

Pat1 Couples mRNA Decay with Ribosome-associated Quality Control

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Article

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Pat1 Couples mRNA Decay with Ribosome-associated Quality Control

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ABSTRACT

Ribosome collisions induced by defective mRNAs trigger mRNA degradation and the ribosome-associated quality control (RQC) pathway, which eliminates the associated nascent peptide. Upon ribosome stalling, collided ribosomes are remodeled by the Ribosome Quality Triggering (RQT) complex, allowing the nascent peptide remaining attached to the released 60S ribosomal subunit to be recognized by the RQC machinery and targeted for degradation. In parallel, defective mRNAs undergo decapping followed by 5'–3' degradation by the exoribonuclease Xrn1. How these pathways are coordinated remains largely unknown. Here, we identify the conserved decapping activator Pat1 as a key regulator linking mRNA decay to ribosome-associated quality control. We show that Pat1 remains associated with decay-committed stalled mRNPs before ribosome dissociation. Separation-of-function *PAT1* mutants uncouple mRNA stabilization from nascent peptide degradation, and efficient nascent peptide degradation in one *PAT1* mutant correlates with enhanced association of the RQT helicase Slh1 and reduced ribosome occupancy at the stalling site. Together, our study identifies Pat1 as the molecular link that coordinates decay-committed mRNPs with RQT-mediated ribosome remodeling and efficient RQC-dependent degradation of nascent peptides.

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1 INTRODUCTION

2
3 Translation-coupled quality control pathways monitor aberrant translation events,
4 promoting the elimination of defective mRNAs together with the aberrant nascent proteins they
5 produce, thereby preserving proteome integrity ¹⁻³. Over the past two decades, numerous
6 translation-coupled quality control pathways have been studied ⁴⁻¹⁰ and were notably found to
7 be conserved from yeast to mammals ^{2,11,12}. Various defects, including chemical damage to
8 mRNAs (truncation, depurination, oxidative damage or nucleobase dimers), stable secondary
9 structures, stretches of rare codons, or errors in mRNA processing, can induce ribosome stalling
10 and collisions during translation ^{2,6,7,13-15}. Ribosome collisions activate surveillance pathways
11 that promote degradation of the defective mRNA and its associated nascent peptide (NP), while
12 enabling ribosome recycling ^{1,13,16-19}. In *S. cerevisiae*, consecutive CGA arginine codons
13 efficiently induce ribosome stalling and are therefore widely used to activate these surveillance
14 pathways ^{2,5,6,9,18,20,21}.

15 On these problematic mRNAs, ribosome collisions generate a unique interface that is
16 recognized by the E3 ubiquitin ligase Hel2, which marks collided ribosomes with K63-linked
17 ubiquitin chains ^{11,17,18}. This signal recruits the Ribosome Quality Trigger (RQT) complex,
18 composed of the RNA helicase Slh1 and its cofactors Cue3 and Rqt4 ^{10,22,23}. RQT promotes
19 dissociation of the leading stalled ribosome, releasing a 60S subunit that remains associated
20 with the NP. The resulting 60S–NP complex is engaged by the Ribosome-associated Quality
21 Control (RQC) machinery, in which Rqc2 promotes recruitment of the E3 ubiquitin ligase Ltn1
22 to ubiquitinate the NP ^{4,7,24}. Cdc48 subsequently extracts the ubiquitinated peptide from the 60S
23 ribosomal subunit and delivers it to the proteasome for degradation ^{7,25,26}. In addition, Rqc2 can
24 catalyze the synthesis of CAT tails, thereby facilitating ubiquitination of NPs whose lysine
25 residues remain buried within the ribosomal exit tunnel ^{8,27}.

26 These studies have established a detailed mechanistic framework for ribosome
27 dissociation and NP degradation. However, whether mRNA decay and the RQC pathway are
28 coordinated remains an open question. Because RQC requires prior ribosome dissociation
29 whereas mRNA decay is initiated on the same transcript, these processes are likely coordinated
30 in space and time. Whether components of the mRNA decay machinery actively participate in
31 this coordination remains unknown.

32 The mechanisms governing mRNA degradation during ribosome stalling have also been
33 extensively investigated. No-Go mRNA Decay (NGD) was originally described as a
34 surveillance pathway triggered by severe translation elongation blocks, such as those caused by

1 stable stem-loop structures or clusters of rare codons, leading to endonucleolytic cleavage of
2 the defective transcript^{13,28}. Subsequent studies identified the SMR-domain protein Cue2 as the
3 endonuclease responsible for this cleavage^{19,29}. Cue2 cleaves mRNAs in a Hel2-dependent
4 manner, generating 5' and 3' RNA fragments³⁰. The resulting 3' fragment is degraded by Xrn1,
5 whereas stalled ribosomes remaining on the 5' fragment are rescued by Dom34–Hbs1, allowing
6 degradation of the transcript by the Ski–exosome complex^{19,31–33}. More recently, however,
7 accumulating evidence has indicated that Cue2-mediated cleavage is not the predominant
8 response to ribosome collisions. Instead, many collided mRNAs are eliminated through
9 decapping followed by Xrn1-mediated 5'–3' degradation^{19,34,35}.

10 Although these pathways are now well established, whether decapping and 5'–3' mRNA
11 degradation are mechanistically coordinated with RQT-mediated ribosome remodeling and
12 subsequent RQC remains unknown. To address this question, we screened mutants defective in
13 general mRNA decay using reporters containing consecutive CGA codons. Using this
14 approach, we identified the conserved decapping activator Pat1, a key factor in 5'–3' mRNA
15 decay^{36,37}, as a regulator of ribosome-associated quality control. Analysis of separation-of-
16 function *PAT1* mutants revealed an unexpected role for Pat1 in coordinating mRNA decay with
17 ribosome dissociation and Rqc2-dependent peptide degradation.

1 RESULTS

3 **Pat1 is required for efficient NP clearance during ribosome stalling**

4 We initially hypothesized that stabilization of defective mRNAs would permit
5 additional rounds of translation, thereby increasing ribosome collisions and potentially
6 overwhelming ribosome-associated quality control, resulting in NP accumulation. To test this
7 hypothesis, we used a modified *C. albicans* *URA3* reporter HA_URA as a control and an
8 identical construct containing four consecutive CGA codons HA_URA(CGA)₄, which
9 efficiently induces ribosome stalling and collisions (Fig. 1a).

10 We first examined mutants affecting general 5'–3' mRNA decay, including
11 components of the Ccr4–Caf1 deadenylase complex, the associated Not4 and Not5 factors,
12 and the decapping activators Lsm1 and Pat1³⁷⁻⁴⁰. Northern blot analysis revealed
13 accumulation of the characteristic 3' RNA decay intermediate, particularly in *lsm1Δ* and *pat1Δ*
14 mutants (Fig. 1b). These fragments correspond to ribosome-protected RNA regions generated
15 when Xrn1-mediated 5'–3' degradation is blocked by stalled ribosomes^{6,19,33,41,42}. Consistent
16 with this interpretation, no corresponding 5' RNA fragments were detected using a 5' probe
17 (Supplementary Figs. 1a-b), nor were 3' fragments observed when decapping was abolished
18 in a *dcp2* mutant (Supplementary Fig. 1c).

19 Western blot analysis of the same strains revealed a striking result. While several
20 mutants impaired mRNA decay, only *pat1Δ* exhibited a robust accumulation of stalled NPs
21 together with an increase in full-length (FL) translation products (Fig. 1c). The presence of FL
22 protein most likely reflects translational bypass of the four CGA codons followed by normal
23 translation termination. Consistent with this interpretation, extending the stall sequence to
24 twelve consecutive CGA codons completely abolished FL protein production (Supplementary
25 Fig. 1d).

26 The specificity of the *pat1Δ* phenotype became even clearer upon analysis of
27 additional mRNA decay mutants. Despite their established roles in mRNA turnover, *lsm1Δ*,
28 *lsm6Δ*, *not4Δ*, *not5Δ*, *ccr4Δ*, and *cue2Δ* mutants failed to accumulate NPs (Fig. 1c;
29 Supplementary Fig. 1e). Likewise, depletion of Dcp2, Ccr4/Caf1 or Dhh1 using auxin-
30 inducible degrons did not reproduce the *pat1Δ* phenotype (Supplementary Figs. 2 c-d-e). Thus,
31 impaired mRNA decay alone is insufficient to compromise NP clearance. Particularly
32 noteworthy was the absence of NP accumulation in *lsm1Δ*, despite the well-established
33 functional partnership between Lsm1 and Pat1 during decapping activation³⁷, suggesting that

Pat1 contributes to NP surveillance through an additional function independent of its canonical role in mRNA decay.

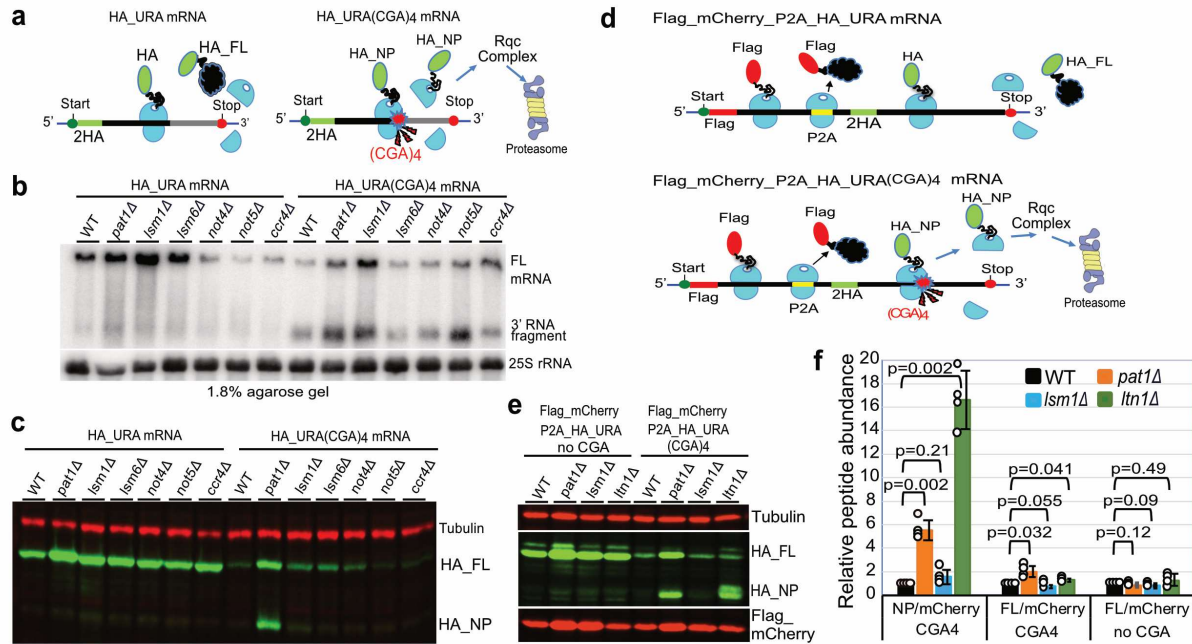


Fig.1. PAT1 deletion specifically affects the accumulation of nascent peptide upon ribosome stalling. **a** Schematic drawing of reporter mRNAs derived from *candida albicans* *URA3* gene. HA_URA mRNA serves as a control, and HA_URA(CGA)₄ is a reporter containing a (CGA)₄ insertion, four rare CGA codons, known to induce ribosome stalling during translation (indicated by red sparkles). Ribosome dissociation can be triggered, and a nascent peptide, NP, remaining on the 60S subunit, can be targeted for degradation by the proteasome. Start and stop of translation are indicated, reporters contain HA-tag sequence, thus producing HA-tagged full-length proteins (HA_FL) or HA-tagged nascent peptide (HA_NP). **b** Northern blot showing full-length mRNA (FL mRNA) or 3' RNA fragment derived from HA_URA and HA_URA(CGA)₄ mRNA reporters in WT and mRNA decay mutants. RNAs were detected with prA probe annealing the translational stop region (see Supplementary Fig. 1). 25S rRNA served as a loading control. **c** Western blot analysis using an anti-HA antibody to detect full-length (HA_FL) and nascent peptides (HA_NP). Tubulin detected by anti-tubulin antibody serves as a loading control. **d** Schematic representations of Flag_mCherry_P2A_HA_URA and Flag_mCherry_P2A_HA_URA(CGA)₄ mRNA reporters. From these reporters, the Flag-mCherry output is decoupled from the HA_URA by a P2A self-cleaving peptide sequence (ribosome skipping sequence). Because Flag-mCherry is released before the ribosome encounters the stalling sequence within the HA_URA ORF, its abundance is independent of the presence of a stalling sequence. HA_NP output will be dependent on the activation of a quality control (denoted RQC) in the presence of a (CGA)₄ stalling sequence. **e** Western blot analysis using an anti-HA antibody to detect full-length (HA_FL) and (HA_NP). Flag_mCherry, used as a proxy for monitoring reporter expression, was detected using an anti-Flag antibody. Tubulin served as a loading control. **f** Ratios of HA_NP and HA_FL to the corresponding Flag-mCherry levels following expression of the (CGA)₄ or control (no CGA) mRNA reporters, based on the experiment shown in (e). Ratios were normalized to the corresponding WT values, which were set to 1. Bars represent the mean ± SD of four independent biological replicates (n = 4), and dots indicate individual experiments. Statistical significance was assessed using a one-sample Student's t-test against the normalized WT value. Exact P values are indicated.

To further validate our reporter system, we next examined mutants defective in the early steps of ribosome collision recognition and remodeling, including *hel2Δ* (*rqt1Δ*), *cue3Δ*, *slh1Δ*, and *cue2Δ*^{10,18,19}. None of these mutations resulted in NP accumulation, with the exception of *pat1Δ* (Supplementary Fig. 1e). Finally, we analyzed mutants affecting the RQC machinery itself. As expected, deletion of *LTN1*, *RQC1*, or *RQC2* caused a marked accumulation of NPs without any detectable effect on RNA levels (Supplementary Figs. 1f-g), consistent with the established role of these factors in NP degradation^{4,7,35}. Notably, NP levels in these mutants were comparable to those observed in *pat1Δ*.

Together, these results identify Pat1 as a general mRNA decay factor whose loss selectively accumulates NPs. This unexpected phenotype suggests that Pat1 performs an additional function during ribosome-associated quality control. This observation prompted us to determine at which stage of the RQT–RQC pathway Pat1 exerts this previously unrecognized activity.

***PAT1* deletion induces NP accumulation at a post-translation initiation step**

We first asked whether the increased abundance of NPs in *pat1Δ* cells simply reflected enhanced reporter expression or increased translation initiation. To distinguish translation initiation from downstream quality control events, we generated a modified set of reporter constructs containing an N-terminal Flag-mCherry-P2A sequence upstream of the HA_URA and HA_URA(CGA)₄ sequences (Fig. 1d). The P2A peptide mediates ribosomal skipping, resulting in release of the nascent peptide while the ribosome continues translation, thereby producing two distinct proteins from a single open reading frame^{19,43,44}. As a result, Flag-mCherry expression is uncoupled from downstream events affecting the NP and therefore serves as a reliable proxy for mRNA abundance and translation initiation. Only the downstream 2HA-tagged NP is subjected to quality control when the four rare CGA codons are present (Fig. 1e-f).

As expected, disruption of the RQC pathway in *ltm1Δ* cells resulted in robust accumulation of the stalled HA-tagged NP without affecting production of the upstream Flag-mCherry protein, thereby validating the reporter strategy (Figs. 1e-f)^{4,7,35}. Importantly, *lsm1Δ* and *pat1Δ* cells produced comparable levels of Flag-mCherry, indicating similar reporter expression. Nevertheless, only *pat1Δ* cells accumulated HA-tagged peptides. Together, these observations demonstrate that the phenotype observed in *pat1Δ* cannot be simply explained

by increased reporter abundance or enhanced translation initiation, but instead reflects a defect occurring downstream of ribosome stalling.

We therefore asked whether Pat1 directly associates with stalled ribosome–mRNA complexes upon ribosome collision.

Pat1 remains associated with decay-committed stalled mRNPs

The selective accumulation of NPs in *pat1Δ* cells suggested that Pat1 might act directly on stalled ribosome–mRNA complexes during ribosome-associated quality control. We therefore asked whether Pat1 remains physically associated with these complexes after defective mRNAs have entered the decay pathway.

To address this question, we immunoprecipitated Flag-tagged NPs expressed from the Flag_URA(CGA)₁₂ reporter. Because insertion of twelve consecutive CGA codons effectively abolishes translational bypass and FL peptide production (Supplementary Fig. 1d), this reporter selectively enriches stalled ribosome-associated complexes. It therefore provides an optimal substrate for analyzing Pat1-containing surveillance intermediates.

Immunoprecipitation of the Flag-tagged NP revealed robust co-purification of the characteristic 3' RNA decay fragment (Fig. 2a). Detection of both 18S rRNA and the large ribosomal subunit protein Rpl15 further demonstrated that these RNA fragments remain associated with stalled ribosomes (Figs. 2a-b). Notably, co-immunoprecipitation of the NP, 3' RNA fragment and ribosomes was particularly prominent in *pat1Δ* cell extracts (Figs. 2a-b), consistent with the potential accumulation of stalled translation complexes in this mutant.

We next asked whether Pat1 itself remains associated with these stalled complexes. Immunoprecipitation of the Flag-tagged NP from wild-type cells expressing the Flag-CGA₁₂ mRNA reporter resulted in clear co-purification of Pat1 (Fig. 2b). In contrast, pulled-down Pat1 was markedly reduced when the Flag-tagged peptide was expressed from the non-stalling Flag-NoCGA reporter (Fig. 2b), in accordance with abundant and free Flag-tagged peptides released by canonical translation termination.

To further investigate this association, we performed reciprocal immunoprecipitation using Flag-tagged Pat1 and, for comparison, Flag-tagged Lsm1 under ribosome-stalling conditions. Both proteins efficiently co-immunoprecipitated the 3' RNA decay fragment detected by a probe specific for the stalling sequence (Fig. 2c), whereas no corresponding 5' RNA fragments were detected using a probe specific for the 5' region. Thus, reciprocal immunoprecipitation independently confirms that Pat1 remains associated with decay-committed stalled mRNPs. The role of Lsm1 is addressed in the next subsection.

Together, these findings identify a previously unrecognized decay-committed surveillance intermediate in which partially degraded mRNAs, stalled ribosomes and NPs remain physically associated with Pat1. Although our data do not establish whether Pat1 interacts directly with stalled ribosomes or through other components of the mRNP, they demonstrate that Pat1 persists on stalled translation complexes after initiation of mRNA decay.

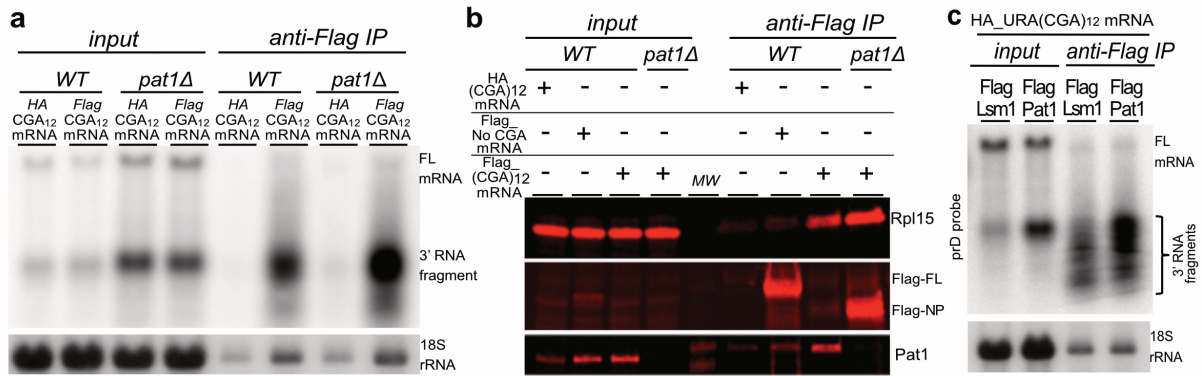


Fig. 2. The 3' RNA fragment remains associated with the ribosome-bound nascent peptide and with the mRNA decapping activator Pat1. **a** Analysis of 3' RNA fragment enrichment following immunoprecipitation of Flag-tagged peptides (anti-Flag IP). Northern blot analysis of input and anti-Flag-immunoprecipitated RNAs from WT and *pat1Δ* cells expressing HA_URA(CGA)₁₂ and Flag_URA(CGA)₁₂ mRNA reporters (HA_CGA₁₂, and Flag_CGA₁₂ respectively). Full-length mRNA (FL mRNA) and 3' RNA fragments were detected using probe prA (see Supplementary Fig. 1). The 18S rRNA serves as a control for ribosome recovery in the input and immunoprecipitated fractions. **b** Western blot analysis of input and anti-Flag-immunoprecipitated proteins. Expression from WT and *pat1Δ* cells expressing of the HA_URA(CGA)₁₂ (denoted HA_CGA₁₂), Flag_URA (denoted Flag_No CGA), or Flag_URA(CGA)₁₂ (denoted Flag_CGA₁₂) mRNA reporters. Membranes were probed for Flag-tagged full-length and NPs (Flag_FL and Flag_NP), Pat1 (the antibody recognizes the C-terminal region of Pat1) and the 60S ribosomal protein Rpl15. **c** Northern blot analysis of RNAs co-immunoprecipitated with Flag-tagged Pat1 (Flag_Pat1) or Flag-tagged Lsm1 (Flag_Lsm1) from cells expressing the HA_URA(CGA)₁₂ mRNA reporter. Full-length mRNA and the associated 3' RNA fragments were detected using probe prD, which hybridizes within the (CGA)_n region (see Supplementary Fig. 1). The 18S rRNA serves as a control for ribosome recovery in the input and immunoprecipitated fractions.

Pat1 closely associates with stalled ribosomes

The persistence of Pat1 on decay-committed stalled mRNPs prompted us to determine whether this association extends to collided ribosomes themselves. To distinguish interactions mediated by accessible regions of the mRNA from those maintained in close proximity to stalled ribosomes, cell lysates were treated with RNase I before immunoprecipitation. Under these conditions, only ribosome-protected mRNA fragments remain intact^{45,46}. This approach allowed us to test whether Pat1 remains associated with ribosome-protected RNA fragments encompassing the CGA stalling site.

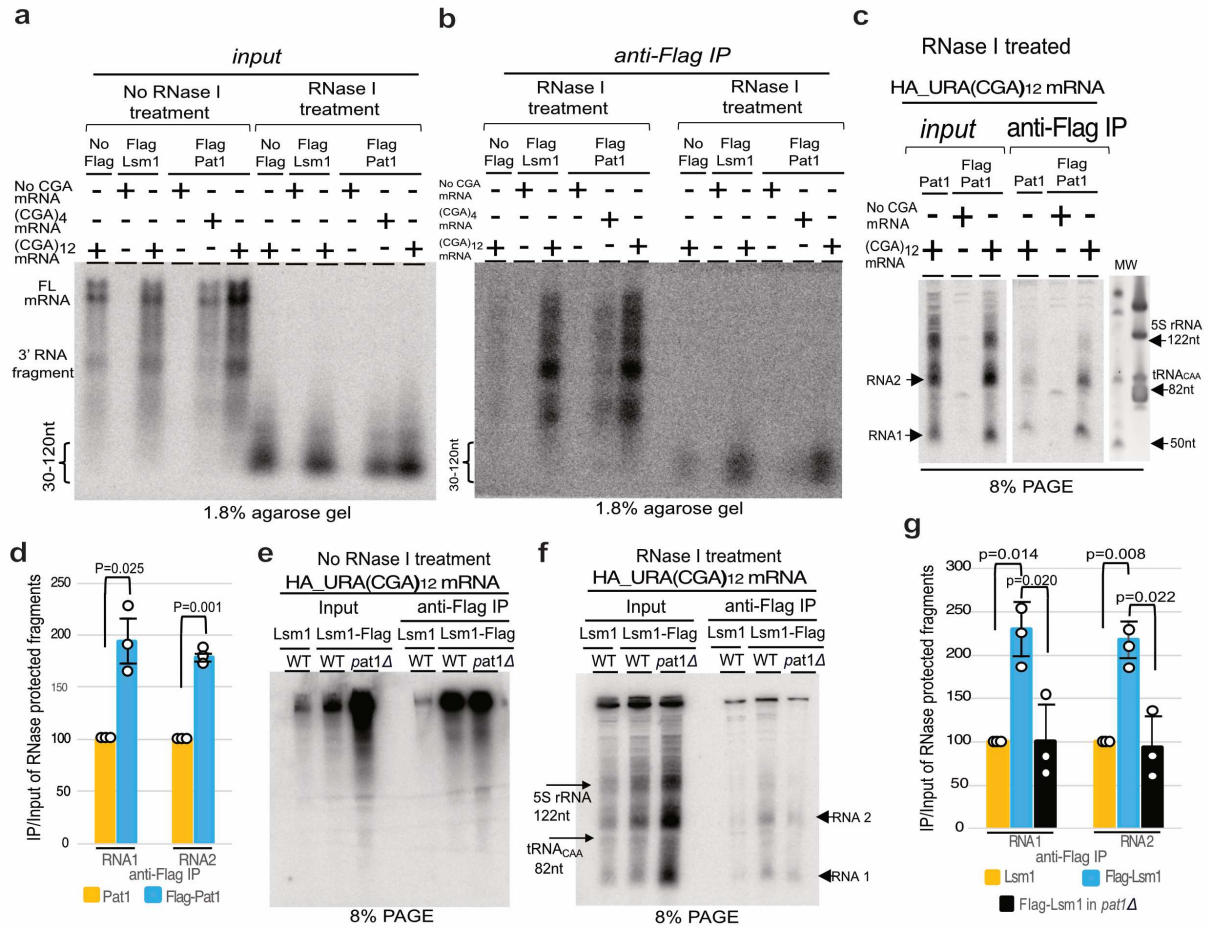


Fig. 3. Pat1 associates with stalled ribosomes. **a, b** Northern blot analysis of total RNA (**a**, Input) and anti-Flag-immunoprecipitated RNA (**b**, anti-Flag IP) from RNase I-treated or untreated cell extracts expressing the HA_URA or HA_URA(CGA)₁₂ reporters (No CGA and (CGA)₁₂, respectively) in the presence of untagged (NoFlag) or tagged proteins (Flag-Pat1 or Flag-Lsm1). Expression of HA_URA(CGA)₄ is shown only in the presence of Flag-Pat1. RNAs were separated on 1.8% agarose gels and hybridized with probe prD specific for the (CGA)_n stalling region. RNase I-resistant (CGA)-containing RNA fragments co-immunoprecipitating with Flag-Pat1 or Flag-Lsm1 are shown in (**b**). **c** High-resolution analysis of the RNase I-protected RNA fragments shown in (**b**). Total (Input) and anti-Flag-immunoprecipitated RNAs from cells expressing untagged Pat1 or Flag-Pat1 were resolved on an 8% polyacrylamide gel and hybridized with probe prD. Two protected (CGA)_n-containing RNA fragments of approximately 60 and 90 nt were designated RNA1 and RNA2, respectively. The 50-nt marker of the low-range ssRNA ladder, 5S rRNA (122 nt), and tRNA_{CAA} (82 nt) are indicated (MW). **d** Quantification of RNA1 and RNA2 enrichment following anti-Flag IP of Flag-Pat1 based on (**c**). IP/Input ratios obtained with untagged Pat1 were set to 100, and Flag-Pat1 values were normalized accordingly. Bars represent the mean $\pm$ SD of three independent biological replicates ($n = 3$), with dots indicating individual experiments. Statistical significance was assessed using a one-sample Student's *t*-test against the normalized control value (100). Exact P values are indicated. **e, f** Northern blot analysis of untreated (**e**) or RNase I-treated (**f**) WT and *pat1Δ* cell extracts expressing HA_URA(CGA)₄ in the presence of untagged or Flag-tagged Lsm1 (Lsm1 and Lsm1-Flag, respectively). Total and immunoprecipitated RNAs were resolved on an 8% polyacrylamide gel and hybridized with probe prD. Co-immunoprecipitated RNase I-protected RNA fragments (RNA1 and RNA2) are labeled as in (**c**). **g** Quantification of RNA1 and RNA2 enrichment following anti-Flag IP of Flag-Lsm1 from WT and *pat1Δ* cells as shown in (**f**). IP/Input ratios were normalized to the corresponding untagged Lsm1 values (set to 100). Statistical analysis was performed as in (**d**) using three independent biological replicates.

1 RNase I digestion generated the expected protected RNA fragments containing the
 2 CGA stalling sequence (Fig. 3a). Remarkably, immunoprecipitation of Flag-tagged Pat1
 3 specifically enriched these ribosome-protected fragments (Fig. 3b), demonstrating that Pat1
 4 remains associated with stalled ribosome complexes even after removal of accessible regions
 5 of the mRNA. High-resolution gel electrophoresis further showed that the recovered fragments
 6 predominantly correspond in size to footprints protected by disomes and trisomes, consistent
 7 with previous ribosome profiling analyses of CGA-induced ribosome collisions (Figs. 3c-d),
 8 ^{33,42,46}.

9 Because Flag-tagged Lsm1 immunoprecipitation also enriched both 3' RNA
 10 fragments (Fig. 2c) and RNase I-protected RNA fragments (Fig. 3b), we next asked whether
 11 this association depended on Pat1. We first confirmed that Pat1 efficiently co-
 12 immunoprecipitates with Lsm1 (Supplementary Figs. 3a-b). Strikingly, enrichment of
 13 ribosome-protected RNA fragments by Lsm1 was markedly reduced in the absence of Pat1
 14 (Figs. 3e-f-g), indicating that the association of Lsm1 with stalled ribosome complexes is
 15 largely mediated through Pat1. Although these experiments do not establish whether Pat1
 16 contacts the ribosome directly or through additional components of the stalled mRNP, these
 17 observations further support a model in which Pat1 is the primary decapping factor maintained
 18 in close proximity to collided ribosomes, even after defective mRNAs have entered the 5'–3'
 19 decay pathway.

21 **Separation-of-function *PAT1* mutants uncouple mRNA decay from NP clearance**

22 The observation that Pat1 remained associated with collided ribosome complexes
 23 suggested that its contribution to ribosome-associated quality control might extend beyond its
 24 canonical role in mRNA decapping. If so, these two activities should be genetically separable.
 25 To test this hypothesis, we generated a series of *PAT1* deletion mutants (Supplementary Fig.
 26 4a) and examined their effects on both mRNA decay and NP accumulation (Figs. 4a-b) ^{47 48}
 27 ^{49,50}.

28 We examined reporter mRNA accumulation under ribosome-stalling conditions induced
 29 by the (CGA)₄ sequence (Fig. 4a and Supplementary Fig. 4b), together with the corresponding
 30 NP and FL protein products by western blot (Fig. 4b and Supplementary Fig. 4c). Two mutants,
 31 designated *pat1-D1* and *pat1-D2*, or simply named *D1* and *D2*, displayed strikingly different
 32 phenotypes (Figs. 4a-b). The *D1* mutant closely phenocopied *pat1Δ*, leading to robust
 33 accumulation of both the NP and the full-length translation product. In contrast, the *D2* mutant
 34 increased accumulation of the FL protein while maintaining low levels of NPs.

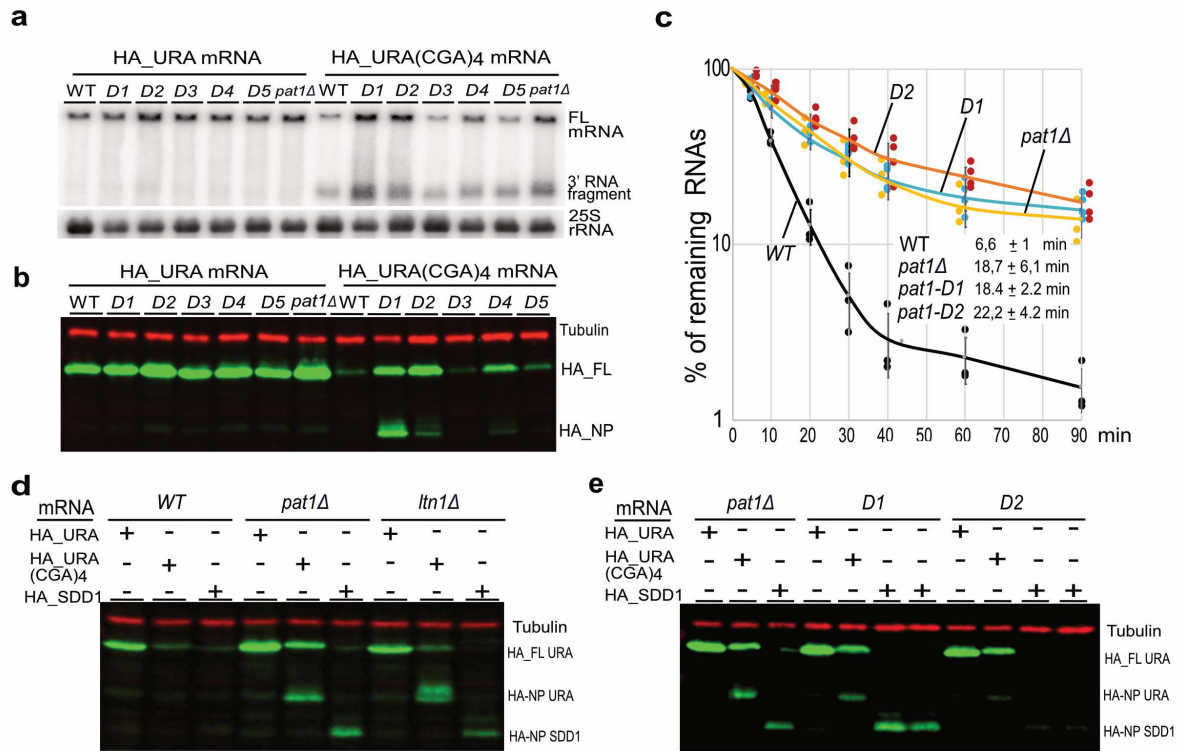


Fig. 4. Separation-of-function *PAT1* mutants differentially affect nascent peptide accumulation. **a** Northern blot analysis of full-length mRNA (FL mRNA) and 3' RNA decay intermediates (3' RNA fragment) derived from the HA_URA and HA_URA(CGA)₄ reporters expressed in wild-type (WT), *pat1Δ*, and cells expressing the Pat1 truncation mutants *D1*–*D5* (see supplementary Figs. 4a-c for mutants *D6*–*D11*). RNAs were detected using probe prA, which anneals near the stop codon. 25S rRNA serves as a loading control. **b** Western blot analysis of HA-tagged full-length (HA_FL) and arrest peptide (HA_NP) products expressed from the HA_URA and HA_URA(CGA)₄ reporters in the same strains as in (a). Proteins were detected with an anti-HA antibody. Tubulin, detected with an anti-tubulin antibody, serves as a loading control. **c** Decay kinetics of the Full-length RNA generated from the HA_URA(CGA)₄ reporter following transcriptional shutoff of cells expressing wild-type Pat1, *pat1Δ*, *pat1* mutants *D1* and *D2*. RNA samples were collected at the indicated time points and analyzed by northern blotting using probe prA (see Supplementary figs. 4d-e). The remaining FL RNA fragment was quantified, normalized to the value at time 0 (set to 100%), and fitted with a one-phase exponential decay model. Dots represent individual biological replicates (n = 3 for WT and *pat1Δ*, and n=4 for *D1* and *D2*). Calculated half-lives (mean ± SD) are indicated. **d** Western blot analysis comparing accumulation of full-length (FL) and nascent peptide (NP) products derived from HA_URA, HA_URA(CGA)₄, and HA_SDD1 reporters (containing a natural ribosome stalling sequence) expressed in WT, *pat1Δ*, and *ltn1Δ* cells. Products were detected using an anti-HA antibody, and tubulin serves as a loading control. **e** Comparative western blot analysis of full-length (FL) and nascent peptide (NP) products derived from the HA_URA, HA_URA(CGA)₄, and HA_SDD1 reporters expressed in *pat1Δ*, *D1*, and *D2* cells. Tubulin serves as a loading control.

To determine whether the different NP levels simply reflect differences in mRNA decay, we measured reporter mRNA stability following transcriptional shutoff (Fig. 4c and Supplementary Figs. 4d-e). We observed that both *D1* and *D2* stabilized the reporter mRNA to a similar extent, resulting in half-lives comparable to those observed in *pat1Δ* cells. Indeed,

mRNA stabilization was even greater in *D2* than in *D1*. Thus, the striking difference in NP accumulation cannot be explained by differential effects on mRNA stability.

Because *D1* and *D2* exhibit nearly identical defects in mRNA decay but markedly different capacities to eliminate NPs, these alleles genetically separate two functions of Pat1. Although CGA-containing reporters have been instrumental in dissecting the molecular mechanisms of translational quality control, they remain artificial substrates. To determine whether the distinct phenotypes observed for *D1* and *D2* also apply to an endogenous RQC substrate, we examined *SDD1*, a well-characterized yeast mRNA that naturally induces ribosome stalling and collisions, thereby activating the RQC pathway¹⁰ (Figs. 4d-e). To this end, we generated a tagged version of *SDD1* and confirmed that *PAT1* deletion leads to the expected accumulation of the Sdd1 NP (Fig. 4d). Notably, the *D1* mutant recapitulated *PAT1* deletion phenotype, whereas the *D2* mutant did not (Fig. 4e). These observations indicate that the functional separation revealed by the *D1* and *D2* alleles extends beyond artificial reporter constructs to a physiological RQC substrate.

In summary, the *D2* mutant uncouples defective mRNA decay from efficient ribosome-associated quality control. These results demonstrate that stabilization of collision-containing mRNAs is not sufficient to impair RQC-dependent NP degradation and reveal that Pat1 possesses a second activity that is mechanistically distinct from its established role in mRNA decapping.

Efficient Rqc2-dependent NP degradation is preserved in the *D2* mutant

We next verified whether the reduced steady-state abundance of NPs in the *D2* mutant reflected more efficient NP degradation. Using our ribosome stalling reporter constructs (Fig. 5a), we measured the half-life of the NP in both the *D1* and *D2* backgrounds (Figs. 5b-c). As expected, the FL protein, interpreted as the product of successful translation termination following bypass of the (CGA)₄ stall site, exhibited similar stability in both mutants (Fig. 5b). In contrast, the NP displayed a markedly shorter half-life in *D2* than in *D1* (Fig. 5c), indicating that its reduced steady-state abundance results from enhanced degradation.

If efficient NP degradation in *D2* reflects productive RQC activity, disruption of the pathway should restore NP accumulation. Consistent with this prediction, deletion of *RQC2* completely restored accumulation of the NP in the *D2* background, yielding levels comparable to those observed in *D1* cells (Fig. 5d)^{7,51}.

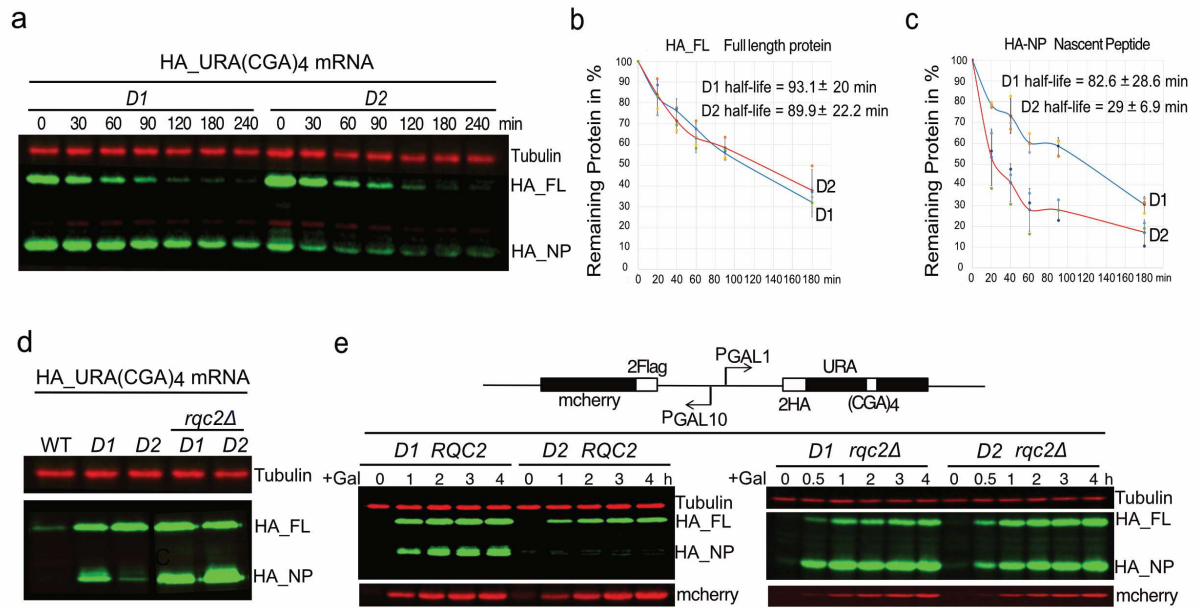


Fig. 5. Rqc2-dependent degradation of nascent peptides distinguishes the *D1* and *D2* mutants. **a** Time-course western blot analysis of the HA-tagged HA_URA(CGA)₄ reporter expressed in *D1* and *D2* cells. Translation was inhibited by cycloheximide addition, and cells were harvested at the indicated time points. Protein extracts were analyzed by western blot using an anti-HA antibody to detect the full-length protein (HA_FL) and nascent peptide (HA_NP). Tubulin, detected with an anti-tubulin antibody, serves as a loading control. **b, c** Quantification of protein decay over time for the full-length protein (HA_FL) (**b**) and nascent peptide (HA_NP) (**c**) based on the experiment shown in (**a**). Protein levels were normalized to the value at time 0 (set to 100%), and one-phase exponential decay curves were fitted to estimate protein half-lives. Data represent the mean $\pm$ SD of three independent biological replicates. **d** Western blot analysis of HA-tagged full-length (HA_FL) and nascent (HA_NP) peptides expressed from the constitutively active HA_URA(CGA)₄ reporter in WT, *D1*, *D2*, *D1 rqc2Δ*, and *D2 rqc2Δ* cells. Products were detected using an anti-HA antibody, and tubulin serves as a loading control. **e** Top, schematic representation of the galactose-inducible dual-reporter construct. The HA_URA(CGA)₄ reporter is expressed from the *GAL1* promoter, whereas a Flag-tagged mCherry reporter is expressed from the divergent *GAL10* promoter and serves as an internal control for galactose induction. Bottom, time-course western blot analysis following galactose induction in *RQC2* (left) and *rqc2Δ* (right) backgrounds expressing the *D1* or *D2* alleles. Products were detected as in (**d**) and Flag-mCherry with an anti-Flag antibody.

Likewise, time-course experiments following GAL inducible reporter expression showed that NPs readily accumulated in the *D2* mutant when the RQC pathway was inactivated by deletion of *RQC2* (Fig. 5e), closely recapitulating the kinetics observed in the *D1* background (Fig. 5e).

Together, these experiments demonstrate that the *D2* mutant retains efficient RQC-dependent NP degradation despite exhibiting a severe defect in mRNA decay. They further establish that the distinct phenotypes of *D1* and *D2* arise from differences in NP degradation rather than from differences in reporter expression, translation, or mRNA stability.

D1 and D2 proteins remain associated with stalled ribosomes

Polysome profile analysis first confirmed that both D1 and D2 factors co-sediment with polysomes, similarly to wild-type Pat1 (Supplementary Fig. 5a). Furthermore, although mRNA decay is impaired in both mutants, as in *pat1Δ* cells, immunoprecipitation of Flag-tagged NPs expressed from the ribosome-stalling reporter demonstrated that both D1 and D2 proteins co-immunoprecipitate with stalled ribosome complexes, similarly to wild-type Pat1 (Supplementary Fig. 5b). Importantly, neither the *D1* nor the *D2* mutation impairs the ability of Pat1 to associate with stalled mRNPs.

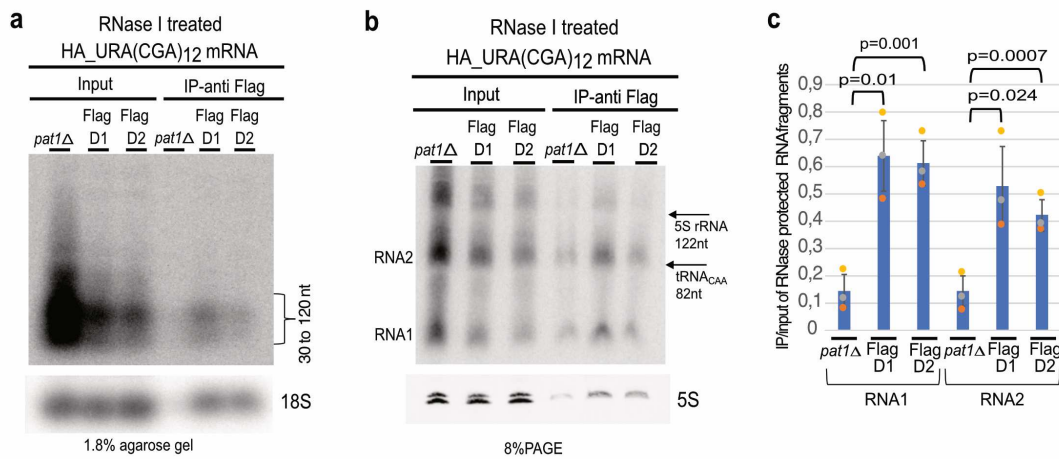


Fig. 6. D1 and D2 mutants remain associated with stalled ribosomes. **a** Northern blot analysis of RNase I-treated cell extracts expressing the HA_URA(CGA)₁₂ reporter in *pat1Δ*, *Flag-D1*, and *Flag-D2* cells. Total (Input) and anti-Flag immunoprecipitated (IP-anti-Flag) RNA were analyzed by northern blot following separation on a 1.8% agarose gel and hybridization with probe prD, which specifically detects the (CGA)_n stalling region. RNase I-protected (CGA)-containing RNA fragments co-immunoprecipitating with *Flag-D1* or *Flag-D2* are shown. 18S rRNA serves as a control for ribosome recovery in both the input and immunoprecipitated fractions. **b** High-resolution analysis of the RNase I-protected RNA fragments shown in (a). Total (Input) and immunoprecipitated (IP-anti-Flag) RNA samples were resolved on an 8% polyacrylamide gel and hybridized with probe prD. Two protected (CGA)_n-containing RNA fragments of approximately 60 nt and 90 nt were designated RNA1 and RNA2, respectively. The positions of 5S rRNA (122 nt) and tRNA_{CAA} (82 nt) are indicated. The 5S rRNA serves as a control for ribosome recovery in the input and immunoprecipitated fractions. **c** Quantification of the IP/Input ratios of RNA1 and RNA2 following anti-Flag immunoprecipitation of *Flag-D1* and *Flag-D2*. Bars represent the mean ± SD of three independent biological replicates (n = 3), and dots represent individual experiments. Statistical significance was assessed using a two-tailed Student's t-test. Exact P values are shown.

To determine whether these interactions are maintained in close proximity to stalled ribosomes, we generated Flag-tagged versions of the D1 and D2 proteins and performed anti-Flag immunoprecipitation following RNase I treatment (Figs. 6a-c). As with the wild-type Pat1, we asked whether the D1 and D2 proteins could enrich ribosome-protected RNA fragments containing the CGA stalling sequence. Input samples confirmed the presence of the

expected RNase I-protected CGA-containing RNA fragments in both mutant backgrounds (Figs. 6a-b). Importantly, immunoprecipitation of either Flag_D1 or Flag_D2 efficiently enriched these ribosome-protected RNA fragments to a similar extent (Fig. 6c), with no significant difference between the two mutants.

Together, these results demonstrate that both D1 and D2 factors retain the ability to associate with stalled ribosome complexes in close proximity to collided ribosomes, similarly to wild-type Pat1. Therefore, the distinct phenotypes of the two mutants cannot be explained by defective recruitment of Pat1 to stalled mRNPs, suggesting that the functional difference between D1 and D2 arises at a subsequent step.

***D1* and *D2 pat1* mutants differ in ribosome occupancy and Slh1 association**

Taken together, our results demonstrate that the *D1* and *D2* mutants similarly impair mRNA decay, remain associated with stalled ribosome complexes, yet exhibit markedly different capacities to promote NP clearance. Because efficient NP degradation requires productive RQT-mediated ribosome dissociation, we next asked whether these mutants differ in their ability to remodel stalled ribosomes.

To address this question, we first examined ribosome occupancy on the 3' RNA decay fragments by polysome profiling following sucrose gradient fractionation (Figs. 7a-b). As shown in Fig. 7c, the 3' RNA fragments displayed distinct sedimentation profiles in the two mutants. In *D1* mutant cells, the fragments were shifted toward heavier polysome fractions, indicating association with a greater number of ribosomes than in *D2* mutant. This increased ribosome occupancy could reflect a mild defect in ribosome dissociation in the *D1* mutant.

To independently assess this difference, we performed an RNase H assay (Fig. 7d) to determine whether the increased ribosome occupancy correlated with longer 5' extensions of the mRNA decay intermediates. Because Xrn1-mediated degradation is blocked by stalled ribosomes, increased ribosome occupancy should result in progressively longer protected 5' extensions, as previously mapped by primer extension³³. In the *D1* mutant (Fig. 7e), similarly to *pat1Δ*, we detected the accumulation of multiple RNA species (RNA1, RNA2, and RNA3; Figs. 7e-f) corresponding to progressively longer 5' RNA extensions. Quantification after normalization to 5S rRNA revealed a significant accumulation of the RNA1 and RNA2 species in *D1* compared with *D2* (Fig. 7f). The approximate sizes of RNA1 and RNA2 (55 nt and 85 nt, respectively) are consistent with the predicted sizes of RNA fragments protected by one or two stalled ribosomes at the CGA sequence (approximately 30 nt and 60 nt, respectively),

together with the additional 25 nt retained upstream of the RNase H cleavage site (Figs. 7d-e)
 33,46. Although these estimates do not fully match the ribosome occupancy inferred from
 polysome profiling (Fig. 7c), probably because the two methods differ in their sensitivity to
 the stability and composition of stalled ribosome complexes in cell extracts, both approaches
 consistently indicate that, unlike *D2*, the *D1* mutant accumulates mRNA decay intermediates
 associated with ribosomes stalled at the CGA sequence.

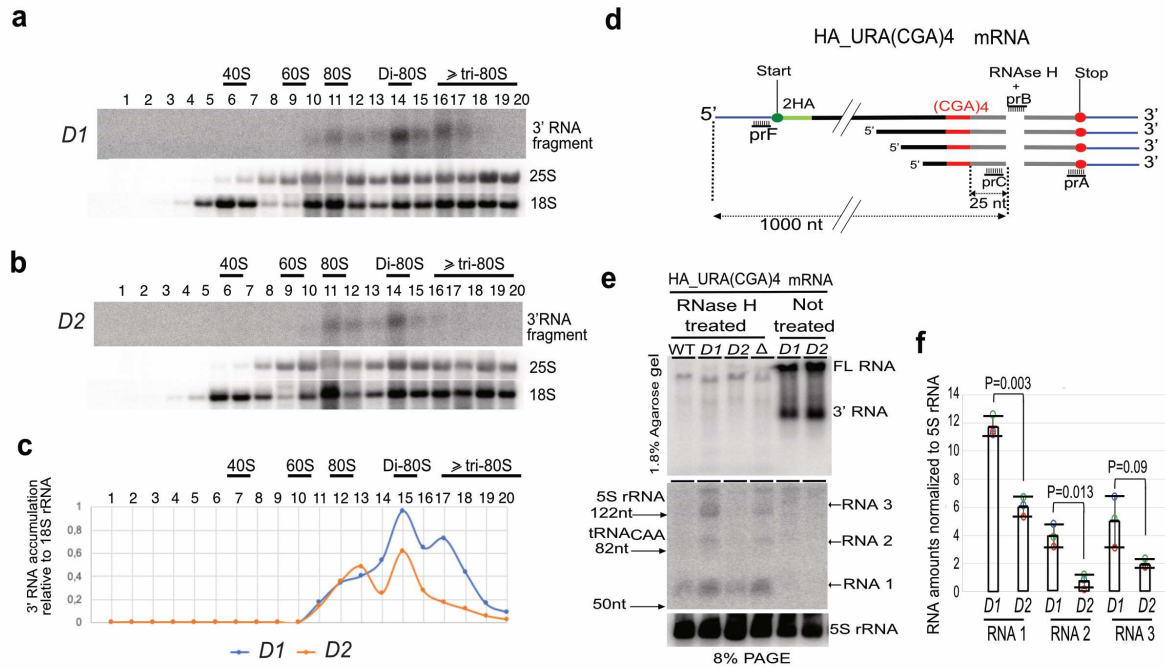


Fig. 7. *D1* and *D2* mutants differ in ribosome occupancy on the 3' RNA decay intermediate. **a, b** Northern blot analysis of RNA isolated from sucrose gradient fractions prepared from *D1* (a) and *D2* (b) cell extracts expressing the HA_URA(CGA)₄ reporter. RNAs were analyzed by northern blot using probe prA, which detects the 3' RNA decay intermediate. The positions of the 40S and 60S ribosomal subunits, 80S monosomes, di-80S, and >tri-80S complexes are indicated. 18S and 25S rRNAs serves as a control for ribosome distribution across the gradient. **c** Quantification of the 3' RNA fragment distribution across sucrose gradient fractions shown in (a,b). RNA signals were normalized to the corresponding 18S rRNA signal in each fraction. **d** Schematic representation of the HA_URA(CGA)₄ reporter and the RNase H mapping strategy used to determine the 5' ends of the 3' RNA decay intermediates. Total RNA was hybridized with the sequence-specific oligonucleotide prB, complementary to a region located 25 nt downstream of the fourth CGA codon, followed by RNase H digestion. **e** RNase H mapping of the 3' RNA decay intermediates in WT, *D1*, *D2*, and Δ (*pat1* Δ) cells expressing the HA_URA(CGA)₄ reporter. Top, northern blot analysis following separation on a 1.8% agarose gel showing full-length mRNA and the 3' RNA decay intermediate before RNase H treatment. Bottom, RNase H cleavage products resolved on an 8% denaturing polyacrylamide gel and detected using probe prC (see supplementary Fig. 1). Three protected RNA species (RNA1, RNA2, and RNA3) of approximately 55, 85, and 120 nt, respectively, were identified. 5S rRNA, detected by hybridization, serves as a loading control. **f** Quantification of RNA1, RNA2, and RNA3 levels in *D1* and *D2* cells following RNase H mapping. RNA signals were normalized to 5S rRNA. Bars represent the mean $\pm$ SD of three independent biological replicates ($n = 3$), and dots represent individual biological replicates. Statistical significance was assessed using a paired two-tailed Student's t-test. Exact P values are indicated.

Because translation of the CGA-containing reporter is comparable in *D1* and *D2*, as demonstrated by the identical production of NPs when *RQC2* is deleted (Fig. 5e), the increased ribosome occupancy observed in *D1* is unlikely to result from increased translation initiation. Instead, these findings suggest a mild defect in ribosome dissociation. We therefore asked whether this phenotype might reflect altered interaction with the RQT machinery.

To address this question, we immunoprecipitated Flag-tagged NPs from the *D1* and *D2* backgrounds (Figs. 8a, c). We examined the association of the RQT components Slh1 and Cue3 with these stalled complexes (Figs. 8a, c).

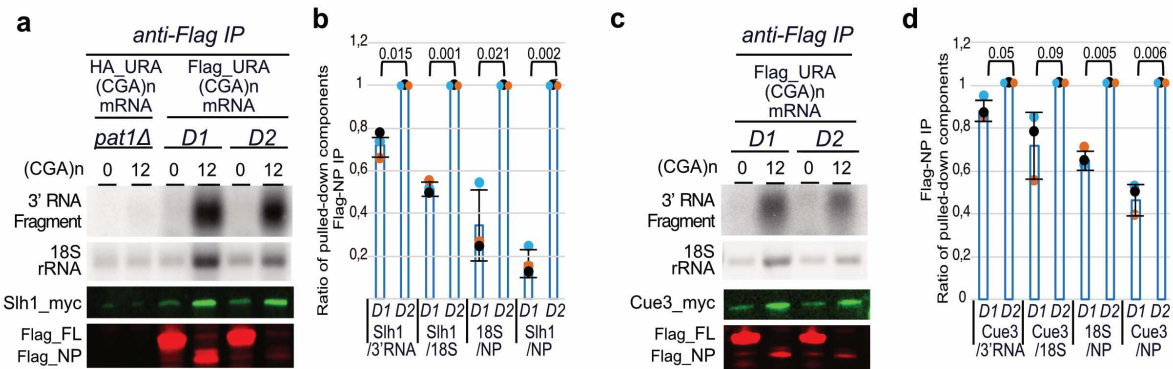


Figure 8. Differential association of Slh1 and Cue3 with stalled ribosome complexes in the *D1* and *D2* mutants. **a** Analysis of stalled ribosome complexes co-immunoprecipitated with the Flag-tagged nascent peptide (Flag-NP) derived from the Flag_URA(CGA)₀ and Flag_URA(CGA)₁₂ reporters expressed in *pat1Δ*, *D1*, and *D2* cells. HA_URA(CGA)₀ and HA_URA(CGA)₁₂ reporters were used as control for Flag_IP specificity in *pat1Δ*. RNAs were analyzed by northern blot using probe prA to detect the 3' RNA decay intermediate, with 18S rRNA serving as a control for ribosome recovery. Co-immunoprecipitated proteins were analyzed by western blot using an anti-Myc antibody to detect Slh1-Myc and an anti-Flag antibody to detect the full-length (Flag_FL) and nascent peptide (Flag_NP) reporter products. **b** Estimation of Slh1 association with stalled ribosome complexes in *D1* and *D2* cells. Signals corresponding to Slh1-Myc, the 3' RNA fragment, 18S rRNA, and Flag_NP were normalized to the corresponding values obtained in *D2* cells (set to 1). The normalized Slh1-Myc/3' RNA fragment, Slh1-Myc/18S rRNA, 18S rRNA/NP and Slh1-Myc/Flag_NP ratios are shown. Bars represent the mean $\pm$ SD of three independent biological replicates ($n = 3$), and dots represent individual biological replicates. Statistical significance was assessed using a one-sample Student's t-test. Exact P values are indicated. **c** Analysis performed as in (a) in the presence of Myc-tagged Cue3. **d** Estimation of Cue3 association with stalled ribosome complexes in *D1* and *D2* cells from experiments as shown in (c), and statistical analysis performed as in (b).

We analyzed the co-immunoprecipitation of Slh1 with NPs, 18S rRNA, and 3' RNA fragments in both mutants (Fig. 8a). Normalization of Slh1 pull-down to co-purified ribosomes (estimated by 18S rRNA levels) or to 3' RNA decay fragments consistently revealed reduced Slh1 association with *D1* stalled mRNPs compared with *D2* (Fig. 8b). This reduced association closely correlates with the increased ribosome occupancy observed in *D1* (Fig. 7), consistent with less efficient RQT-dependent ribosome dissociation. In contrast, the association of the

1 RQT subunit Cue3 with 18S rRNA or 3' RNA decay fragments differed only modestly between
2 D1 and D2 (Fig. 8d), suggesting that Cue3 recruitment is less dependent on Pat1 than the
3 recruitment of Slh1. Taken together, our data suggest that Pat1 promotes a molecular state of
4 decay-committed stalled mRNPs that is permissive for productive RQT-mediated ribosome
5 remodeling, at least in part through efficient association of Slh1, thereby facilitating RQC-
6 dependent clearance of NPs.

DISCUSSION

Ribosome collisions initiate elimination of defective mRNAs and degradation of the associated NPs^{4,35,52,53}. How these decay pathways are coordinated has remained largely unresolved. Here, we identify the conserved decapping activator Pat1 as a key component of this coordination. Our findings reveal that Pat1 remains associated with stalled mRNPs after they have committed to the 5'–3' decay pathway and plays a role that is genetically separable from its established role in mRNA decapping. Rather than acting solely as a decapping activator, Pat1 appears to organize a stage of surveillance in which mRNA degradation and ribosome-associated quality controls remain functionally integrated (Fig. 9).

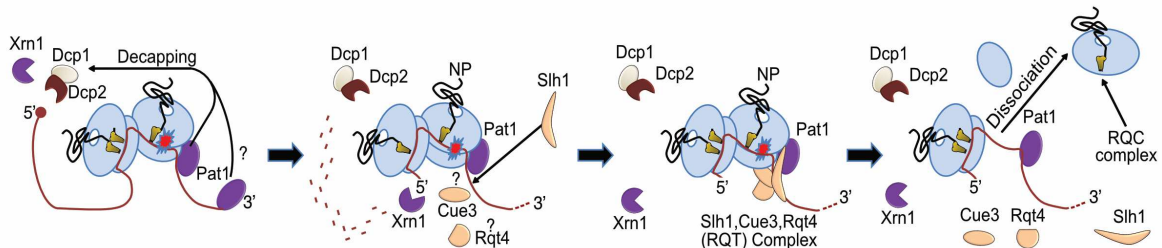


Fig. 9. Model for Pat1-mediated coordination of mRNA decay and ribosome-associated quality control. Pat1 initially functions as a canonical activator of mRNA decapping by promoting Dcp1–Dcp2-mediated removal of the 5' cap from stalled mRNAs. Whether Pat1 activates decapping while primarily associated with the 3' end of the transcript or is already bound to collided ribosomes at this stage remains unknown. The model does not rule out the presence of two Pat1 molecules on the same mRNA. Following decapping, Xrn1 degrades the transcript in the 5'–3' direction, generating a 3' RNA decay intermediate carrying collided ribosomes. Our data indicate that Pat1 remains associated with this decay-committed stalled mRNP. Pat1 then promotes the recruitment or stable association of the RQT helicase Slh1 with collided ribosomes. Cue3 and Rqt4 may associate with ubiquitinated collided ribosomes independently of Pat1, thereby facilitating assembly of the complete RQT complex once Slh1 is recruited. RQT-mediated ribosome dissociation releases a 60S ribosomal subunit carrying the peptidyl-tRNA which is subsequently recognized by the ribosome-associated quality control (RQC) machinery and targeted for proteasomal degradation. Pat1 therefore coordinates mRNA decapping with efficient ribosome dissociation, coupling 5'–3' mRNA decay to nascent peptide (NP) degradation during ribosome-associated quality control. In the absence of Pat1, both mRNA decapping and efficient RQT-dependent ribosome dissociation are impaired. The resulting delay in ribosome splitting may increase the contribution of alternative, RQT-independent ribosome rescue pathways that fail to generate optimal substrates for the RQC machinery, thereby reducing nascent peptide degradation.

Our data indicate that decay-committed mRNPs remain long enough to coordinate transcript degradation with the resolution of collided ribosomes. This conclusion is supported by several independent observations. Pat1 co-purifies with 3' RNA fragments that have already undergone decapping (Fig. 2), demonstrating that mRNA decay can precede, or occur concomitantly with, ribosome dissociation. Moreover, RNase I digestion preserves the

association of Pat1 with ribosome-protected RNA fragments encompassing the CGA stalling site, indicating that Pat1 remains in close proximity to collided ribosomes even after accessible regions of the mRNA have been removed (Fig. 3). Together with previous proteomic studies reporting potential associations between Pat1 and several small ribosomal subunit proteins^{54,55}, these findings support the existence of a decay-committed stalled mRNP that retains Pat1 during ongoing mRNA decay.

The identification of the *D2* separation-of-function allele provides the strongest genetic evidence that Pat1 performs two distinct activities during this quality control (Figs. 4 and 5). Although both *D1* and *D2* mutants impair mRNA decay to a similar extent, only *D1* causes robust accumulation of NPs. Conversely, *D2* retains efficient peptide clearance despite exhibiting reporter stabilization comparable to, or greater than, that observed in *D1* or *pat1Δ* cells. These observations demonstrate that stabilization of collision-containing mRNAs is not sufficient to compromise ribosome-associated quality control. Instead, they genetically separate Pat1-mediated mRNA decay from a second function that specifically promotes efficient elimination of NPs. To our knowledge, this represents the first genetic uncoupling of these two activities.

Importantly, *D1* and *D2* proteins remain associated with polysomes, co-immunoprecipitate stalled NPs and 3' RNA decay intermediates, and efficiently enrich ribosome-protected RNA fragments following RNase I treatment (Fig. 6). Thus, the functional distinction between the two alleles first arises when *D1* exhibits increased ribosome occupancy on the 3' RNA decay fragments relative to *D2*, as demonstrated by both polysome profiling and RNase H protection assays. Second, translation of the CGA-containing reporter is comparable in *D1* and *D2* mutants, as revealed by identical production of stalled NPs when RQC is genetically inactivated (Fig. 5). The increased ribosome occupancy observed in *D1* therefore cannot be attributed to increased translation initiation but instead most likely reflects less efficient ribosome dissociation (Fig. 7). Finally, although both mutants associate with the RQT machinery, normalization to ribosome occupancy consistently reveals increased association of the RQT helicase Slh1 in *D2* (Fig. 8). While these experiments do not demonstrate direct recruitment of Slh1 by Pat1, they strongly suggest that Pat1 promotes a molecular environment that is permissive for productive RQT-mediated remodeling of collided ribosomes.

A likely consequence of inefficient ribosome remodeling in *D1* is the generation of aberrant post-collision intermediates that are sub-optimally processed by the RQC machinery. Consistent with this interpretation, stalled NPs exhibit a substantially longer half-life in *D1* than in *D2* despite comparable levels of reporter translation. Moreover, deletion of *RQC2* completely

restores NP accumulation in *D2*, demonstrating that the reduced steady-state abundance of NPs reflects efficient RQC-dependent degradation rather than reduced synthesis.

The specificity of the *pat1Δ* phenotype further emphasizes that Pat1 performs a function distinct from its canonical role in mRNA decay. Neither impairment of decapping (*dcp2Δ* or *Dcp2* degnon) nor disruption of deadenylation (*ccr4Δ*, or *ccr4* or *ccr4/caf1* degnons) reproduced this phenotype. Likewise, despite its well-established partnership with Pat1 in decapping activation, *lsm1Δ* mutant failed to phenocopy *pat1Δ* mutant. This comparison with Lsm1 is particularly informative because both proteins are associated with decay-committed stalled mRNPs, however their contributions to ribosome surveillance are not equivalent. Lsm1 association with ribosome-protected RNA fragments is largely dependent on Pat1, whereas loss of Lsm1 has little consequence for NP clearance. These findings suggest that Pat1 constitutes the primary interface between the decapping machinery and stalled ribosomes, while the presence of Lsm1 within these complexes likely reflects its interaction with Pat1 rather than an independent role in ribosome-associated quality control.

Our results support a model in which mRNA decay and collided ribosome remodeling remain physically and functionally coordinated long after decapping has begun, indicating that mRNA surveillance and ribosome-associated quality control are not simply parallel outcomes of ribosome collisions^{18,35}. In this model, Pat1 persists on partially degraded transcripts together with stalled ribosomes and creates a molecular environment that favors productive remodeling by the RQT machinery. Although our data do not demonstrate that Pat1 directly recruits Slh1, the increased association of Slh1 with stalled ribosome complexes in the *D2* mutant strongly suggests that Pat1 promotes or stabilizes productive engagement of the RQT machinery. In contrast, Cue3 association did not differ between the *D1* and *D2* mutants, and we were unable to generate a stable tagged version of the third RQT subunit, Rqt4, preventing us from assessing its association with stalled ribosome complexes. To our knowledge, there is currently no evidence excluding the possibility that Cue3 associates with collided ribosomes prior to Slh1 recruitment. Indeed, the RQT complex has been proposed to be recruited to ubiquitinated collided ribosomes following Hel2-dependent K63-linked polyubiquitination, most likely through interaction of the Cue3 CUE domain with ubiquitin²³. This could explain why Cue3 association with stalled ribosomes appears independent of Pat1. It is therefore possible that collided ribosomes carrying Cue3, and potentially Rqt4, represent a pre-assembled intermediate that awaits Slh1 recruitment to complete RQT assembly and initiate ribosome dissociation (Fig. 9). Determining whether this effect reflects direct protein–protein interactions

1 or instead results from Pat1-dependent organization of the stalled mRNP will require future
2 structural and biochemical studies.

3 This work therefore revises the current view of translational surveillance by
4 demonstrating that commitment to mRNA degradation does not terminate quality control on
5 defective translation complexes but instead creates a molecular platform that ensures the
6 coupled elimination of both defective transcripts and their associated NPs. Defining the
7 molecular architecture of this surveillance complex intermediate, comprising Pat1, will be
8 important for understanding how eukaryotic cells preserve transcriptome and proteome
9 integrity during translational stress.

10

METHODS

Strains, plasmids and oligonucleotides

The strains of *S. cerevisiae* used in this study are derivatives of BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or BY4742 (*MATalpha his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Mutant strains were created using PCR-based methods as described in ⁵⁶. Where indicated, genomic mutations were introduced using Cas9-mediated gene targeting as described in ⁵⁷ (See supplementary Table 1 for strain list). Plasmids and oligonucleotides used in this study are described in Supplementary Tables 2 and 3, respectively. Detailed DNA sequences of synthetically generated (twist Bioscience) or PCR-amplified constructs used for plasmid construction or genomic recombination are provided in the Supplementary Information.

Cell cultures for steady-state RNA and protein extractions

Strains were grown either in YP medium contained 2% peptone, 1% yeast extract, and either 2% glucose, or in synthetic minimum media (SD) ⁵⁸. Minimal media was completed for auxotrophy, leucine, tryptophan or uracil was omitted to keep selection for plasmid when necessary. G418 sulfate (*Geneticin, thermofisher*) and ClonNat (*Sigma Aldrich*) were added to YPD agar plates at final concentrations of (200 µg/mL) and (100 µg/mL), respectively, to select for KanMX4 and NatMX6 markers.

Cell culture for mRNA and protein kinetics.

For mRNA and protein appearance kinetics, a preculture grown in synthetic medium lacking leucine was used to inoculate 150 mL of fresh synthetic medium lacking leucine at an initial cell density calculated to reach an OD_{600nm} of 0.6 the following morning. A T₀ sample corresponding to 10 OD units was collected, and gene expression was induced by the addition of galactose to a final concentration of 2%. Samples were subsequently collected at the indicated time points. For mRNA degradation kinetics, precultures grown in synthetic medium without leucine, supplemented with raffinose and galactose, were used to inoculate 150 mL of the same medium and grown to an OD_{600nm} of 0.6. Following collection of the T₀ sample, cells were harvested by centrifugation and resuspended in 10–15 mL of the same medium containing glucose as the sole carbon source to a final OD_{600nm} of 7, and aliquots corresponding to each

time point were collected. For protein decay kinetics, cycloheximide was added to a final concentration of (50 µg/mL). Cells were harvested at the time points indicated for western blot analysis.

Total RNA extraction and northern Blotting.

10 OD_{600nm} of exponentially growing cells were collected by centrifugation. Total RNA was extracted by hot acid phenol/chloroform method, precipitated with ammonium acetate and ethanol as detailed in ³³. RNA samples (5 µg) were separated on 1.8% agarose gel, transferred onto a nylon membrane (*Amersham, Hybond N+*). RNAs were cross-linked by UV irradiation (120 mJ/cm²). The membrane was hybridized overnight at 42°C in Roti-Hybri-Quick® solution (*Carl Roth, #A981.2*) with end-labelled oligodeoxynucleotide probes. The membrane was washed once in 5X SSC, 0.1% SDS, followed by two washes in 1X SSC, 0.1% SDS. Following exposure to phosphor imaging screens, blots were scanned using a Typhoon FLA 9500 or Typhoon 5 and quantified with ImageJ software.

For separation of lower weight RNAs, total RNAs (5–10 µg) was resolved by 8% TBE-urea polyacrylamide gel. The procedures for electrophoresis and subsequent electro-transfer are described in ⁵⁹.

RNase H cleavage of total RNA.

Twenty micrograms of total RNA were mixed with 1 µL of an RNase H-specific primer (50 µM) and 2 µL of hybridization buffer (0.1 M Tris-HCl, pH 7.5, 10 mM EDTA, 0.5 M NaCl) in a final volume of 20 µL. Samples were denatured at 85°C for 5 min and then allowed to cool passively to 37°C to promote primer hybridization. Subsequently, 5 µL of 10× RNase H buffer, 1 µL of RNase H (5,000 U/mL; *New England Biolabs, #M0297L*), and 24 µL of RNase-free water were added. Reactions were incubated for 1 h at 37°C. RNA was recovered by acid phenol extraction followed by ethanol precipitation in the presence of 20 µg glycogen. Pellets were washed with 70% ethanol, air-dried for 30 min, and resuspended in 20 µL of RNA loading buffer. Cleavage products were analyzed by northern blotting following separation on agarose or polyacrylamide gels as described above.

Protein extraction and western blotting

Protein extraction, SDS-PAGE and western blotting were carried out as described previously (Sinturel et al., 2015). The following primary and secondary antibodies were used: anti-HA mouse (Sigma-Aldrich #H9658 2ML); anti-FLAG rabbit (Sigma-Aldrich #F7425 2MG) and mouse (Sigma-Aldrich #F3165); anti-Myc rabbit (Sigma-Aldrich #SAB4301136), anti-Pat1 rabbit (generous gift from F. Lacroute). Secondary antibodies conjugated to IR dye 800 or dye 700 (LI-COR Bioscience-US). The membranes were scanned with Li-COR Odyssey Imager and quantified using Image Studio™ software. The western blots were probed with anti-tubulin rabbit (Abcam #184970) or anti-Rpl15 rabbit (Sigma-Aldrich #SAB 2109183) as a loading control. When the horseradish peroxidase-coupled secondary antibody (HRP) (Sigma-Aldrich #A9044) was used, the signals were revealed using an electrochemiluminescence reagent (ClarityWestern ECL, Biorad) and recorded using the ChemiDoc Imaging System (Biorad).

Polysome profile analysis.

Cultures were grown to exponential phase and harvested at an OD_{600nm} of 0.8. The 100 mL cell culture was quickly cooled in an ice-water bath and washed with buffer (20 mM HEPES, pH 7.5). After being transferred to FastPrep® tubes (ref. 3469-11), the cell pellet was snap-frozen in liquid nitrogen and stored at -80°C before being disrupted in lysis buffer (20 mM HEPES, pH 7.5, 110 mM KOAc, 2 mM MgCl₂, 0.5% Triton X-100, 0.1% Tween-20, 1 mM DTT) containing heparin (0.5 mg/mL), cycloheximide (50 µg/mL), one protease inhibitor tablet (Sigma-Aldrich #S8830), and 2.5 µL RNase inhibitor (Sigma-Aldrich, #03335402001) per tube. An equal volume of zirconium beads (Fisher scientific, ref. 11079105z) was added. Cells were incubated on ice for 15 min and then lysed by two 30-s cycles at speed 6 in a FastPrep® homogenizer. Lysates were recovered by centrifugation and clarified by two successive centrifugation steps (10,000 rpm, 5 min, 4°C). Samples corresponding to 20 OD_{260nm} units were loaded onto linear 10–50% sucrose gradients, which were centrifuged at 39,000 rpm for 3H at 4°C in a Kontron SW41 rotor. Polysome profiles were generated by continuous monitoring of absorbance at 254 nm using an ISCO fraction collector. Gradients were collected into 20 fractions. Protein samples were recovered by adding TCA to each fraction to a final concentration 10% v/v, mixed thoroughly, incubated at -20°C overnight, and precipitated by centrifugation. The precipitated protein pellet was rinsed using 100% acetone by centrifugation

and dissolved in 1 x Laemmli Sample Buffer to prepare the protein samples for western blotting. Total RNA was recovered from each gradient fraction, by the addition of 0,5 mL isopropanol and precipitation overnight at -20°C. RNA was pelleted by centrifugation for 20 min at 4°C and was extracted by hot acid phenol/chloroform method, and precipitated with ammonium acetate and ethanol.

Immunoprecipitation.

Yeast cells were grown as described in the polysome profiling section, with the exception that heparin was omitted. Following cell lysis, half of each sample was treated with 4 µL of RNase I (50 U/µL; *New England Biolabs*, #M0243L) for 30 min at 30°C. Input aliquots (10 µL for protein analysis and 20 µL for RNA analysis) were snap-frozen. A 500-µL aliquot of clarified lysate was incubated overnight at 4°C on a rotating wheel (8 rpm) with pre-equilibrated anti-FLAG M2 magnetic beads (*Sigma-Aldrich*, #M8823). 40 µL of beads were used for arrest-product immunoprecipitations, whereas 30 µL were used for immunoprecipitations of Pat1, Lsm1 and the D1 and D2 mutants. Following incubation, the beads were magnetically separated and washed three times with ice-cold lysis buffer and once with last wash buffer (100 mM NH₄OAc, 2 mM MgCl₂, 0.02% Tween-20) for 5 min at 4°C. Aliquots were then collected for protein and RNA analyses. Samples for protein analysis were resuspended in 40 µL of 1× Laemmli buffer and heated at 95°C for 5 min. RNA was extracted by hot acid phenol/chloroform method, precipitated with ammonium acetate and ethanol.

Statistical testing

All statistical analyses and graphing were performed using Microsoft Excel. As described in the individual figure legends, unpaired t-tests (two-tailed) or one-sample t-tests were applied to the raw data. Exact p-value are indicated in the figures.

Author contributions

L.B., C.T. and G.F. conceived and designed the study. L.B. prepared the manuscript and figures with input from all authors. All authors reviewed, edited and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Data availability

Source data are provided with this manuscript and include uncropped western blots, northern blots, and the data underlying all calculations and statistical analyses. Supplementary Figures, Supplementary Tables and a sequence file are provided in the Supplementary Information.

End notes

Supplementary Information: This manuscript includes Supplementary Information comprising five Supplementary Figures, three Supplementary Tables, and the sequences of constructs generated in this study.

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