in Edc3-mediated decay of *YRA1* pre-mRNA and *RPS28B* mRNA are functionally interchangeable. We have previously shown that Edc3-mediated *YRA1* pre-mRNA decay is controlled through five functionally distinct elements (A, B, C, D, and E) located in the *YRA1* intron (24). Elements A and B control Edc3 substrate specificity, and elements C, D, and E repress *YRA1* pre-mRNA translation, thus ensuring that this transcript is degraded by Edc3-mediated decay but not by translation-dependent NMD. Furthermore, these five elements appear to perform at least partially redundant functions. A combination of elements A and C or B, C, and D is sufficient to

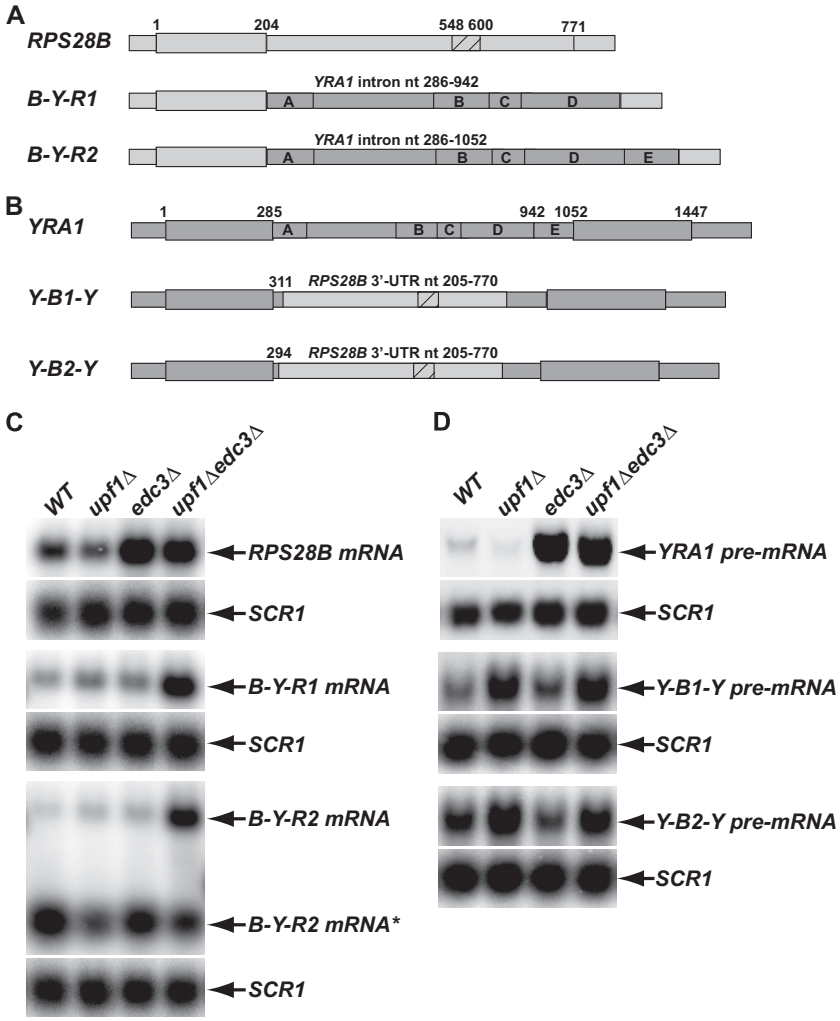

FIG 2 *cis*-regulatory elements involved in the decay of *YRA1* pre-mRNA and *RPS28B* mRNA have different functions. A set of chimeric alleles encompassing segments of the *RPS28B* and *YRA1* genes was constructed, and each of the chimeric alleles was individually introduced into wild-type (HFY114), *upf1*Δ (HFY871), *edc3*Δ (CFY25), and *upf1*Δ *edc3*Δ (SYY158) strains. Total RNA was isolated from each of the resulting strains, and the steady-state levels of transcripts encoded by each of the chimeric alleles in the strains were analyzed by Northern blotting, using random-primed probes specific for each of the transcripts or *SCR1* transcripts, with the latter serving as a loading control. (A) Schematic representation of structural features of *RPS28B* mRNA and the *RPS28B* chimeric alleles harboring *YRA1* intronic sequences in the 3′ UTR. (B) Schematic representation of the structural features of *YRA1* pre-mRNA and *YRA1* chimeric alleles harboring *RPS28B* 3′ UTR sequences within their intron regions. In both panels A and B, enlarged rectangles denote coding regions, hatched boxes represent the *RPS28B* 3′ UTR regulatory element, and dark rectangles indicate *YRA1* intron sequences. The numbers refer to nucleotides. The *cis*-regulatory elements of the *YRA1* intron are labeled A, B, C, D, and E. (C and D) Transcripts were subjected to Northern analysis as for Fig. 1. (C) Analysis of transcripts encoded by the chimeric alleles shown in panel A. The *B-Y-R2* allele produces two different mRNAs, and the spliced form is indicated by an asterisk. WT, wild type. (D) Analysis of transcripts encoded by the chimeric alleles shown in panel B.

trigger robust Edc3-mediated decay. In contrast to the complexity of the *YRA1 cis*-acting elements, Edc3-mediated decay of *RPS28B* mRNA is controlled through a single 53-nt element located in the 3′ UTR of *RPS28B* mRNA (8).

We constructed two sets of chimeric alleles between the *RPS28B* and *YRA1* genes and analyzed the decay phenotypes of the transcripts encoded by these chimeric alleles in wild-type, *upf1*Δ, *edc3*Δ, and *upf1*Δ *edc3*Δ cells (Fig. 2). We included *upf1*Δ cells in these analyses because the chimeric transcripts may lose Edc3 regulation and become sensitive to NMD. In the first set of chimeric alleles, we replaced the *RPS28B* 3′ UTR, from nt 205 to 770, with two different *YRA1* intron fragments (Fig. 2A). One of the fragments covered *YRA1* intron sequences from nt 286 to 942 and contained the intronic elements A, B, C, and D (allele *B-Y-R1*). The second fragment includes the entire *YRA1* intron and contains all five of the intronic elements (allele *B-Y-R2*). In the second set of chimeric constructs, we replaced two largely overlapping *YRA1* intron regions, nt 312 to 942 (allele *Y-B1-Y*) and 295 to 942 (allele *Y-B2-Y*), with *RPS28B* 3′ UTR sequences from nt 205 to 770 (Fig. 2B). Northern blotting analyses showed that although the endogenous *YRA1* pre-mRNA and *RPS28B* mRNA are both substrates for Edc3-mediated decay, each of the chimeric transcripts lost its Edc3 substrate specificity (Fig. 2C and D). Chimeric *RPS28B* mRNAs containing *YRA1* intronic sequences in their 3′ UTRs were degraded by either NMD or Edc3-mediated decay (Fig. 2C, *B-Y-R1* and *B-Y-R2* mRNAs). Chimeric *YRA1* pre-mRNAs con-

taining *RPS28B* 3′ UTR sequences in their introns were degraded exclusively by NMD (Fig. 2D, *Y-B1-Y* and *Y-B2-Y* pre-mRNAs). These data show that the *cis*-regulatory elements of the *YRA1* intron and the *RPS28B* 3′ UTR cannot substitute for each other and suggest that the elements most likely have distinct regulatory functions. Consistent with these functional results, bioinformatic analysis did not reveal any linear motifs or secondary structures that were common to the *RPS28B* 3′ UTR element and any of the *YRA1* intronic elements (data not shown).

RPS28B mRNA is degraded by a translation-dependent mechanism. We have previously shown that Edc3-mediated *YRA1* pre-mRNA decay is independent of translation and, in fact, requires active translational repression (24). This conclusion is based on the following observations: (i) in steady state, the majority of *YRA1* pre-mRNAs are not associated with translating ribosomes in wild-type yeast cells; (ii) *cis* inhibition of *YRA1* pre-mRNA translation and *trans* inhibition of general translation have little or no effect on Edc3-mediated *YRA1* pre-mRNA decay; and (iii) the *YRA1* intron contains specific sequence elements that inhibit the transcript's susceptibility to NMD.

Since Edc3-mediated decay of the *YRA1* pre-mRNA and the *RPB28B* mRNA manifests notable differences in the respective Edc3 requirements and *cis*-regulatory elements (see above), we reasoned that these two transcripts may also differ with respect to the involvement of translation in their degradation. To assess whether Edc3-mediated *RPS28B* mRNA decay requires translation, we carried out four different experiments. In the first experiment, we analyzed the translation status of *RPS28B* mRNA in wild-type and *edc3*Δ cells, utilizing sucrose gradient fractionation and Northern blotting. We found that in both wild-type and *edc3*Δ cells, the vast majority (74 to 84%) of *RPS28B* mRNA was associated with polyribosome fractions (Fig. 3A). As a control, we analyzed the translation status of *RPS28B* mRNA harboring a *cis*-inhibitory stem-loop structure in its 5′ UTR (*SL-RPS28B* mRNA). As expected, this transcript behaved differently from the endogenous wild-type *RPS28B* mRNA. In both wild-type and *edc3*Δ cells, the majority (67 to 68%) of *SL-RPS28B* mRNA was located in the messenger ribonucleoprotein (mRNP) fractions (see Fig. S1 in the supplemental material). In the second experiment, we introduced nonsense mutations into the coding region of *RPS28B* mRNA and analyzed the sensitivity of the resulting transcripts to NMD. We found that nonsense mutations located in the 5′-proximal coding region at codon positions 11, 27, and 42 all triggered the transcript's degradation by NMD, i.e., their decay was reversed by deletion of *UPF1*. In contrast, nonsense mutations located in the 3′-proximal coding region at codon position 58 did not trigger the transcript's degradation by NMD (Fig. 3B). Importantly, inserting nonsense mutations at codon positions 20, 40, and 60 in exon 1 of *YRA1-DE2* pre-mRNA, a transcript degraded by translation-independent Edc3-mediated decay (7), did not trigger that transcript's degradation by NMD (see Fig. S2 in the supplemental material). In the third experiment, we analyzed the effect of blocking translation elongation on *RPS28B* mRNA decay. We found that treating wild-type yeast cells, as well as *edc3*Δ cells, with cycloheximide (an inhibitor of translation elongation) for 20 or 40 min stabilized *RPS28B* mRNA (Fig. 3C). In this respect, *RPS28B* mRNA behaved similarly to *RPS28A* mRNA and nonsense mutation-containing mRNAs but differently from *YRA1* pre-mRNA. Treatment with cycloheximide stabilized *RPS28A* mRNA (Fig. 3C) and nonsense mutation-containing mRNAs but had no effect on *YRA1* pre-mRNA decay (24). In the final experiment, we constructed an *HA-CUP1-RPS28B* reporter gene and analyzed the expression of its encoded mRNA and protein in wild-type and *edc3*Δ cells. This reporter gene codes for a transcript that is almost identical to that of the endogenous *RPS28B* mRNA except that the latter mRNA's 67-amino-acid-encoding region was replaced by a region encoding 94-amino-acid triple HA and Cup1. *HA-CUP1-RPS28B* mRNA exhibited Edc3-dependent decay similar to that of wild-type *RPS28B* mRNA, accumulating to about 2-fold-higher levels in *edc3*Δ cells than in wild-type cells (Fig. 3D). Importantly, protein expression from this reporter transcript was detected in both wild-type and *edc3*Δ cells (Fig. 3D). This result is in sharp contrast to our previous analyses of *YRA1* pre-mRNA reporter genes, where, despite high levels of expression of the *YRA1* pre-mRNA reporter transcripts, no protein expression was detected in both wild-type and *edc3*Δ cells (24). Altogether, these results indicate that *RPS28B* mRNA is engaged in translation and, in contrast to *YRA1* pre-mRNA, is likely degraded through a translation-dependent mechanism.

Edc3-mediated RPS28B mRNA degradation requires the Rps28 protein expressed from both the RPS28A and RPS28B genes. Edc3-mediated *RPS28B* mRNA decay is an integral part of a negative-feedback circuit that autoregulates *RPS28B* expression (8). A previous study suggested that two orthologous proteins, Rps28a and Rps28b, encoded by the *RPS28A* and *RPS28B* genes, respectively, are both involved in *RPS28B* autoregulation (8). Rps28a and Rps28b share almost identical amino acid sequences except at the third codon position, where an asparagine in Rps28a replaces a serine present in Rps28b. To further define the roles of Rps28a and Rps28b in Edc3-mediated *RPS28B* mRNA decay, we generated deletions of the *RPS28A* and *RPS28B* genes and analyzed the effects of these deletions on *RPS28B* mRNA decay. Compared to wild-type cells, deletion of *RPS28A* resulted in increased steady-state accumulation of *RPS28B* mRNA (about 1.6-fold) (Fig. 4A) and a decreased decay rate for the transcript (a half-life of 12 min versus 4 min) (Fig. 4B), effects that were similar to those of deleting *EDC3* (Fig. 4A and B). These results indicate that Edc3-mediated *RPS28B* mRNA decay requires the Rps28a protein encoded by the *RPS28A* gene.

To address the role of Rps28b in Edc3-mediated *RPS28B* mRNA decay, we utilized a different strategy. We generated a set of chimeric *RPS28B* alleles in which the *RPS28B* coding region was replaced by the sequences of the *PGK1* or *YRA1* coding region (alleles *B-P-B* and *B-Y-B*) or by sequences that are complementary to the *RPS28B* coding region (allele *B-B′-B*), and analyzed the decay phenotypes of transcripts generated from these chimeric alleles in *EDC3* and *edc3*Δ cells in *RPS28B* and *rps28b*Δ backgrounds (Fig. 4C). In the *RPS28B* background, each of the three chimeric transcripts exhibited Edc3-dependent decay (Fig. 4C, bottom left). In contrast, in the *rps28b*Δ background, none of the chimeric transcripts exhibited Edc3-dependent decay (Fig. 4C, bottom middle). These results indicate that Edc3-mediated *RPS28B* mRNA decay also requires the Rps28b protein encoded by the *RPS28B* gene.

We also generated a chimeric allele, *B-A-B*, in which the *RPS28B* coding region was replaced by the coding sequences of *RPS28A* and analyzed the decay phenotypes of the transcript generated from this chimeric allele in *EDC3* and *edc3*Δ cells in *rps28b*Δ backgrounds (Fig. 4C). In contrast to the other chimeric transcripts described above, the chimeric *B-A-B* transcript exhib-

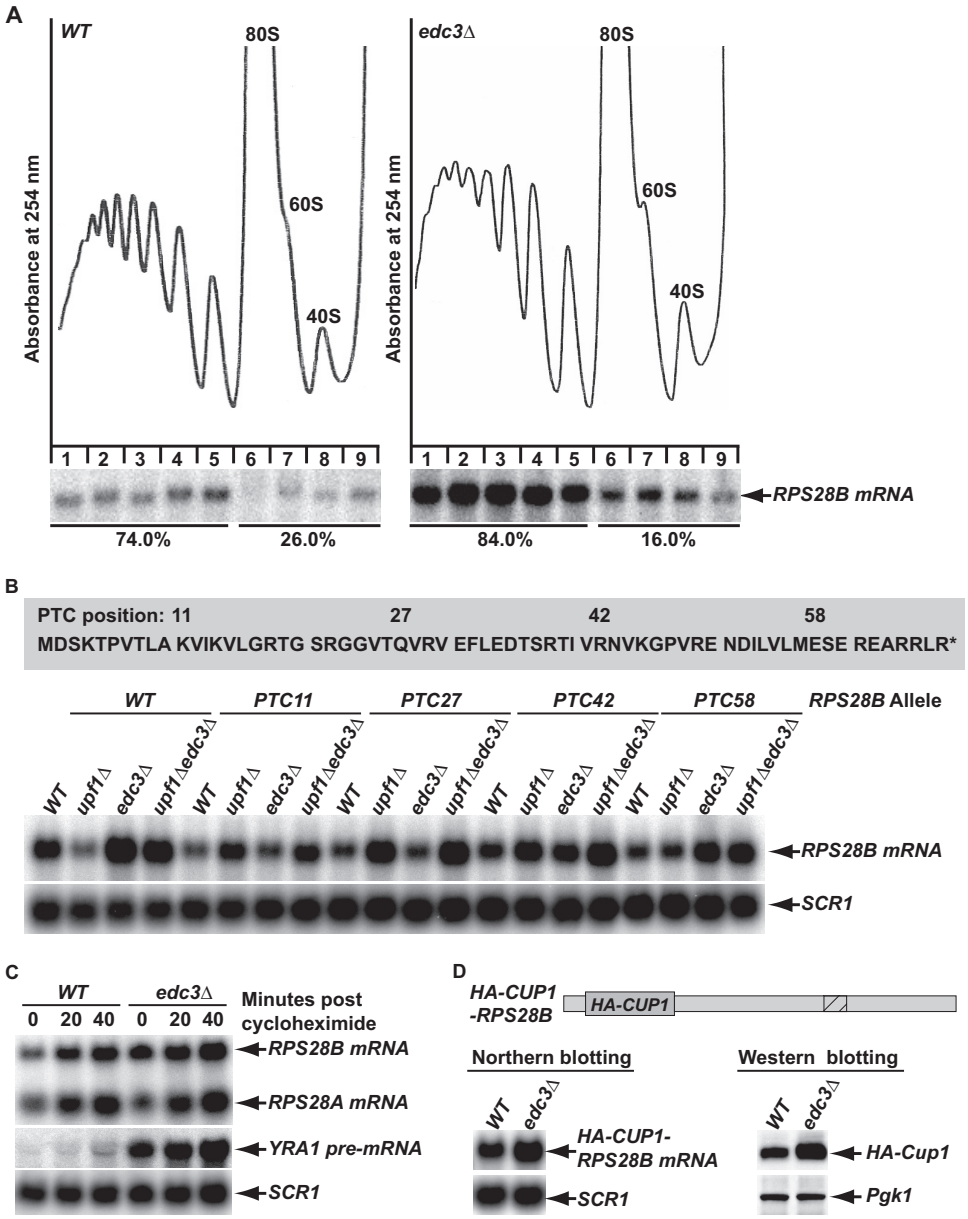

FIG 3 Edc3-mediated *RPS28B* mRNA decay requires translation. (A) Analysis of the translation status of *RPS28B* mRNA in wild-type (HFY114) and *edc3*Δ (CFY25) cells. Wild-type and *edc3*Δ cells were grown in YEPD medium at 30°C, and whole-cell extracts were prepared. The polyribosomal association of *RPS28B* mRNA in these cells was analyzed by sucrose gradient fractionation and Northern blotting. (Top) Absorbance tracings at 254 nm. (Bottom) Northern blots of individual gradient fractions. The blots were hybridized with an oligonucleotide complementary to the *RPS28B* transcript. (B) *RPS28B* mRNA is sensitive to nonsense mutations introduced into its early coding region. Nonsense mutations were introduced into the indicated codon positions of the *RPS28B* gene. The resulting alleles were individually introduced into wild-type (CFY159), *upf1*Δ (CFY322), *edc3*Δ (CFY161), and *upf1*Δ *edc3*Δ (CFY324) strains with an *rps28b*Δ background. Total RNA was isolated from each of the resulting strains, and the steady-state levels of the *RPS28B* transcript encoded by each of the nonsense-containing alleles were analyzed by Northern blotting, using random-primed probes specific for the *RPS28B* or *SCR1* transcripts. PTC, premature termination codon. (C) *RPS28B* mRNA is stabilized by treating cells with cycloheximide. Wild-type (HFY114) and *edc3*Δ (CFY25) cells were grown in YEPD medium at 30°C, and cycloheximide was added to the cell cultures. Cells were harvested at the indicated times. Total RNA was isolated from the collected cells, and the steady-state levels of *RPS28B* mRNA in the cells at each time point were analyzed by Northern blotting, using an oligonucleotide probe that hybridizes to both the *RPS28A* and *RPS28B* transcripts and a random-primed probe specific for the *SCR1* transcript. (D) Analysis of mRNA and protein expression from the *HA-CUP1-RPS28B* reporter gene. The *RPS28B* coding region was replaced by *HA-CUP1* coding sequences, and the resulting reporter gene was introduced into wild-type (HFY114) and *edc3*Δ (CFY25) cells. mRNA and protein expression from the reporter gene were analyzed by Northern or Western blotting, respectively. The Northern blots were hybridized to random-primed probes specific for the *CUP1* and *SCR1* transcripts, with the latter serving as a loading control. The Western blots were probed with monoclonal antibodies targeted to the HA epitope or Pgk1, with the latter serving as a loading control.

ited Edc3-dependent decay in cells having an *rps28b*Δ background (Fig. 4C, bottom right). Since only Rps28a was expressed in the *rps28b*Δ cells, the observed Edc3-dependent decay of the *B-A-B* transcript shows that Rps28a is sufficient to trigger Edc3-mediated *RPS28B* mRNA decay. To assess whether Rps28b is also sufficient for Edc3-mediated *RPS2B* mRNA decay, we introduced a modified exogenous *RPS28B* allele into *EDC3* and *edc3*Δ cells in an *rps28a*Δ background and analyzed the levels of accumulation of

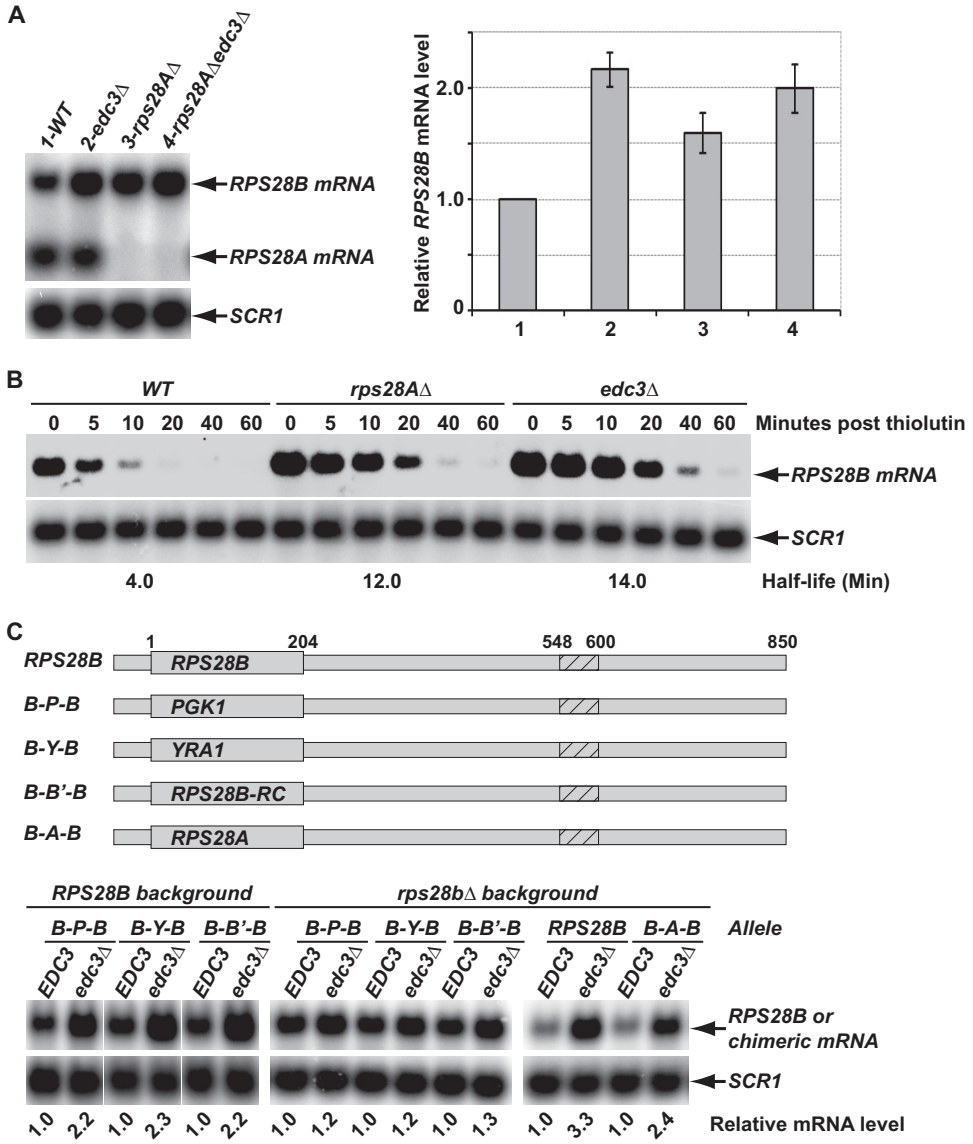

FIG 4 Edc3-mediated *RPS28B* mRNA decay requires the Rps28 protein expressed from the *RPS28A* and *RPS28B* genes. (A) Deletion of *RPS28A* leads to an increased steady-state level of *RPS28B* mRNA. Wild-type (HFY114), *edc3*Δ (CFY25), *rps28a*Δ (CFY98), and *rps28a*Δ *edc3*Δ (CFY96) cells were grown in YEPD medium at 30°C, and the steady-state levels of *RPS28B* mRNA in the cells were analyzed by Northern blotting as described in the legend to Fig. 3C. The relative levels of *RPS28B* mRNAs derived from the Northern blot (left) are depicted in the bar graph (right). The error bars indicate standard deviations of the means of three independent experiments. (B) Deletion of *RPS28A* decreases the *RPS28B* mRNA decay rate. Wild-type (HFY114), *rps28a*Δ (CFY98), and *edc3*Δ (CFY25) cells were grown in YEPD medium at 30°C. Thiolutin was added to each culture, cells were collected at the indicated time points, and the levels of *RPS28B* mRNA at each time point were analyzed by Northern blotting, using random-primed probes specific for *RPS28B* or *SCR1* transcripts. (C) Deletion of *RPS28B* abolishes Edc3-mediated decay of chimeric *RPS28B* transcripts. A set of *RPS28B* chimeric alleles was constructed (schematics are shown at the top), and the alleles were individually introduced into *EDC3* and *edc3*Δ cells in *RPS28B* and *rps28b*Δ backgrounds (HFY114 and CFY25, and CFY159 and CFY161, respectively). The steady-state levels of the transcript encoded by each of these chimeric alleles in the different strains were analyzed by Northern blotting, with different probes used for hybridization with specific transcripts and strain backgrounds. In the *rps28b*Δ background, all chimeric transcripts were detected using a random-primed probe specific for the *RPS28B* transcript. In the *RPS28B* background, the B-P-B and B-Y-B chimeric mRNAs were detected using random-primed probes specific for the *PGK1* and *YRA1* transcripts, respectively; the B-B′-B chimeric mRNA was detected using an oligonucleotide probe complementary to its coding region. In the schematic, the *RPS28B* 3′ UTR regulatory element is indicated by the hatched boxes, and RC denotes reverse complement.

the endogenous *RPS28B* mRNA in the resulting cells. The modified *RPS28B* allele is almost identical to the wild-type allele but contains a 321-nt deletion in its 3′ UTR. We found that introduction of this modified *RPS28B* allele into *EDC3* cells restores Edc3-mediated decay of the endogenous *RPS28B* mRNA (see Fig. S3 in the supplemental material). This result indicates that Rps28b is also sufficient to trigger Edc3-mediated *RPS28B* mRNA decay. Together, these results indicate that Edc3-mediated *RPS28B* mRNA decay requires either the Rps28a or Rps28b protein expressed from the *RPS28A* and *RPS28B* genes, i.e., the two Rps28 protein isoforms appear to have indistinguishable roles in Edc3-mediated *RPS28B* mRNA decay.

Rps28b interacts directly with Edc3 but indirectly with Dcp1. Rps28b has been shown to exhibit two-hybrid interactions with

Dcp1 and Edc3 (17). To dissect the role of Rps28b in Edc3-mediated *RPS28B* mRNA decay, we sought to identify its direct interacting partners and to delineate protein domains involved in these interactions. We assayed binary interactions between Rps28b and Dcp1, Dcp2, Edc3, or itself in the yeast two-hybrid system. In a wild-type tester strain, Rps28b indeed interacted with both Dcp1 and Edc3. In contrast, no interaction was detected between Rps28b and Dcp2 or itself (Fig. 5A).

Dcp1, Dcp2, and Edc3 interact with each other and appear to form a complex *in vivo* (15, 16, 36). To assess whether the observed Dcp1-Rps28b and Edc3-Rps28b interactions may be bridged through Dcp1- and Edc3-interacting partners of the decapping complex, we generated yeast two-hybrid tester strains harboring complete deletions of *EDC3* or a C-terminally truncated *dcp2-N245* allele and assayed binary interactions between Rps28b and Dcp1 or Edc3 in these strains. The *dcp2-N245* allele encodes only the N-terminal first 245 amino acids of Dcp2 and lacks the C-terminal 725 amino acids, including the Edc3 binding site (reference 37 and data not shown). We were unable to generate tester strains harboring a complete deletion of Dcp1 or Dcp2 because the two genes were essential in this strain background. Deleting Edc3 from the tester strain did not affect the Edc3-Rps28b interaction but eliminated the Dcp1-Rps28b interaction (Fig. 5A). Similarly, deleting the C-terminal 725 amino acids of Dcp2 also did not affect the Edc3-Rps28b interaction but eliminated the Dcp1-Rps28b interaction (Fig. 5A). These results indicate that the observed Edc3-Rps28b interaction is likely direct. In contrast, the observed Dcp1-Rps28b interaction is indirect and appears to be bridged by both Edc3 and Dcp2.

To map the Rps28b-interacting domain of Edc3, we generated a panel of DNA fragments covering different coding regions of *EDC3* and analyzed the interaction of each of these encoded Edc3 fragments with Rps28b in the two-hybrid system (Fig. 5B). Since Edc3 can self-associate (20), we eliminated the potential bridged molecular interactions by endogenous Edc3 by carrying out our initial mapping analysis in an *edc3*Δ tester strain and then further validating our observations in the corresponding wild-type strain. In the *edc3*Δ strain (Fig. 5B, bottom right), Rps28b interacted with Edc3 fragments containing an internal region between the N-terminal Lsm domain and the C-terminal YjeF-N domain (Fig. 5B, constructs 2 and 4). In fact, Rps28b interacted strongly with this internal Edc3 fragment alone (Fig. 5B, construct 6). Furthermore, Rps28b interacted with two smaller fragments from the C-terminal region of this Edc3 internal fragment (Fig. 5B, constructs 7 and 8). Rps28b also interacted with each of these Edc3 fragments in the wild-type tester strain (Fig. 5B), validating our observations from the *edc3*Δ strain. Interestingly, in the wild-type strain, Rps28b interacted with one additional Edc3 fragment containing the C-terminal YjeF-N domain (Fig. 5B, construct 3). Since this Edc3 fragment can self-associate (20) (data not shown) and exhibited no interaction with Rps28b in the *edc3*Δ strain, the observed interaction between Rps28b and this Edc3 fragment in the wild-type strain must be bridged by endogenous Edc3. Collectively, these results indicated that Rps28b binds to a small internal segment of Edc3. This segment is located between the FDF and the YjeF-N domains and does not appear to overlap either of the domains.

Edc3, but not Rps28b, binds to the 3′ UTR element in RPS28B mRNA. Rps28b regulates its own level of expression through Edc3-mediated *RPS28B* mRNA decay (8) (see above). Based on observations that Rps28b interacts with the decapping

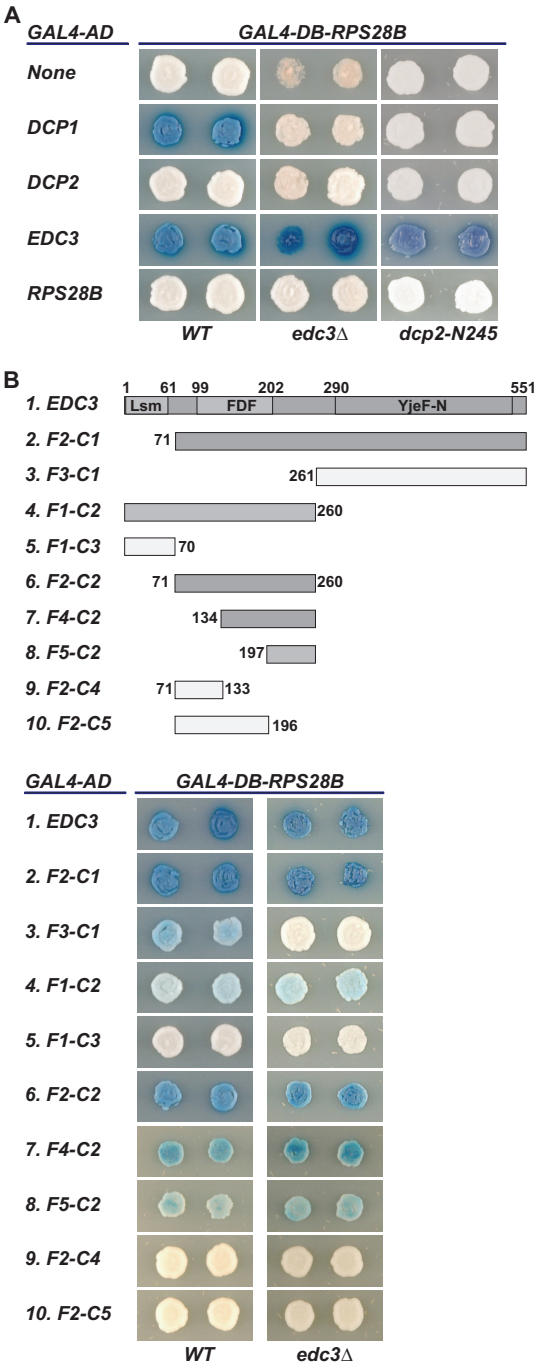

FIG 5 Rps28b binds directly to Edc3 but not to Dcp1. (A) Analysis of direct two-hybrid interactions between Rps28b and Dcp1, Dcp2, Edc3, or itself in the wild-type tester strain GGY1::171 or isogenic tester strains harboring *edc3*Δ (SYY1773) or *dcp2-N245* (SYY2390) alleles. (B) Analysis of two-hybrid interactions between Rps28b and different fragments of Edc3 in the wild-type tester strain or the tester strain harboring an *edc3*Δ allele. (Top) Schematic representation of the Edc3 domain structure and *EDC3* fragments used in the experiment. In both panels A and B, a DNA fragment encoding Rps28b was fused to *GAL4*(*DB*). This *GAL4-DB-RPS28B* fusion was used in the yeast two-hybrid system to test for Rps28b interaction with full-length Dcp1, Dcp2, Edc3, and Rps28 (A) or fragments of Edc3 (B) fused to *GAL4*(*AD*). In each case, a *GAL4*(*DB*) fusion and a *GAL4*(*AD*) fusion were cotransformed into the indicated tester strains. Individual transformants were selected, and qualitative β-galactosidase activity was determined on X-Gal-containing plates. Blue colony color indicates an interaction, and white colony color indicates no interaction.

factors Dcp1 and Edc3 in the two-hybrid assay (17) and apparently also binds to the *RPS28B* 3′ UTR decay-regulatory element in the three-hybrid assay (8), a model for Rps28b function in Edc3-mediated *RPS28B* mRNA decay was proposed (8). In this model, when its protein level reaches a threshold, excess Rps28b binds directly to the *RPS28B* 3′ UTR element and, through its interaction with Dcp1 and Edc3, recruits the decapping enzyme to trigger decapping of *RPS28B* mRNA.

We made intriguing observations that are at odds with the function proposed for Rps28b in Edc3-mediated *RPS28B* mRNA decay. As described above, Rps28b binds to an internal segment of Edc3 (Fig. 5B, construct 6), yet a large deletion covering this internal Edc3 segment has no effect on Edc3-mediated *RPS28B* mRNA decay (Fig. 1, allele Δ*FDF*). This result suggests that Rps28b-Edc3 interaction does not serve to recruit the decapping complex but instead may serve to regulate Edc3 activity. This raises the possibility that Rps28b does not directly bind the *RPS28B* 3′ UTR element and that Edc3 is engaged directly in binding of the element. To test this idea, we analyzed the binding of Edc3 and Rps28b to the *RPS28B* 3′ UTR element in the yeast three-hybrid system. We carried out this analysis in the wild-type strain, as well as in yeast strains harboring a complete deletion of *EDC3* or a C-terminally truncated *dcp2-N245* allele, and utilized a mutant *RPS28B* 3′ UTR element as a control. The mutant element contains three nucleotide substitutions in the conserved tetraloop structure and was previously shown to be deficient in Rps28b binding in the three-hybrid assay (8). Edc3 interacted with the *RPS28B* 3′ UTR element in all three yeast tester strains but exhibited no interaction with the mutant element in each of these strains (Fig. 6A). Rps28b interacted with the *RPS28B* 3′ UTR element in the wild-type and *dcp2-N245* strains but exhibited no interaction with this element in the *edc3*Δ strain (Fig. 6A). Based on these results, we conclude that (i) Edc3 binds to the *RPS28B* 3′ UTR element, (ii) Rps28 does not bind directly to the *RPS28B* 3′ UTR element but can associate with the element through bridging by Edc3, and (iii) Edc3 binding to the *RPS28B* 3′ UTR element does not require its association with Dcp2.

To map the Edc3 protein domain(s) involved in binding to the *RPS28B* 3′ UTR element, we generated a set of *EDC3* deletions and analyzed the effects of these deletions on Edc3 binding to this *cis*-regulatory element in the three-hybrid assay. We carried out this analysis in the wild-type tester strain. Deletion of either the Lsm domain or the YjeF-N domain eliminated Edc3 binding to the *RPS28B* 3′ UTR element (Fig. 6B, alleles *ND70* and *CD260*). In contrast, a large internal deletion that eliminates the FDF domain and the Rps28b binding region of Edc3 did not affect Edc3 binding to the *RPS28B* 3′ UTR element (Fig. 6B, allele Δ*FDF*). These results indicate that Edc3 binding to the *RPS28B* 3′ UTR element requires both the Lsm domain and the YjeF-N domain of Edc3.

Edc3-mediated RPS28 mRNA decay requires dimerization of Edc3. Our yeast three-hybrid analysis established that Edc3 binds directly to the *RPS28B* 3′ UTR element (Fig. 6). Edc3 binding to this regulatory element most likely serves to recruit the decapping enzyme to *RPS28B* mRNA. To delineate Edc3's effector domain(s) and its downstream effector molecules, we analyzed tethered functions of full-length Edc3 and different Edc3 domains in the decay of *RPS28B* mRNA. In this experiment, we generated an *RPS28B-MS2* allele in which a 3′ UTR fragment covering the *RPS28B* regulatory-element region was replaced by two tandem MS2 coat protein binding sites (Fig. 7A). We first confirmed that the transcript encoded by this *RPS28B-MS2* allele is no longer degraded by Edc3-mediated decay, as the transcript accumulated to a higher level (about 2-fold) than the wild-type *RPS28B* mRNA in *EDC3* cells and was insensitive to deletion of *EDC3* (data not shown). We then introduced the *RPS28B-MS2* allele into *EDC3* and *edc3*Δ cells in *DCP1/DCP2*, *dcp1*Δ, and *dcp2*Δ backgrounds and analyzed the steady-state levels of the encoded *RPS28B-MS2* mRNA in these cells in the presence of different MS2 coat protein-Edc3 fusion proteins (Fig. 7B).

Tethering different Edc3 polypeptides to *RPS28B-MS2* mRNA yielded several distinct phenotypes in cells with a *DCP1/DCP2* background (Fig. 7C). Tethering full-length Edc3 promoted *RPS28B-MS2* mRNA decay in both *EDC3* and *edc3*Δ cells, indicating that the tethered full-length protein can elicit its decay activity independent of endogenous Edc3. Tethering an Edc3 fragment (CD261) containing the Lsm and the FDF domains but lacking the YjeF-N domain did not have a significant effect on *RPS28B-MS2* mRNA decay in either *EDC3* or *edc3*Δ cells, indicating that the polypeptide most likely lacks at least one essential effector function and is unable to elicit any decay activity regardless of the Edc3 status of the cell. Interestingly, tethering Edc3 fragments containing the YjeF-N domain but lacking the Lsm domain (N70), or both the Lsm and FDF domains (ND260), was inconsequential for *RPS28B-MS2* mRNA decay in *edc3*Δ cells but promoted *RPS28B-MS2* mRNA decay in *EDC3* cells. This observation indicates that these two polypeptides most likely lack a decay-inducing activity but retain at least one essential effector function of full-length Edc3 and are able to recruit endogenous Edc3 to promote *RPS28B-MS2* mRNA decay. Together, these results show that Edc3-mediated *RPS28B* mRNA decay requires the effector functions encoded by the Lsm domain and the YjeF-N domain of Edc3 and that the YjeF-N domain must function by promoting the dimerization of Edc3.

Tethering different Edc3 polypeptides to *RPS28B-MS2* mRNA yielded identical phenotypes in *dcp1*Δ and *dcp2*Δ backgrounds (Fig. 7D and E). Regardless of the Edc3 status in the cell, none of the tethered Edc3 fragments, including the full-length protein, enhanced *RPS28B-MS2* mRNA decay. This result indicates that tethered Edc3 executes its function through the Dcp1/Dcp2 decapping enzyme. We also analyzed the effect of tethering Rps28a on *RPS28B-MS2* mRNA decay in the cells described above and found that tethering Rps28a did not enhance *RPS28B-MS2* mRNA decay in these cells. This provides additional evidence against the Rps28b recruitment model for Edc3-mediated *RPS28B* mRNA decay (8).

DISCUSSION

A molecular mechanism for Edc3-mediated RPS28B mRNA decay: ribosomal proteins Rps28a and Rps28b regulate Edc3 function in mRNA decapping. Decay of *RPS28B* mRNA is triggered by negative feedback of its product, serves to autoregulate *RPS28B* expression, and is controlled by Edc3 and a single regulatory element located in the mRNA's 3′ UTR (8). Here, we have elucidated details of the mechanism controlling Edc3-mediated *RPS28B* mRNA decay. Unlike *YRA1* pre-mRNA, the only other known substrate of Edc3-mediated decay, our experiments reveal that *RPS28B* mRNA is degraded by a translation-dependent mechanism. This conclusion is supported by experiments demonstrating that (i) *RPS28B* mRNA is associated with polyribosomes in both wild-type and *edc3*Δ cells (Fig. 3A), (ii) *RPS28B* mRNA levels

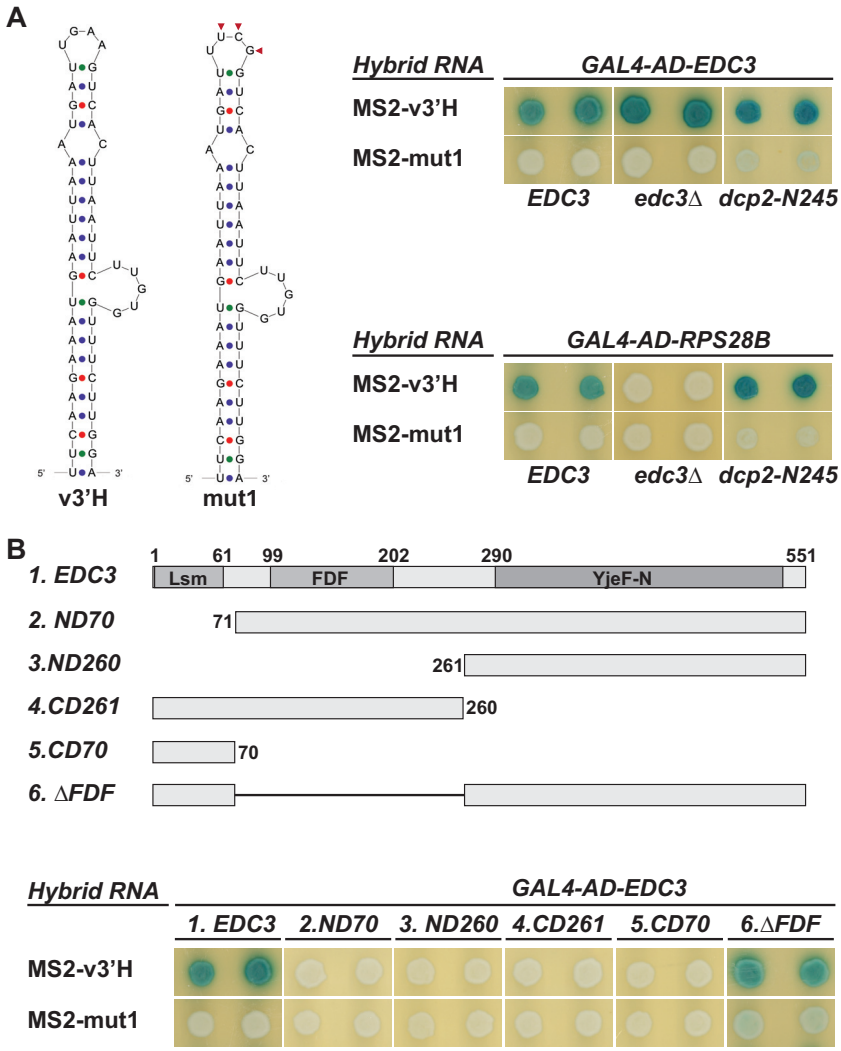

FIG 6 Edc3, but not Rps28b, binds directly to the *RPS28B* 3′ UTR regulatory element. (A) Analysis of the binding of Edc3 and Rps28b to the *RPS28B* 3′ UTR element using the three-hybrid assay in a wild-type tester strain (YBZ1) and tester strains harboring an *edc3*Δ (SYY2691) or *dcp2-N245* (SYY2693) allele. The primary sequences and secondary structures of the wild-type and mutant *RPS28B* 3′ UTR elements used in these analyses are shown on the left. Mutated nucleotides are indicated by red triangles. (B) Analysis of the binding of different Edc3 fragments to the *RPS28B* 3′ UTR element using the three-hybrid assay in the wild-type tester strain YBZ1. A schematic representation of Edc3 domain structure and *edc3* fragments used in the analysis is shown at the top. In both panels A and B, DNA fragments encoding the wild-type (v3′H) and mutant (mut1) *RPS28B* 3′ UTR elements were fused to MS2 binding sites. The resulting hybrid RNAs were used for testing the interaction with full-length Edc3 or Rpd28b (A) and different fragments of Edc3 (B) fused to *GAL4*(*AD*) in the yeast three-hybrid system. In each case, a hybrid RNA and a *GAL4*(*AD*) fusion were cotransformed into the indicated tester strains. Individual transformants were selected, and qualitative β-galactosidase activity was determined on X-Gal-containing plates. Blue colony color indicates an interaction, and white colony color indicates no interaction.

are sensitive to early nonsense codons in the mRNA's coding region (Fig. 3B) and to treatment of cells with the translation elongation inhibitor cycloheximide (Fig. 3C), and (iii) the *HA-CUP1-RPS28B* reporter gene produces a transcript that is translated in both wild-type and *edc3*Δ cells and exhibits Edc3-dependent decay (Fig. 3D).

Edc3-mediated *RPS28B* mRNA decay also requires either of two orthologous ribosomal proteins, Rps28a and Rps28b. Both proteins play a positive role in this pathway, as deletion of their respective genes effectively inhibits Edc3-mediated *RPS28B* mRNA decay (Fig. 4). Consistent with their difference of only a single amino acid, the two proteins appear to have identical functions, with either one capable of promoting *RPS28B* mRNA decay (Fig. 4C; see Fig. S3 in the supplemental material). Data shown in Fig. 5A and 6A demonstrate that previously observed molecular interactions between Rps28b and Dcp1 (17) or the *RPS28B* 3′ UTR element (8) are both indirect and bridged by Edc3. Instead, we found that Rps28b binds directly to Edc3 but not to Dcp1 (Fig. 5A) and it is Edc3, not Rps28b, that binds the *RPS28B* 3′ UTR regulatory element (Fig. 6). Consistent with these conclusions, tethering Edc3 enhanced *RPS28B-MS2* mRNA decay, but tethering Rps28a did not (Fig. 7C). These observations argue against the proposed role of Rps28b-Edc3 interaction in recruitment of the Dcp1/Dcp2 decapping enzyme (8) and suggest instead that interaction with Rps28b most likely regulates Edc3's ability to bind the *RPS28B* mRNA 3′ UTR regulatory element. Since Edc3 binds directly to Dcp2 (10, 37) and the effector function of tethered Edc3 is dependent on both subunits of the decapping enzyme (Fig. 7C),

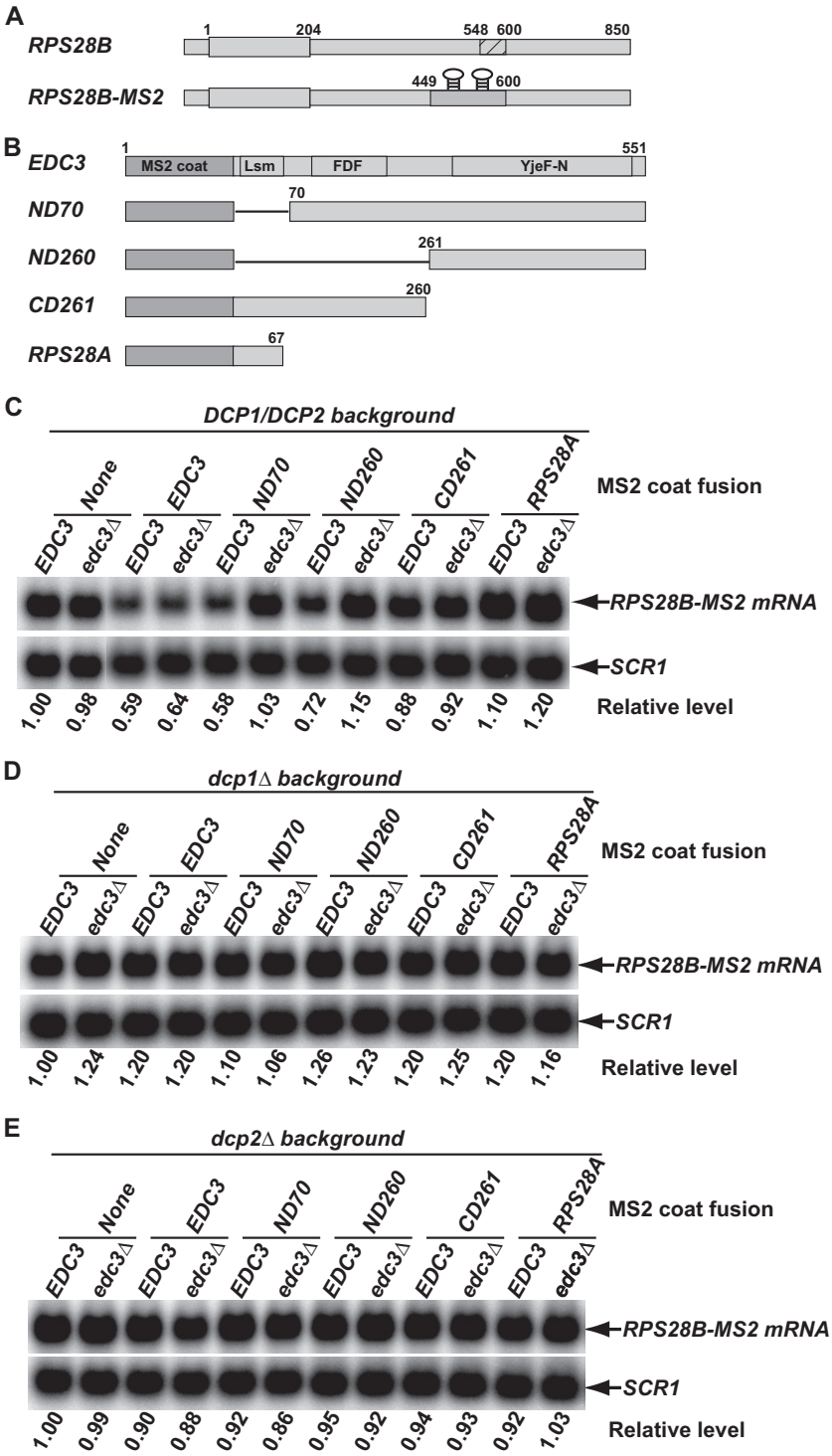

FIG 7 Analysis of the effect of tethering Edc3 or Rps28a on *RPS28B* mRNA decay. A DNA fragment containing two MS2 coat protein binding sites was inserted into the 3′ UTR originally containing the *cis*-regulatory element of the *RPS28B* gene. The resulting *RPS28B-MS2* allele and each of the individual alleles harboring Edc3 or Rps28a MS2 coat protein fusions were cointroduced into *EDC3* and *edc3*Δ cells in *DCP1/DCP2* (CFY159 and CFY161), *dcp1*Δ (HFY1067 and SYY2420), and *dcp2*Δ (CFY1016 and CFY1052) backgrounds. The steady-state levels of the transcript encoded by the *RPS28B-MS2* allele in these cells were analyzed by Northern blotting, using an oligonucleotide probe complementary to the MS2 coat protein binding sites. The blots were also hybridized to a random-primed probe specific for *SCR1* RNA to serve as loading controls. The relative levels of *RPS28B-MS2* mRNA in the cells in different strain backgrounds were normalized to the respective *EDC3* cells harboring the empty vector only. (A) Schematic representations of the *RPS28B* and *RPS28B-MS2* alleles. (B) Schematic representations of the Edc3 and Rps28a MS2 coat protein fusions used in the experiment. (C to E) Analysis of the effects of tethering Edc3 or Rps28a on *RPS28B* mRNA decay in *EDC3* and *edc3*Δ cells in *DCP1 DCP2* (C), *dcp1*Δ (D), and *dcp2*Δ (E) backgrounds.

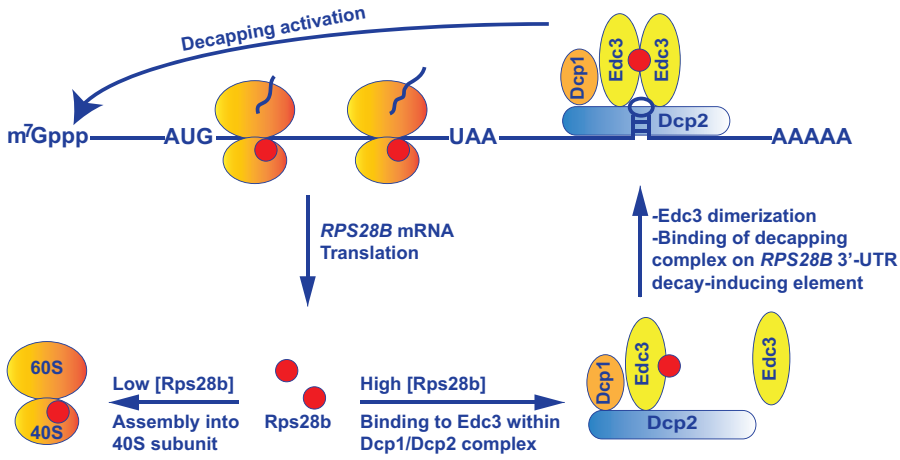

FIG 8 Revised model for Edc3-mediated *RPS28B* mRNA decay. *RPS28B* mRNA is translated in a conventional manner to produce molecules of Rps28b protein that are usually assembled into ribosomal 40S subunits. However, once the Rps28b protein concentration reaches a certain threshold, the excess Rps28b molecules that are produced bind to Edc3 within the Dcp1/Dcp2 complex. Rps28b binding to Edc3 promotes Edc3 dimerization and leads to the formation of an active decapping complex. This active decapping complex possesses unique substrate specificity for *RPS28B* mRNA, binds to the *RPS28B* 3′ UTR decay-inducing element, and catalyzes the removal of the cap structure from the *RPS28B* mRNA. The molecules and ribosomal subunits are not drawn to scale.

Rps28b-Edc3 interaction most likely targets the Dcp1/Dcp2 complex directly to *RPS28B* mRNA.

Collectively, our findings lead to a revised and expanded model for Edc3-mediated *RPS28B* mRNA decay (Fig. 8). In this model, *RPS28B* mRNA actively engaged in translation produces excess Rps28b that then binds to Edc3, most likely in a Dcp1/Dcp2 complex. Rps28b binding to Edc3 is thought to trigger the ability of Edc3 to self-associate, leading to the recruitment of one additional Edc3 molecule to the decapping complex. This newly assembled decapping complex must exhibit unique specificity for the *RPS28B* 3′ UTR element, and its binding to the element is postulated to target the Dcp1/Dcp2 decapping enzyme to *RPS28B* mRNA and to catalyze decapping of that mRNA. The two-hybrid analyses supporting this model indicate that Rps28b can form complexes with Dcp1, Dcp2, and Edc3 in yeast nuclei (Fig. 5A). This phenomenon is reminiscent of extraribosomal functions observed for yeast Rpl30 and human L13a and S3 ribosomal proteins (38–40), i.e., the regulatory functions of Rps28a and Rps28b most likely occur outside the yeast ribosome.

Edc3-mediated *RPS28B* mRNA decay is triggered by the excess Rps28 protein (8) (Fig. 4) and requires a physical interaction between Rps28 and Edc3 (25) (Fig. 5A). Paradoxically, we found that deleting a large internal Edc3 fragment that includes the Rps28 binding site (allele Δ*FDF*) had no effect on Edc3-mediated *RPS28B* mRNA decay (Fig. 1) or Edc3 binding to the *RPS28B* 3′ UTR element (Fig. 6B). One possible explanation of this unanticipated observation is that the large internal Edc3 fragment likely encodes two functionally antagonistic regulatory elements for Edc3-mediated *RPS28B* mRNA decay. A positive regulatory element may function to promote Edc3 decay activity, and a negative regulatory element could function to inhibit Edc3 decay activity. The positive regulatory element could promote Edc3-mediated *RPS28B* mRNA decay largely by counteracting or alleviating the inhibitory activity of the negative regulatory element. The *edc3* Δ*FDF* allele likely eliminates both the negative and positive regulatory elements, thus rendering the mutant protein constitutively active and independent of the function of the positive regulator Rps28 for Edc3-mediated *RPS28B* mRNA decay. The Rps28 binding motif identified by recent biochemical experiments (25) and our two-hybrid analyses (Fig. 5B) most likely represents the positive regulatory element located in the large internal Edc3 fragment. Consistent with this interpretation, deleting the Rps28 binding motif from the large internal fragment did not promote *RPS28B* mRNA decay. This mutant Edc3 protein likely retains the negative regulatory element but lacks the positive regulatory element for Rps28 binding and thus can no longer be activated by Rps28 to promote *RPS28B* mRNA decay.

Edc3 is directly involved in the recognition of mRNA substrates for decapping. Despite the limited number of transcripts regulated by Edc3 (7, 8), the factor is thought to be a general activator of mRNA decapping (2, 10), a role principally reflecting its ability to activate the Dcp1/Dcp2 decapping enzyme *in vitro* (10, 37, 41). Our experiments establish that, at least for *RPS28B* mRNA, Edc3 most likely functions as a direct substrate specificity factor for the decapping enzyme. This conclusion follows from two important observations. First, Edc3 binds to the *RPS28B* 3′ UTR regulatory element in the three-hybrid assay (Fig. 6A) and, second, tethered Edc3 can promote the efficient degradation of *RPS28B*-MS2 mRNA (Fig. 7C).

Considering that *YRA1* pre-mRNA and *RPS28B* mRNA are the only known Edc3 substrates (7, 8, 24), it is surprising that the two transcripts manifest notable mechanistic differences in their respective decay pathways, including the following. First, *YRA1* pre-mRNA decay is independent of translation and requires active translational repression (24). In contrast, *RPS28B* mRNA decay requires translation (Fig. 3). Second, the *cis*-regulatory elements involved in the decay of *YRA1* pre-mRNA and *RPS28B* mRNA exhibit significant differences in complexity, structure, and function. *YRA1* pre-mRNA decay is controlled by a set of functionally distinct yet partially redundant elements located in the *YRA1* intron (24) while *RPS28B* mRNA decay is controlled by a single element located in its 3′ UTR (8). The regulatory elements in these two transcripts do not share apparent sequence similarity and are not functionally interchangeable (Fig. 2). Third, decay of *YRA1* pre-mRNA or *RPS28B* mRNA requires different domains of Edc3. *YRA1* pre-mRNA decay requires only the Lsm domain of Edc3

and is independent of the activity of the protein's Rps28b binding motif (Fig. 1) (25). In contrast, *RPS28B* mRNA decay requires both the Lsm and the YjeF-N domains of Edc3 (Fig. 1) and is also dependent on the activity of the protein's Rps28 binding motif (25). These observations suggest that Edc3 uses its different functional domains or different combinations of these domains to identify its substrate mRNAs and that its Lsm domain may well be directly involved in recognition of the two ERE elements that we previously identified in the *YRA1* intron (24).

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