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Identification of the Rps28 binding motif from yeast Edc3 involved in the autoregulatory feedback loop controlling *RPS28B* mRNA decay

Olga Kolesnikova¹, Régis Back², Marc Graille^{2,3} and Bertrand Séraphin^{1,*}

¹Equipe Labellisée La Ligue, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Centre National de la Recherche Scientifique (CNRS) UMR 7104/Institut National de la Santé et de la Recherche Médicale (INSERM) U964/Université de Strasbourg, 67404 Illkirch, France, ²Ecole Polytechnique, Laboratoire de Biochimie, CNRS UMR7654, 91128 Palaiseau Cedex, France and ³Institut de Biochimie et Biophysique Moléculaire et Cellulaire (IBBMC), CNRS, UMR8619, Bat 430, Université Paris Sud, 91405 Orsay Cedex, France

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, the Edc3 protein was previously reported to participate in the auto-regulatory feedback loop controlling the level of the *RPS28B* messenger RNA (mRNA). We show here that Edc3 binds directly and tightly to the globular core of Rps28 ribosomal protein. This binding occurs through a motif that is present exclusively in Edc3 proteins from yeast belonging to the *Saccharomycetaceae* phylum. Functional analyses indicate that the ability of Edc3 to interact with Rps28 is not required for its general function and for its role in the regulation of the *YRA1* pre-mRNA decay. In contrast, this interaction appears to be exclusively required for the auto-regulatory mechanism controlling the *RPS28B* mRNA decay. These observations suggest a plausible model for the evolutionary appearance of a Rps28 binding motif in Edc3.

INTRODUCTION

Regulation of messenger RNA (mRNA) decay rates is an important mechanism to determine the abundance of cellular transcripts. Generally, eukaryotic mRNA degradation starts with the shortening of their poly(A)-tail. This is subsequently followed by decapping and 5'–3' exonucleolytic degradation. Alternatively, 3'–5' degradation by the exosome may take place after deadenylation (1–3). mRNAs as well as proteins involved in translation repression and mRNA decay assemble in cytoplasmic granules, often referred to as processing bodies (P-bodies), in which some transcript degradation was shown to occur (4–7). However, formation of these assemblies is not a prerequisite for initiation of mRNA

decay, and some mRNA decay was shown to occur co-translationally (8). In yeast, decapping is mediated by Dcp2, but the latter needs to be stimulated by additional factors such as Lsm1-7 complex, Dhh1, Edc1-3, Scd6 and Pat1 (9–15). Dhh1 and Pat1 were reported to promote decapping mainly through translational repression (16), whereas Edc1-3 proteins were shown to activate Dcp1/Dcp2 enzyme directly (15,17,18). It has become evident that the control of mRNA stability and the control of mRNA translation are interconnected, although little is known about the physical and functional relationships between factors of mRNA degradation and the translation machinery. Interestingly, Edc3 protein was shown to interact with ribosomal protein S28 (Rps28) in yeast. This association is involved in an auto-regulatory mechanism controlling the production of Rps28 (19) but could be involved in other processes, in particular if Edc3 was able to interact with ribosome bound Rps28. Furthermore, molecular details of the mode of interaction of these two factors remain unclear.

Edc3 protein is a conserved eukaryotic factor that interacts genetically and/or physically with many proteins involved in mRNA decay (14,17,20,21). Edc3 is composed of three conserved domains, each being responsible for interaction with factors involved in mRNA decay: an N-terminal Sm-like domain was reported to bind Dcp1 and/or Dcp2 (17,21,22), an internal FDF motif was shown to bind Dhh1 (20), whereas its C-terminal YjeF-N domain was shown to promote homodimerization (23,24). The multi-domain organization of Edc3 was shown to contribute to its function as scaffold for decapping proteins during P-body assembly (23). In the yeast *Saccharomyces cerevisiae*, the *EDC3* gene is not essential, and its inactivation does not result in detectable growth defect. Its deletion, however, enhances the growth phenotypes, and mRNA decay defects resulting from point mutation in the decapping enzyme Dcp2 or

*To whom correspondence should be addressed. Tel: +33 3 88 65 33 36; Fax: +33 3 88 65 33 37; Email: seraphin@igbmc.fr

its close co-factor Dcp1 (25). Additional genetic screens also demonstrated that Edc3 acts synergistically with Scd6 and Pbp1 to control mRNA decapping (14). Transcriptome analyses revealed that *EDC3* deletion causes accumulation of two specific RNAs: the *RPS28B* mRNA and the *YRA1* pre-mRNA (19,26). Yra1 is an mRNA export factor encoded by a split gene. Excess Yra1 promotes cytoplasmic export of the Yra1 pre-mRNA, which, once in cytoplasm, is degraded by 5'-3' decay mechanism dependent on Edc3 and on specific sequences in the *YRA1* intron (19,26,27). The Rps28 protein is a highly conserved archaea- and eukaryote-specific component of the small ribosomal subunit (28–31). Nuclear magnetic resonance studies of archaeal Rps28 proteins revealed that the protein contains four β -strands, forming compact globular oligonucleotide/oligosaccharide binding (OB) fold followed by a non-structured C-terminal tail (28,31). X-ray structure of the yeast ribosome indicates that eukaryotic Rps28 proteins adopt the same overall fold, even though the region equivalent to the unstructured archaeal C-terminal tail is stabilized through interaction with other components of the small ribosomal subunit (29). Rps28 binds near the exit site of the mRNA and has been shown to crosslink with the mRNA (32). In yeast, Rps28 protein is encoded by two genes: *RPS28A* and *RPS28B*, which produce polypeptides differing by a single residue that can substitute for one another in the ribosome. When present in excess, the Rps28 protein (the pool of Rps28a and Rps28b) binds to a conserved hairpin structure present in the 3' untranslated region (UTR) of the *RPS28B* mRNA and, in a manner dependent on Edc3, recruits the decapping machinery (19). The molecular details of this mechanism remain however unclear, as the mode of interaction of Rps28 with the *RPS28B* mRNA and Edc3 are currently unknown. It is also unclear whether this regulation is widely conserved in eukaryotes.

Here, we identify a region in yeast Edc3 that is necessary and sufficient for binding Rps28. Deletion of this region has no effect on yeast growth rate or accumulation of Edc3 in P-bodies, indicating that it is dispensable for the general RNA decay function of this factor. In contrast, this sequence is required for efficient degradation of the *RPS28B* mRNA. Degradation of *YRA1* pre-mRNA, the second Edc3-regulated transcript, is not affected by deletion of this region, indicating that the mechanism of Edc3 action on *YRA1* pre-mRNA is independent of its interaction with Rps28.

MATERIALS AND METHODS

Strains and plasmids

Yeast strains used in this study are listed in Table 1. All strains except BSY2475 are in the BMA64 background (33). The BSY2475 strain was derived from the MAV203 strain (Invitrogen) by PCR-based disruption of *EDC3*. Oligonucleotides OBS4144 and OBS4149 were used to amplify nourseothricine (NAT) resistance gene with flanking regions of yeast *EDC3* gene. BSY2474 was constructed from BSY1664 using the same PCR product.

BSY2831 strain was obtained by crossing strains BSY2474 and BSY2067. The construction of plasmids used in this study is described in the Supplementary Materials and Methods. These plasmids are listed in Supplementary Table S1. Oligonucleotides used for these constructions or for probing northern blots are listed in Supplementary Table S2.

Cell growth conditions

For growth analysis, cells were grown in exponential phase in synthetic complete (SC) medium lacking tryptophan complemented with 2% glucose. Cultures were diluted to the optical density at 600 nm (OD_{600}) 0.1 with sterile water. Five microlitres of each culture as well as 5 μ l of two 10-fold serial dilutions were plated on SC medium lacking tryptophan. Cell growth was monitored after 48 h at 25, 30 or 37°C. For microscopy, cells were grown at 30°C in SC medium lacking tryptophan and uracil complemented with 2% glucose, until an OD_{600} 0.3 was reached. Cells were subsequently harvested by centrifugation, washed, resuspended in SC medium lacking tryptophan and uracil supplemented, or not, with a carbon source. Cells were incubated in a shaker at 30°C for 10 min before analysis. For total RNA isolation, yeasts were grown at 30°C in SC medium lacking tryptophan complemented with 2% galactose until an OD_{600} of 0.8–1 was reached. For western blot analysis, 1 ml of corresponding cell cultures was further grown till $OD_{600} \geq 2$. For *RPS28B* mRNA half-life analysis, cells were grown in SC medium lacking tryptophan and uracil, supplemented with glucose. Cells were grown at 30°C until OD_{600} reached 0.8–1. Cultures were concentrated 10 times, doxycycline was added to the growth media to give a 40 μ g/ml final concentration and 1 ml aliquots of cells were taken at the time points indicated for total RNA isolation.

Protein purification, protein–protein interaction assay and protein detection

Recombinant proteins were (co-)expressed in BL21 Codon+ cells grown overnight in 100 ml of auto-induction media at 37°C. Cells were harvested by centrifugation, resuspended in 5 ml of lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl and 10 mM imidazole (pH 8.0) and sonicated. Two millilitres of cleared cell lysate was incubated with 100 μ l of Ni-NTA agarose beads at 4°C with rotation for ~1 h. Beads were washed with 500 μ l of 50 mM Tris-HCl, 300 mM NaCl and 20 mM imidazole (pH 8.0) three times. Specifically bound proteins were eluted with 250 μ l of 50 mM Tris-HCl, 300 mM NaCl and 200 mM imidazole (pH 8.0). Eluted proteins were separated in 12% Tris-Tricine gel and visualized with Coomassie-blue staining. Details of the analyses of protein interaction by size-exclusion chromatography with multi-angle laser light scattering measurements are described in the Supplementary Materials and Methods.

For western blot analysis of proteinA-tagged Edc3 proteins, total yeast extract was prepared as described previously (34). For detection, peroxidase-anti-peroxidase (3:10 000, Sigma) was used. For a loading control, the

Table 1. Yeast strains used in this study

Strain	Genotype	Origin
BMA64	MAT α ura3-1, delta trp1, ade2-1, leu2-3,112, his3-11,15	(33)
BSY2474	MAT α ura3-1, Δ trp1, ade 2-1, leu2-3,112, his3-11,15, can1-100, Δ edc3::NAT ^R	This work
BSY2475	MAT α leu2-3, 112, trp1-901, his3- Δ 200, ade2-101, gal4 Δ , gal80 Δ , SPAL10::URA3, GAL1::lacZ, HIS3UAS GAL1::HIS3@LYS2, can1 ^R , cyh2 ^R , Δ edc3::NAT ^R	This work
BSY2587	MAT α ura3-1, Δ trp1, ade2-1, leu2-3,112, his3-11,15, Δ scd6::Kan ^R	Gift of C. Gaudon
BSY2596	MAT α ura3-1, Δ trp1, ade2-1, leu2-3,112, his3-11,15, Δ edc3::NAT ^R , Δ scd6::Kan ^R	Gift of C. Gaudon
BSY2067	MAT α ura3-1, Δ trp1, ade 2-1, leu2-3,112, his3-11,15, can1-100, Δ rps28B::HIS	Gift of T. van den Elzen
BSY2831	MAT α ura3-1, Δ trp1, ade 2-1, leu2-3,112, his3-11,15, can1-100, Δ edc3::NAT ^R , Δ rps28B::HIS	This work

endogenous Stm1 proteins were detected using polyclonal anti-Stm1 antibody (1:1000, kind gift of Françoise Wyers).

Two-hybrid analysis

To avoid interference from the endogenous copy of Edc3, the BSY2475 strain was used. Beta-galactosidase activity was measured using Beta-Glo Assay system (Promega).

Microscopy

Microscopy analyses were performed as described previously (35). All images were acquired using Leica Microsystems Heidelberg GmbH microscope, using an objective HCX PL APO CS 63.0 \times 1.40 OIL UV. ImageJ software was used to adjust all images to equal contrast ranges.

Northern blot analysis

Total RNA was extracted by a hot phenol method (36). Usually, 15 μ g of total RNA were loaded per lane on a 3% formaldehyde agarose gel. Nucleic acids were transferred to the Hybond N membrane by passive transfer overnight. Random-prime labeling with NEBlot kit (NEB) was used to generate probes specific to *RPS28* and *YRA1* mRNA. The Yra1-specific PCR product was obtained with OBS4425 and OBS4426, whereas the Rps28-specific PCR product was obtained with OBS4427 and OBS4262. As a loading control, the signal recognition particle 7S RNA (*SCR1*), a stable RNA polymerase III transcript, was detected by a labeled probe generated by random priming. The amount of *YRA1* mRNA and pre-mRNA, as well as *RPS28A* and *RPS28B* mRNA was quantified using the Typhoon Phosphoimager. Mean values of the ratios of these species and standard deviation were calculated from three independent experiments. For *RPS28B* mRNA half-life analysis, 6 μ g of total RNA were loaded on gel and detected as indicated earlier in the text. The quantity of *RPS28B* mRNA normalized for the loading control was plotted as a function of time and fitted for exponential decay. Mean values of half-lives and standard deviations were calculated from two independent experiments.

RESULTS

A new conserved motif in yeast Edc3 proteins

Amino acid sequence of Edc3 from different species (*S.cerevisiae*, Human, Mouse and *Drosophila*) were

reported to share significant conservation (25). This early analysis was improved with the identification of three conserved motifs and domains: Lsm, FDF and YjeF-N. The availability of additional putative Edc3 sequences deduced from genome-sequencing projects lead us to perform new Edc3 sequence alignments. Although confirming the conservation of the Lsm domain, FDF motif and YjeF-N domain, those revealed the presence of a conserved region located between the FDF motif and the YjeF-N domain in Edc3 proteins from various *Saccharomycetaceae* species, but not from the other subdivisions of the *Saccharomycetales* phylum or more divergent species (Figure 1). This correlated with the previous report of the presence of a conserved hairpin in the 3' UTR of the *RPS28B* mRNA in the former group of organisms (19). This additional conserved region of Edc3, which corresponds to amino acids 209–222 of the *S.cerevisiae* protein, is hereafter referred to as the RB motif (for Rps28 Binding motif). The presence of this motif in a specific subset of Edc3 proteins suggested that it could be involved in the auto-regulatory feedback loop that controls the stability of the *RPS28B* mRNA, for example by mediating interaction with Rps28 or facilitating interaction of the latter with the hairpin structure present in the *RPS28B* mRNA 3' UTR.

Recombinant Edc3 protein binds directly Rps28, and this interaction requires region 201–231 of Edc3

Experimental evidences supporting an interaction between Edc3 and Rps28 were derived from two-hybrid and co-immunoprecipitation analyses (19). These data did not indicate whether Rps28 and Edc3 interact directly, possibly in a manner stabilized by protein and/or RNA partners, or whether their interaction was exclusively bridged by other factor(s). To study the role of the newly defined Edc3 motif, we, thus, first sought to develop a more direct protein interaction assays. For this purpose, we constructed plasmids to overexpress Edc3, either alone or together with Rps28a in *Escherichia coli* (Figure 2A). Although plasmids encoding full-length Edc3 produced insoluble protein (data not shown), a construct carrying an operon encoding a 6His-tagged Rps28 protein and Edc3 lacking its C-terminal YjeF-N domain [Edc3(1–277)] expressed both proteins in a soluble form (Figure 2B). Moreover, affinity chromatography on Ni-NTA beads demonstrated a co-purification of recombinant Rps28 and Edc3 (Figure 2B). A control construct lacking the 6His-Rps28

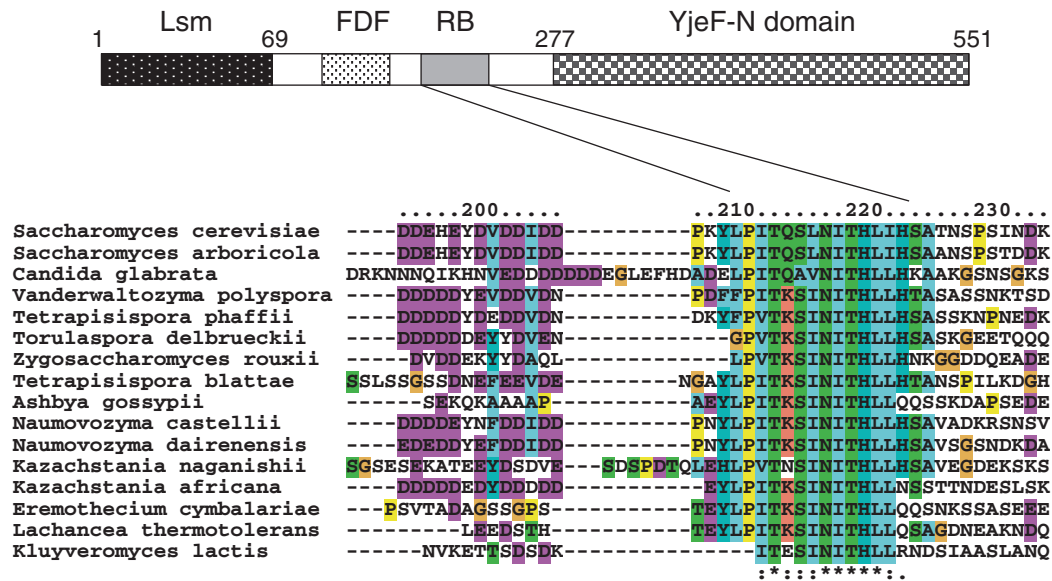


Figure 1. Localization of a new conserved motif in yeast Edc3 protein. Upper part: Domain architecture of *Saccharomycetaceae* Edc3 protein composed of an Lsm domain, a FDF motif and an YjeF-N domain. Numbers above the schematic representation of the protein indicate the amino acid positions of domain boundaries for the *S. cerevisiae* protein. Lower part: Amino acid alignment of Edc3 proteins from several *Saccharomycetaceae* species encompassing the RB motif. The RB motif is often preceded by an acidic region that is not absolutely conserved.

demonstrated that Edc3(1–277) did not bind non-specifically to Ni-NTA beads. These results indicate that Rps28 and Edc3 interact directly forming a stable complex without requirement for additional RNA or protein partners, and further that the YjeF-N domain of Edc3 is not required for this interaction.

Using this assay, we tested deletion derivatives of Rps28 and/or Edc3 to define sequences required or dispensable for heterodimer formation. Nuclear magnetic resonance and X-ray crystallography studies have shown that archaeal and eukaryotic Rps28 proteins contain four β -strands, forming compact globular part, followed by a less structured C-terminal tail (28,29,31). Deletion of the eight residues forming this tail in yeast Rps28 did not affect its association with Edc3 (Figure 2B). This indicates that Edc3 interacts with the globular OB fold of Rps28. An Edc3 construct covering residues 1–231 interacted well with Rps28, indicating that none of the residues located between the RB motif and the YjeF-N domain are required for this interaction. Similarly, deletion of the Lsm domain of Edc3 (construct 89–231) did not affect the formation of the Edc3-Rps28 heterodimer. In contrast, only background level of an Edc3(1–277) variant lacking residues 201–231 [Edc3(1–277- Δ RB)] were recovered with Rps28 (Figure 2B), indicating that this region, encompassing the RB motif, is required for heterodimer formation.

We used two-hybrid assay as a second strategy to validate these results *in vivo*. As Edc3 is known to multimerize (23,24), we preventively deleted the *edc3* gene in the host strain MAV203 to avoid any interference from wild-type Edc3 protein encoded by the endogenous chromosomal gene. We analysed the interaction of

full-length wild-type Edc3, or derivative Edc3 lacking residues 201–231, fused to the *GAL4* activation domain with either Dcp2, Dhh1 and Rps28 proteins fused to *GAL4* DNA-binding domain. Interactions were monitored by β -galactosidase production. Wild-type Edc3 interacted with Dcp2, Rps28 and Dhh1 as expected (23,37) (Figure 2C). Deletion of residues 201–231 of Edc3 resulted in only slightly lower β -galactosidase production when Dcp2 or Dhh1 were present but reduced this activity to background level with Rps28 (Figure 2C). Altogether, these data demonstrated that Edc3 binds directly the Rps28 core, and that this interaction requires amino acids 201–231 of Edc3.

The Edc3(201–231) fragment is sufficient for interaction with Rps28

The 201–231 region of Edc3, encompassing the conserved RB motif, was too small by itself to test whether it contains the necessary information to bind Rps28, using the co-purification strategy described earlier in the text. To assay whether this region is sufficient for interaction with Rps28, we constructed operons encoding a 6His-tagged GST carrier protein fused to amino acids 201–231 of Edc3 and untagged Rps28 (Figure 3A). Chromatography on Ni-NTA revealed co-purification of Rps28 with the 6His-GST-Edc3(201–231) fusion (Figure 3B). Untagged Rps28 by itself was not retained on the Ni-NTA resin when co-expressed with 6His-GST, indicating that Edc3 fragment 201–231 is responsible for interaction with ribosomal protein. Consistent with the results reported earlier in the text, a C-terminally truncated Rps28 behaved like the full-length protein (Figure 3B). We conclude that residues 201–231 of Edc3

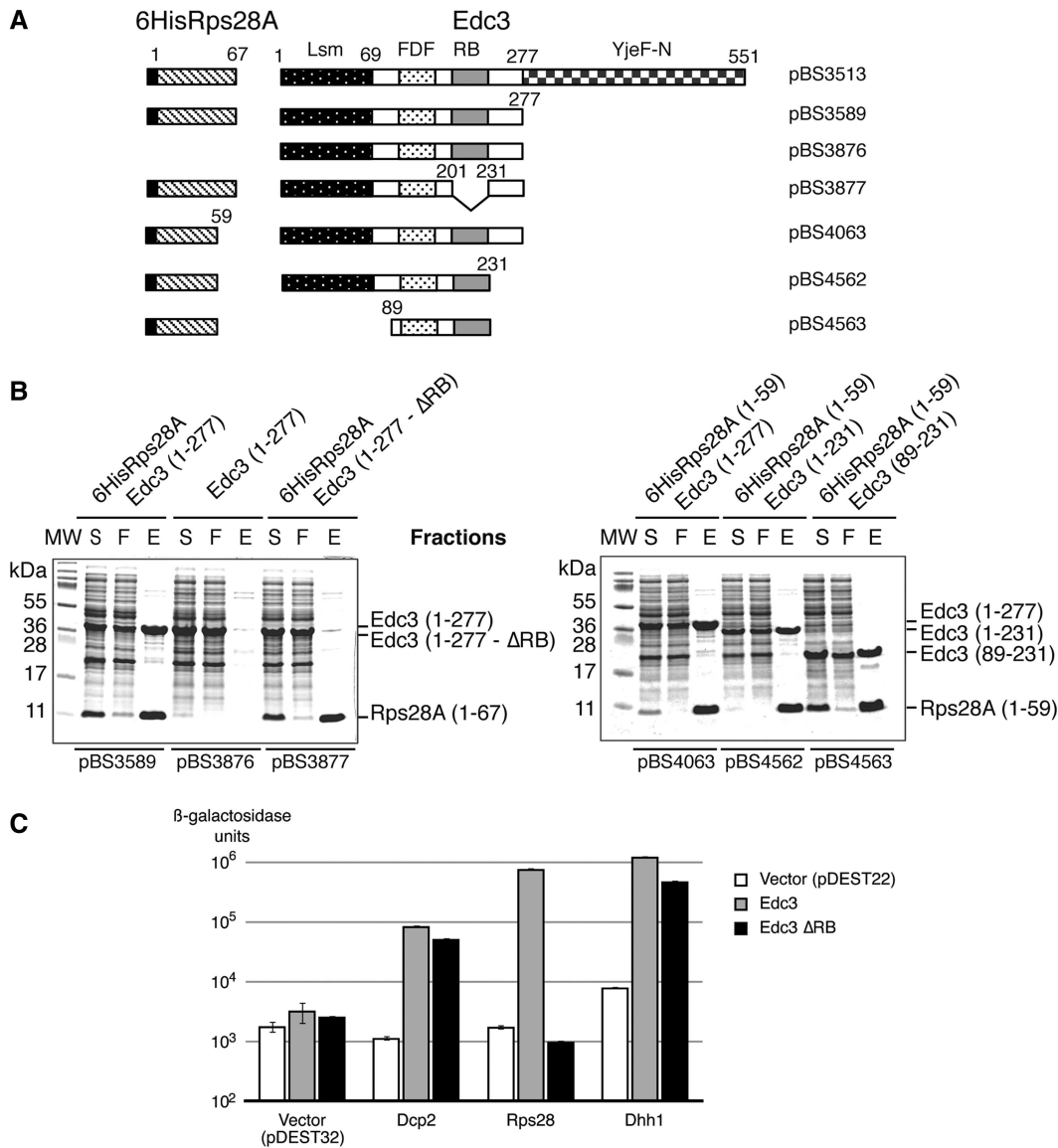


Figure 2. The RB motif of Edc3 is needed for interaction with Rps28a protein. (A) Schematic representation of the operon constructs used for expressing variants of *S. cerevisiae* Edc3 and Rps28a in *E. coli*. A 6His tag was fused to the N-terminus of Rps28a. Numbers above the schematic representation of the proteins correspond to the amino acid boundaries. Pattern codes for Edc3 domains are as in Figure 1. (B) Coomassie blue-stained Tris-Tricine-SDS-PAGE showing co-purification of recombinant Edc3 and Rps28a proteins. Supernatants of lysed *E. coli* cells expressing recombinant yeast 6His-Rps28a and Edc3 protein fragments were incubated with Ni-NTA beads and subsequently washed before elution of bound proteins with imidazole. S, supernatant of lysed cells; F, flow through; E, elution. (C) Beta-galactosidase activity measurements to monitor interactions in the two-hybrid assay. Full-length wild-type Edc3 and derivative Edc3ΔRB were fused to the *GAL4* activation domain, whereas Dcp2, Dhh1 and Rps28 proteins were fused to the *GAL4* DNA-binding domain. In each case, the matching vector was used as negative control.

are necessary and sufficient to interact with the core of Rps28 protein.

We next checked that the observed complex formation was not due to formation of soluble aggregates between both partners. For this purpose, we purified the untagged Rps28a protein as well as the Rps28a-Edc3(201–231) complex lacking the His-GST tag to analyse their quaternary structure in solution using size exclusion chromatography coupled online to light scattering (Figure 3C). This yielded calculated molecular weights of 7142 Da for free Rps28 (theoretical molecular weight of 7592 Da) and of

10836 Da for the Rps28a-Edc3(201–231) complex (theoretical molecular weight of 11267 Da). Interestingly, the molecular weight difference (3694 Da) between the Rps28a-Edc3(201–231) complex and free Rps28 corresponds to the molecular weight of the isolated Edc3(201–231) peptide (3693 Da theoretical), confirming complex formation. This further indicates that Rps28a and the Edc3(201–231) region interact with a 1:1 stoichiometry to form a heterodimeric complex in solution, and that this complex is stable as it resists to a 3-steps purification protocol.

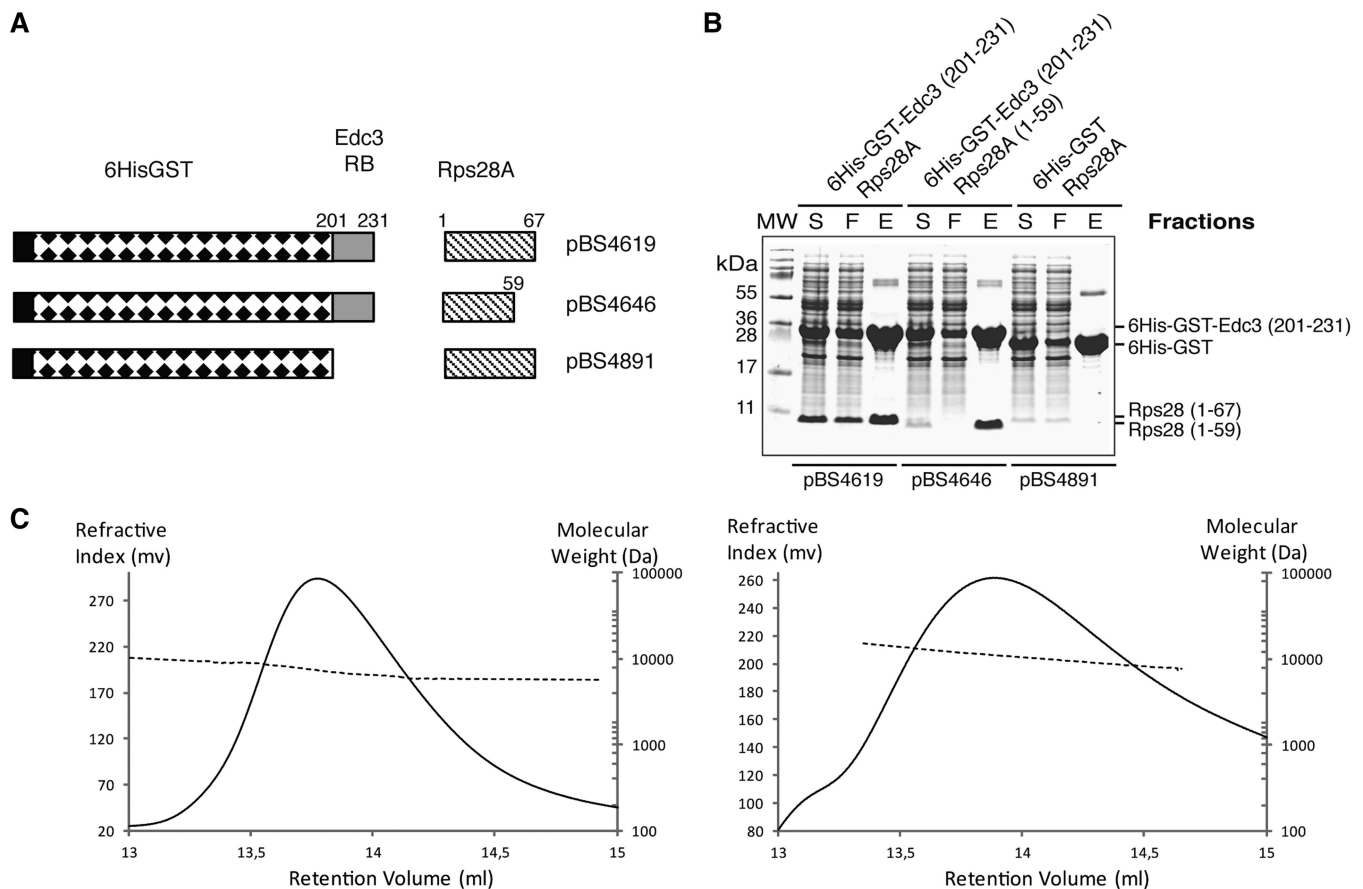


Figure 3. The RB motif of Edc3 is sufficient for interaction with Rps28a protein. (A) Schematic representation of the operon constructs used to express the RB domain of Edc3 protein (fragment 201–231) fused downstream of a 6His-GST carrier protein and Rps28a protein in *E. coli*. Numbers above the schematic representation of the proteins correspond to the amino acid boundaries. (B) Coomassie blue-stained Tris-Tricine-SDS-PAGE showing co-purification of recombinant yeast proteins. Supernatants of lysed *E. coli* cells expressing recombinant 6His-GST-Edc3(201–231) and Rps28A, 6His-GST-Edc3(201–231) and Rps28A(1–59), or 6His-GST and Rps28A, were incubated with Ni-NTA beads and subsequently washed before elution of bound proteins with imidazole. S, supernatant of lysed cells; F, flow through; E, eluate. Rps28A expressed with 6His-GST accumulates to a lower level, probably as a result of instability, in the absence of its partner 6His-GST-Edc3(201–231). Yet, the protein can be detected [compare with lanes where Rps28A(1–59) is present]. Furthermore, Rps28A(1–59) also accumulates at a low level; yet, its interaction with Edc3(201–231) can readily be detected, indicating that the absence of RPS28A in the eluate fraction when it is expressed with 6His-GST results from a lack of interaction. Consistently, when expressed with 6His-GST, Rps28A remains in the flow-through fraction. (C) Size-exclusion chromatograms of Rps28a (left panel) and Rps28-Edc3(201–231) complex (right panel) are shown. For clarity, only the refractive index (RI, solid line, left y-axis) for the eluted samples and the molecular mass calculated from light scattering (right y-axis, dashed line, logarithmic scale) are shown.

The Rps28 binding motif of Edc3 is not required for the general activity of Edc3 nor for its targeting to P-bodies

To analyse the functional role of interaction between Edc3 and Rps28, we constructed a centromeric plasmid carrying the *EDC3* gene in which residues 201–231 were deleted. To facilitate protein-level monitoring, a Protein A tag (protA) was fused to the C-terminus of the protein (*EDC3ARB-ProtA* construct). A plasmid encoding protA tagged wild-type Edc3 was built to serve as a positive control, whereas the vector without insert served as a negative control. We tested the ability of Edc3 Δ RB-protA protein to complement the slow growth phenotype resulting from the poor RNA decay in a *Δedc3Δscd6* strain (14). Indeed, deletion of *edc3* by itself has no impact on cell division or RNA decay (25). In the BMA yeast strain background, the reduced growth rate of the *Δedc3Δscd6* strain is especially apparent at 37°C (Figure 4A). Introduction of the

wild-type *EDC3* gene in this strain restored a growth comparable with that of *Δscd6* strain, which was itself equivalent to a wild-type strain (Figure 4A, expression of untagged Edc3 protein had the same effect, data not shown). Expression of Edc3 Δ RB-protA fusion restored the growth rate of the *Δedc3Δscd6* strain almost to the wild-type level, indicating that the mutant gene is functional and that interaction between Edc3 and Rps28 is not prerequisite for normal Edc3 function. Consistent with this efficient complementation, western blot analysis confirmed that Edc3 Δ RB-protA and Edc3-protA accumulate to the same level in cells (data not shown).

To test another potential general role of the Edc3-Rps28 interaction, we analysed the effect of deleting Edc3 residues 201–231 upon P-bodies assembly. Indeed, a strong reduction of microscopically visible P-bodies on glucose deprivation was observed in a *Δedc3* strain (23). We analysed the co-localization of Dcp2-GFP with

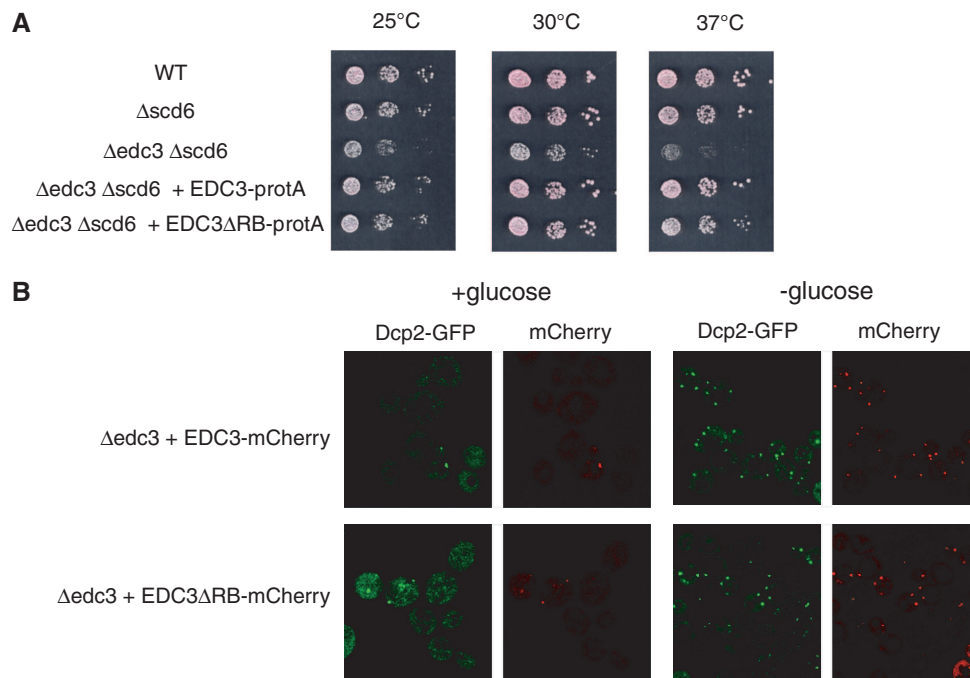


Figure 4. Deletion of the RB motif does not affect the general function of Edc3 or its intracellular location. **(A)** Assaying Edc3 activity by complementation of the slow growth phenotype of the $\Delta edc3 \Delta scd6$ strain at various temperatures. Ten-fold serial dilutions of $\Delta edc3 \Delta scd6$ strain transformed with a control empty vector (vector), a plasmid expressing wild-type Edc3 fused to the protA tag (Edc3-protA), or with a plasmid expressing Edc3 Δ RB-protA were spotted on -TRP selective media and incubated at 25, 30 or 37°C for 2 days. As positive controls, the isogenic wild-type and $\Delta scd6$ strains transformed with a control empty vector were spotted on the same plate. **(B)** Intracellular localization of Dcp2-GFP and Edc3-mCherry or Edc3 Δ RB-mCherry proteins in $\Delta edc3$ strain during exponential growth in selective media lacking uracil and tryptophan with or without glucose.

Edc3-mCherry fusions, using either wild-type Edc3 or Edc3 Δ RB. No difference in P-bodies number and/or pattern was observed upon expression of either wild-type or mutant Edc3 (Figure 4B). This indicates that interaction of Edc3 with Rps28 is not needed to address Edc3 to P-bodies.

The region 201–231 of Edc3 is needed for the regulation of the RPS28B mRNA decay

Deletion of the *EDC3* gene blocks the degradation of two specific RNAs: the *RPS28B* mRNA and the *YRA1* pre-mRNA (19,26). We analysed whether the deletion of the Edc3 RB motif influenced the stability of these RNAs. Total RNA was extracted from $\Delta edc3$ cells transformed either with an empty vector, with a vector coding for the wild-type Edc3-protA fusion, or with Edc3 Δ RB-protA. As a control, total RNA from wild-type cells transformed with an empty vector was used. We observed that deletion of the RB motif of Edc3 that abrogates interaction of the latter with Rps28 significantly stabilized the *RPS28B* mRNA (Figure 5A, left panel). Quantification revealed that deletion of the *edc3* gene led to more than 2-fold (2.8 ± 0.29) increase of the amount of *RPS28B* mRNA relative to *RPS28A* mRNA compared with the wild-type strain. Expression of the wild-type Edc3 restored a normal ratio of *RPS28A* versus *RPS28B* mRNA (1.3 ± 0.25). Deletion of residues 201–231 of Edc3 resulted in a significant accumulation of the *RPS28B* mRNA, although this effect was not as strong as in the complete absence of the

protein (2.2 ± 0.36 -fold increase compared with 2.8 ± 0.29 -fold). Western blot analysis of total protein extract from yeast cells indicated that the Edc3 Δ RB-protA protein was expressed at least as well as the wild-type factor excluding the possibility that lower levels of the former was responsible for the poor *RPS28B* mRNA degradation (Figure 5B).

Analysis of the *YRA1* pre-mRNA degradation gave opposite results. Deletion of the *edc3* gene resulted in an almost 4-fold (3.7 ± 1.86) increase in the amount of *YRA1* pre-mRNA relative to the mRNA compared with the wild-type strain (Figure 5A, right panel). Expression of wild-type Edc3-protA led to an almost complete restoration of the *YRA1* pre-mRNA/*YRA1* mRNA ratio (1.1 ± 0.66). Expression of Edc3 Δ RB-protA had the same effect (1.2 ± 0.42), indicating that interaction between Edc3 and Rps28 is not needed for the degradation of the *YRA1* pre-mRNA. This observation demonstrates further that the effect of the removal of residues 201–231 of Edc3 on *RPS28B* mRNA was highly specific.

To verify that the observed changes in *RPS28B* mRNA steady-state level are related to autoregulation of its stability, we analysed the half-life of *RPS28B* mRNA using a strain where the endogenous *RPS28B* and *EDC3* genes were deleted. Cells were transformed with a vector bearing *RPS28B* gene under a tetracycline-repressible transcription activator (19) and either a vector coding for wild-type EDC3-protA fusion or a vector coding for EDC3 Δ RB-protA fusion. Total RNA was isolated at different time points after doxycycline-induced

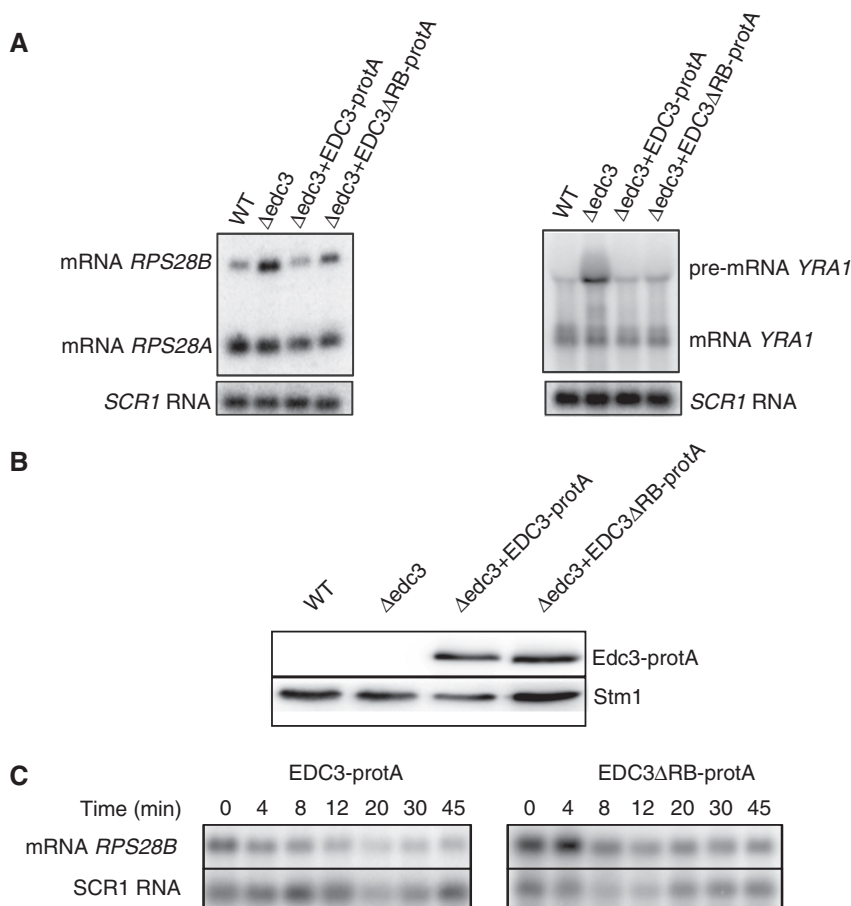


Figure 5. The motif of Edc3 is required for regulation of the *RPS28B* mRNA degradation but not for the decay of the *YRA1* pre-mRNA. (A) Northern blot analysis of total yeast RNA isolated from the $\Delta edc3$ strain transformed with empty vector (vector), with a plasmid expressing Edc3-protA, or with a plasmid expressing Edc3 Δ RB-protA. The isogenic wild-type strain transformed with empty vector was included as a positive control. Left panel: hybridization with a Rps28 probe that reveals both the *RPS28A* and *RPS28B* mRNAs. Right panel: hybridization with a Yra1 probe to detect the *YRA1* mRNA and pre-mRNA. To control for equal loading, the blots were hybridized with a probe recognizing the *SCR1* RNA. (B) Western blot analysis of total protein extract from yeast cells used for total RNA isolation for northern blot hybridization. Presence of protA tagged Edc3 was analysed with peroxidase-anti-peroxidase complexes. Antibodies against Stm1 were used to control for equal loading. (C) Northern blot analysis of total yeast RNA isolated from the $\Delta edc3Arps28B$ strain transformed with a vector bearing *RPS28B* gene under a tetracycline-repressible transcription activator and a plasmid expressing Edc3-protA, or a plasmid expressing Edc3 Δ RB-protA. Total RNA was isolated at indicated time points after addition of doxycycline. Hybridization was done with a Rps28 probe and a Scr1 probe (which serves as a loading control).

transcriptional repression of *RPS28B* and analysed by northern blotting. The results obtained (Figure 5C) clearly show the increase of the half-life time of *RPS28B* mRNA in the strain expressing EDC3 Δ RB-protA fusion (22.5 ± 2.1 min compared with 8 ± 1 min in the strain expressing the wild-type EDC3-protA fusion). These changes are similar to those reported when the autoregulation loop of *RPS28B* mRNA level was identified (19). These data indicate that the interaction between Rps28 and Edc3 mediated by RB motif is implicated in this autoregulatory process.

DISCUSSION

We have identified a novel motif within yeast Edc3 protein that is required and sufficient for binding to the ribosomal protein Rps28. Moreover, we have shown that Rps28 and Edc3 interact directly, forming a stable heterodimeric complex in the absence of other specific RNA or protein

partners. *In vivo* analyses demonstrated that this region of Edc3 is crucial for the auto-regulatory feedback loop that controls the *RPS28B* mRNA level, but that it is dispensable for other functions of Edc3, including its ability to regulate the *YRA1* mRNA level by inducing degradation of *YRA1* pre-mRNA. The latter result indicates that the two processes in which Edc3 participates to the auto-regulation of RNA levels by controlling their decay rates rest on different mechanisms involving different protein-protein interaction interfaces. The binding of Edc3 to Rps28 will ensure a rapid induction of *RPS28B* mRNA decay if free Rps28 protein starts to accumulate.

One can wonder whether the ability of Edc3 to interact with Rps28 extends to situation where the latter is incorporated in ribosomes. Our data indicate that Edc3 interacts with the Rps28 core rather than its C-terminal tail. Interaction of Edc3 with ribosome-bound Rps28 may be limited by the presence of Rps5 and ribosomal RNA that mask large share of the Rps28 core surface (29).

If decapping was shown to occur in part on polysome-associated mRNAs (8) and several mRNA decay factors were reported to sediment in polysomes (38,39), the presence of Edc3 in polysomes was, to the best of our knowledge, never reported. We also failed to detect such an association by polysome analyses or pull-down experiments (data not shown). Thus, it is probable that Rps28 present in ribosome is not accessible to Edc3, even if we cannot exclude that such association may take place temporarily or under specific conditions. If it would occur, association of Edc3 with ribosome-bound Rps28 would be restricted to a small group of organisms, arguing against a general role for such an interaction in mRNA decay. Detailed structural characterization of the Edc3-Rps28 dimer would provide more insights into this issue.

The limited conservation of the region of Edc3 involved in Rps28 binding suggests that it was acquired recently during evolution. This would explain its exclusive presence in proteins encoded by *Saccharomycetaceae* species where it would have been co-opted to finely tune the Rps28 protein production. Given the small size of the RB motif, one can easily imagine how it may naturally have evolved by incremental selection of residues improving the binding affinity in an otherwise poorly conserved linker region. Other events of auto-regulation of yeast ribosomal protein production, either through modulation of translation, splicing and/or control of transcription termination are known (40–42). The frequent observations of such mechanisms suggest that they evolve rapidly, taking advantage of the ability of proteins and RNA to create new interaction interfaces from short linear stretches of amino acid and oligonucleotide sequences. Their recursive occurrence indicates that finely tuning the production of ribosome subunits provides a key evolutionary advantage to yeast cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including [43–45].

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