ATP hydrolysis by UPF1 is required for efficient translation termination at premature stop codons

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Nonsense-mediated mRNA decay (NMD) represents a eukaryotic quality control pathway that recognizes and rapidly degrades transcripts harbouring nonsense mutations to limit accumulation of non-functional and potentially toxic truncated polypeptides. A critical component of the NMD machinery is UPF1, an RNA helicase whose ATPase activity is essential for NMD, but for which the precise function and site of action remain unclear. We provide evidence that ATP hydrolysis by UPF1 is required for efficient translation termination and ribosome release at a premature termination codon. UPF1 ATPase mutants accumulate 3' RNA decay fragments harbouring a ribosome stalled during premature termination that impedes complete degradation of the mRNA. The ability of UPF1 to impinge on premature termination, moreover, requires ATP-binding, RNA-binding and NMD cofactors UPF2 and UPF3. Our results reveal that ATP hydrolysis by UPF1 modulates a functional interaction between the NMD machinery and terminating ribosomes necessary for targeting substrates to accelerated degradation.
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quality control checkpoints exist during gene expression to
detect and eliminate intermediates lacking integrity or
functionality. For messenger RNAs (mRNAs) harbouring
a nonsense mutation, premature translation termination
precludes the synthesis of a full-length polypeptide and relegates
the transcript to rapid degradation via the nonsense-mediated
mRNA decay (NMD) pathway\(^1\)\(^-\)\(^3\). Three proteins, UPF1,
UPF2 and UPF3, comprise the core components of the NMD
machinery and are highly conserved from yeast to humans.
UPF1, an RNA-dependent ATPase and member of the SF1 family
of RNA helicases, is the only core NMD factor to exhibit catalytic
activity and serves as the central driver for targeting nonsense
codon-containing mRNA to NMD. Critically, mutation of
conserved aspartate and glutamate residues within motif II of
the UPF1 helicase domain prevents ATP hydrolysis and renders
the NMD pathway inactive\(^4\). While the ATP-binding
and hydrolysis cycle of human UPF1 has been implicated in
limiting the association of UPF1 with non-target mRNAs\(^5\)\(^-\)\(^6\) and
promoting disassembly of proteins from substrates after targeting
to NMD\(^7\)\(^-\)\(^8\), the precise site of action and function of ATP
hydrolysis by UPF1 remain unknown.

The cascade of events during NMD that culminates in the
accelerated degradation of a nonsense-containing mRNA begins
with a prematurely terminating ribosome. The key question
of how the NMD machinery monitors translation and distinguishes
between normal and premature termination has been the focus of
intense scrutiny. While it is now generally assumed that the NMD
machinery communicates with a terminating ribosome through
characterized interactions between UPF1 and eukaryotic release
factors, eRF1 and eRF3 (refs 1,9–11), it has been debated how this
interaction is initially established and what events must
subsequently transpire to promote accelerated decay of the
mRNA. Indeed, while it has been proposed that premature
translation termination is inherently aberrant and sufficient to
recruit UPF1 to the translation machinery, recent global
RNA-binding assays reveal that UPF1 interacts with both
normal and nonsense-containing mRNAs and that it binds
transcripts in a translation-independent manner\(^12\)\(^-\)\(^15\). Moreover,
although evidence in support of a role for UPF1 in influencing the
efficiency of translation termination at nonsense codons has been
presented\(^4\)\(^-\)\(^16\), the mechanism(s) by which this occurs remain to
be established. Finally, despite the fact that cofactors UPF2 and
UPF3 are critical in targeting most nonsense-containing mRNA
to NMD, their precise functions in the pathway are still ill
defined. Thus, our mechanistic understanding of the
requirements and consequences of UPF1 interaction with the
ribosome on targeting an mRNA to NMD is far from complete.

Herein, we provide evidence that ATP hydrolysis by UPF1 is
required for efficient translation termination and ribosome
release at premature termination codons. ATPase-deficient
mutants of UPF1 in yeast accumulate 3′ RNA decay intermediates
bound by ribosomes stalled during premature translation
termination that, in turn, impose an impediment to the complete
5′ → 3′ exonucleolytic degradation of the mRNA. We show that
ATP binding and RNA binding by UPF1 and the NMD cofactors
UPF2 and UPF3 are required for the function of UPF1
on termination, and provide evidence that in this context,
UPF2 functions independently of its ability to induce structural
rearrangements within UPF1 that activate its ATP hydrolysis and
helicase activities. Our results demonstrate a functional interac-
tion between UPF1 and terminating ribosomes \textit{in vivo}, and reveal
a direct role for ATP hydrolysis in modulating the efficiency of
translation termination on NMD substrates. These data add to
the growing body of evidence that the stability of both normal
and aberrant mRNAs is tightly controlled by events directly
impacting the efficiency of mRNA translation.

\section*{Results}

3′ RNA decay intermediates accumulate in UPF1 ATPase mutants.

UPF1 mutants with defects in ATP hydrolysis (DE572AA), ATP
binding (K436E) or RNA binding (RR793AA; Fig. 1a)\(^6\) were examined to evaluate the role of these biochemical activities in
targeting nonsense-containing mRNA to the NMD pathway in
yeast. The steady-state abundance of \textit{PGK1} reporter mRNA
harbouring a nonsense or premature termination codon (PTC; at
position 344; Fig. 1b, top) was monitored in cells deleted for
endogenous \textit{UPF1} (\textit{upf1Δ}) and expressing comparable levels of
plasmid encoded wild-type or mutant \textit{UPF1} (Supplementary
Fig. 1a). Consistent with targeting of the nonsense-containing
reporter to NMD, \textit{PGK1} mRNA abundance was elevated ~4-fold
in cells lacking UPF1 (Fig. 1b; compare full-length RNA in lanes 1
and 2). Moreover, in the presence of all three UPF1 mutants,
reporter mRNA abundance was similar to \textit{upf1Δ} cells, confirming
observations that these mutations inactivate UPF1 and disable the
NMD pathway\(^4\).

In cells expressing ATPase-deficient UPF1, a rapidly migrating
RNA species was detected that was not present in the ATP- or
RNA-binding mutants, or in the complete absence of \textit{UPF1}
(Fig. 1b, arrow). This RNA species corresponds to the 3′ end
of \textit{PGK1} mRNA since the probe hybridizes to a unique region
within the reporter 3′ untranslated region (UTR), and a probe
complementary to the mRNA 5′ end upstream of the PTC failed
to detect this species (Supplementary Fig. 1b). Appearance of
the smaller RNA species was also dependent on premature transla-
tion termination, as \textit{PGK1} reporter mRNA lacking the nonsense
codon failed to generate a similar band (Fig. 1c).

Our data reveal distinct functional consequences for inactiva-
tion of ATP-binding versus ATP hydrolysis activities of UPF1 on
the accumulation of the 3′ RNA fragment (compare DE572AA
and K436E mutants; Fig. 1b). This observation is unexpected
given that corresponding mutations in human UPF1 (that is,
DE636AA and K498A) behave similarly in reports implicating
the protein in disassembly of protein complexes from NMD
substrates\(^7\) and in NMD target discrimination\(^2\). We addressed
this anomaly by analysing additional missense mutations at lysine
436 reported to disrupt ATP binding by UPF1 (ref. 4). In all cases,
substitution of K436 inactivated UPF1 and eliminated targeting of
substrates to NMD (Supplementary Fig. 1c). Consistent with
our results when glutamic acid was introduced at this position
(that is, K436E), replacement of this residue with either proline
or glutamine failed to yield a faster migrating 3′ RNA species.
In contrast, insertion of alanine (that is, K436A) led to the
appearance of a 3′ RNA fragment similar in size to that formed in
cells harbouring ATPase-deficient UPF1 (Supplementary Fig. 1d).
Although a direct comparison of the ATP-binding and hydrolysis
activities for these mutants is lacking, one simple interpretation
of these data is that the UPF1 K436A mutant, while devoid of ATP
hydrolysis activity, retains residual \textit{in vivo} ATP-binding capacity
(Supplementary Discussion). Consistent with a diminished yet
functional ability to bind ATP, the abundance of the 3′ RNA
fragment in the K436A mutant was 2.5-fold less than that for the
DE572AA mutant, of which the latter exhibits virtually identical
\textit{in vitro} ATP binding kinetics as wild-type UPF1 (ref. 17).

To characterize the nature of the RNA product accumulating
in UPF1 ATP hydrolysis mutants, reporter mRNAs harbouring
a nonsense mutation at a variety of codon positions were analysed
(Fig. 1d, top). Notably, 3′ RNA fragments of variable size were
detected for mRNA reporters in the presence of ATPase-deficient
UPF1, with sizes coincident with the length of RNA extending
from the nonsense codon to the mRNA 3′ end (Fig. 1d, bottom). The
accumulation of 3′ RNA products was independent of
the mRNA analysed, as nonsense-containing \textit{GFP} mRNA also gave
rise to a similar PTC position-dependent pattern of 3′ RNA
fragments (Supplementary Fig. 2). The appearance of 3′ RNA fragments, whose size is dictated by nonsense codon position, suggests that in the absence of ATP hydrolysis by UPF1, a block to decay of nonsense-containing mRNA occurs at or near the site of premature translation termination.

Degradation of mRNA by either the canonical decay pathway or by NMD in yeast proceeds via decapping of the RNA 5′ end followed by 5′→3′ digestion catalysed by the cytoplasmic exoribonuclease XRN1 (refs 18,19). Processive activity of XRN1 in vivo is inhibited by strong RNA secondary structure20 or by translocating ribosomes during the process of co-translational mRNA decay21,22. Critically, 3′ RNA species that accrue in UPF1 ATPase mutants were lost in cells lacking XRN1 (xrn1Δ;Fig. 2a), indicating that the fragments represent bona fide products of 5′→3′ exonucleolytic activity. Moreover, mRNA translation is required for accumulation of the 3′ RNA decay product (Fig. 2b) as demonstrated by loss of the fragment from PGK1 reporter mRNA harbouring a stable stemloop structure in its 5′ UTR that represses translation >100-fold23 (Supplementary Fig. 3) but does not impede XRN1 activity18.

3′ RNA decay intermediates are ribosome bound. Our data suggest that the 3′ RNA fragments that arise from nonsense-containing mRNA in ATPase-deficient UPF1 mutants accumulate due to a block in 5′→3′ degradation caused by a ribosome stalled during premature translation termination. To evaluate directly whether the 3′ RNA decay products are ribosome bound, lysates from cells expressing one of three nonsense codon-containing PGK1 reporter mRNAs were subjected to sucrose density gradient centrifugation (that is, polyribosome analysis). Using this method, ribosome-free RNA (that is, RNP) was separated from transcripts associated with one, or multiple, ribosomes24 (Fig. 3a). Analysis of RNA isolated from gradient fractions showed that for each of the nonsense-containing reporter mRNAs, a 3′ RNA species cosedimented precisely with 80S monosomes (Fig. 3b, lane 6), and its size corresponded to the 3′ RNA decay fragment observed at steady state. Moreover, 3′ RNA fragments of increasing size were detected in progressively denser polyribosome fractions, indicative of a build-up of a mounting number of ribosomes along the transcript and co-translational decay of the nonsense-containing reporter mRNA21 (Fig. 3b, lanes 7–12 and Supplementary Fig. 4a). Notably, 3′ RNA fragments were not readily detected in RNP fractions (Fig. 3b, lanes 1–3), as would be expected if they were ribosome-free, RNA–protein complexes that failed to be disassembled.

Our data demonstrate that 3′ RNA products of nonsense-containing mRNAs are associated with a single ribosome bound

Figure 1 | Accumulation of a 3′ RNA fragment from nonsense-containing mRNA in UPF1 ATP hydrolysis mutants. (a) Schematic diagram of point mutations within the C-terminal helicase domain of UPF1 that impair ATP binding (K436E), ATP hydrolysis (DE572AA) or RNA binding (RR793AA). The cysteine/histidine-rich domain (CH) within the N-terminus of UPF1 is indicated. (b–d) Northern blot analysis of PGK1 reporter mRNA in upf1Δ cells (−) complemented with wild-type (WT) or mutant UPF1 using a probe complementary to the mRNA 3′ end. Reporter mRNA either harboured a PTC within its 416 codon open reading frame (b–d) or lacked a PTC (c – ). Full-length reporter mRNA (FL) and 3′ RNA fragments (arrow) are indicated. RNA levels were normalized to NMD-insensitive SCR1 RNA. Results are representative of three independent experiments. Nt, nucleotide.
at or near the site of premature termination, and indicate that in the presence of ATPase-deficient UPF1, translation is impeded such that ribosomes fail to terminate properly and display prolonged association with the mRNA. Consistent with a conclusion that the 3′ RNA fragments accumulate as a consequence of a defect in translation termination and a stalled ribosome presenting a block to complete transcript degradation, depletion of RLI1, an ATP-binding cassette-type ATPase required for stimulating peptide release and ribosome subunit dissociation, revealed a requirement for RNA binding and NMD cofactors UPF2 (Fig. 4c; also see Supplementary Discussion). Together, these data implicate both RNA binding and an interaction with NMD machinery. Moreover, the role for UPF2 depended on its ability to induce structural rearrangements within UPF1 that stimulate ATPase activity of the NMD factor UPF1 (Supplementary Fig. 4b–d; also see Supplementary Discussion).

Figure 3 | 3′ RNA decay fragments are ribosome bound. (a) Ultraviolet absorbance trace from polyribosome analysis of cells expressing ATPase-deficient UPF1 and one of three PTC-containing PGK1 reporter mRNAs; ribosome-free RNA (RNP), 40S, 60S and 80S ribosomal subunits, and polyribosomes are indicated. (b) Northern blot analysis of PTC-containing PGK1 reporter mRNA from each gradient fraction. Full-length reporter mRNA (FL) and 3′ RNA fragments (arrow) indicated. RNA levels were normalized to NMD-insensitive SCR1 RNA. Results are representative of three independent experiments. Nt, nucleotide.

Requirements for UPF1 activity on terminating ribosomes. Biochemical and structural studies on yeast and human UPF1 implicate both RNA binding and an interaction with NMD cofactors UPF2 and UPF3 in stimulating its ATPase and helicase activities27–29. In light of the fact that UPF1 DE572AA is unable to catalyse ATP hydrolysis, we evaluated whether these activators are therefore required for the accumulation of 3′ RNA decay fragments from nonsense-containing reporter mRNAs in the mutant. Introduction of secondary mutations into ATPase-deficient UPF1 that render the protein deficient in RNA binding (that is, RR793AA)4 almost completely abolished accumulation of the 3′ decay fragment (Fig. 4a), indicating that an interaction between UPF1 and its RNA substrate is a prerequisite for its ability to inhibit translation termination. We also found that 3′ RNA decay fragments failed to accumulate in UPF1 ATPase mutants lacking UPF2, UPF3 or both (Fig. 4b), validating the function of ATP hydrolysis by UPF1 on translation termination as a bona fide activity of the NMD machinery. Moreover, the role for UPF2 depended on its ability to interact with the N-terminal cysteine/histidine-rich domain of UPF1, as deletion of the domain (that is, ΔCH) or introduction of a point mutation in this region that disrupts the interaction (that is, C62Y)30 abolished accumulation of the 3′ RNA decay fragment in UPF1 ATPase mutants (Supplementary Fig. 5a,b). Importantly, the function of UPF2 was distinct from its ability to induce structural rearrangements within UPF1 that stimulate ATPase activity in vitro, since a mutation that alleviates allosteric regulation by UPF2 (that is, UPF1 F131E)29 failed to restore accumulation of the 3′ RNA decay fragment in cells lacking UPF2 (Fig. 4c; also see Supplementary Discussion). Together, these data reveal a requirement for RNA binding and NMD cofactors UPF2...
Figure 5 | ATP hydrolysis by UPF1 promotes efficient translation termination on nonsense-containing mRNAs. Premature translation termination allows UPF1 to remain associated with transcripts at sites downstream of the PTC and in a 3’ UTR length-dependent manner. ATP binding by UPF1, in conjunction with cofactors UPF2 and UPF3, promotes association of the NMD machinery with a terminating ribosome, mediated through interaction with translation release factors eRF1 and eRF3. For wild-type UPF1, ATP hydrolysis promotes rapid disassembly of the trimeric complex (ribosome, RNA and UPF proteins) leading to efficient translation termination and release of the ribosome and NMD factors from the RNA. How this event is communicated to the decay machinery to accelerate decapping and turnover of the mRNA remains unclear (bottom left; Fast). Failure to catalyze ATP hydrolysis leads to a defect in translation termination and a kinetic stall in ribosome release, presenting a block to XRN1-mediated 5’ → 3’ exonucleolytic decay and, critically, failure to trigger rapid turnover of the mRNA (bottom right; Slow).

Discussion

We present a number of observations that collectively serve to demonstrate that RNA fragments seen in cells expressing ATPase-deficient UPF1 are 3’ decay products of nonsense-containing mRNA harbouring a single ribosome bound at or near the PTC that accumulate as a result of a block to 5’ → 3’ exonucleolytic digestion by XRN1. These 3’ RNA decay fragments provide clear evidence that UPF1 directly influences translation termination on nonsense-containing mRNAs in vivo and reveal a site of action and novel function for ATP hydrolysis by UPF1 in NMD.

Our discovery that ATP hydrolysis by UPF1 plays a critical role in translation termination and our description of events required for this activity provide mechanistic insight into NMD substrate discrimination and degradation (Fig. 5). We found that RNA binding is necessary for mutant UPF1 to cause 3’ RNA fragment accumulation (Fig. 4a), and suggest that the binding of UPF1 to mRNA represents an early and, perhaps, initial step in NMD that, in turn, serves to facilitate interaction between the NMD machinery and the terminating ribosome. A view in which the initial association between UPF1 and its substrate is mediated through direct RNA binding is, however, contrary to models that posit that UPF1 is recruited to transcripts by elongating or terminating ribosomes (for a review, see ref. 1). Notwithstanding, recent global RNA-binding studies support the premise that UPF1 associates with transcripts in a translation-independent manner14,15. Moreover, a paucity of UPF1-binding sites within mRNA coding regions and at stop codons in cells undergoing

and UPF3 in modulating the function of UPF1 on premature translation termination, and suggests that, in vivo, UPF2 plays an additional role in regulating UPF1 that extends beyond inducing conformational changes necessary for stimulating UPF1 helicase activity8,28,29.
It has been proposed that 3’ decay intermediates observed for UPF1 mutants deficient in either ATP binding or ATP hydrolysis in human cells accumulate due to a block in decay caused by proteins (including UPF1 itself) that failed to be remodelled from the RNA downstream of the PTC. While ATPase mutants lack 5’→3’ helicase activity, we offer three lines of evidence in support of the interpretation that it is a stalled ribosome and not general RNA-binding proteins retained on the RNA that blocks XRN1-mediated exonucleolytic decay of the RNA. First, 3’ RNA fragments found in ATPase-deficient UPF1 in yeast co sediment with 80S monosomes, consistent with these decay intermediates being bound by a single ribosome. Second, 3’ RNA decay fragments are coincident with the length of the mRNA from the PTC to 3’ end and are generally homogenous in size, indicating that the block to 5’→3’ decay occurs at a fixed site at or near the premature termination event. Interestingly, global RNA-binding analysis of wild-type or ATPase-deficient UPF1 in mammalian cells by crosslinking and immunoprecipitation show that UPF1 is positioned throughout mRNA 3’ UTRs, predicting that 3’ decay fragments arising from UPF1 blockage would be heterogeneous in size. Third, while we and others have established that 5’→3’ degradation by XRN1 can be efficiently impeded by a ribosome, we are unaware of evidence demonstrating that UPF1, or any other RNA-bound protein (independent of an association with a stable RNA structure), is sufficient to inhibit XRN1 activity either in vivo or in vitro.

We note that the 3’ RNA decay fragments observed in ATPase mutants were absent in cells harbouring wild-type UPF1 (Fig. 1b), and attribute this to differences in the activity of wild-type and ATPase-deficient UPF1 on a terminating ribosome. We propose that both wild-type and mutant UPF1 proteins impinge on premature termination events, but that in the presence of catalytically inactive UPF1 does the complex persist to an extent sufficient to block XRN1 activity and accumulate 3’ decay fragments detectable by northern blot. Consistent with wild-type UPF1 also engaging with the translation machinery during termination, impaired ribosome release at premature termination codons dependent on UPF1 has been detected in yeast extracts using toeprint assays.

Our data place ATP hydrolysis by UPF1 at a pivotal point in the NMD pathway — after a functional complex between UPF1 (in conjunction with UPF2 and UPF3), the mRNA and a terminating ribosome has been established, and centred around events at the ribosomal A site that ultimately communicate information which leads to accelerated turnover of the mRNA. It remains to be determined whether the energy released upon ATP hydrolysis by UPF1 promotes efficient termination by stimulating peptide release or the subsequent recycling of ribosomes from the mRNA.

Notwithstanding, the inability of ATPase-deficient UPF1 to target nonsense-containing mRNA to accelerated decay indicates that, regardless of its precise role, UPF1 function in translation termination is monitored and critical to mediate accelerated decay of the transcript. Thus, an important goal for the field will be to elucidate how modulation of termination by UPF1 is communicated to the degradation machinery, which in yeast leads to accelerated decapping of the mRNA 5’ end. Despite the lack of a complete molecular understanding, the function of UPF1 on the terminating ribosome adds to the growing list of events monitored at the ribosomal A site, including the rate of cognate tRNA occupancy and binding of molecular mimics (that is, DOM34 and SK17), which lead to profound effects on mRNA stability for both normal and aberrant RNA transcripts. Thus, it is becoming increasingly clear that the ribosome acts not only as a molecular decoder but also as an exquisite sensor coupling perturbations in the efficiency of translation of an mRNA to its stability.
Methods

Yeast culture and standard methods. Yeast strains used in this study are listed in Supplementary Table 1. Yeast cultures were grown at 30 °C with shaking at 250 r.p.m. in synthetic medium supplemented with appropriate amino acids and either 2% glucose (SD) or 2% galactose and 1% sucrose (SGS). RNA isolation, polyribosome analysis and northern blotting were performed as described previously15.

Plasmid construction. Plasmids and oligodeoxynucleotides used in this study are listed in Supplementary Tables 2 and 3, respectively. Plasmid-encoded PGAL1-3HA-RLI1 depletion experiments, (Zymo Research; D4002), and transformed into wild-type yeast (yKB154). For Fidelity DNA polymerase (NEB; M0530S) and oKB1123/oKB1124. PCR products were generated by site-directed mutagenesis of either pKB556 or pKB598, using oKB823/oKB824 (DE572AA) (pKB645) or pKB598 as a template. The CH domain was then excised by digestion with SspI, and the resulting linearized plasmid recligated to generate pKB638. To construct pULF1-SCH DE572AA (pKB645), pKB638 was used as a template for site-directed mutagenesis using oKB823/oKB824.

Protein isolation and western blot analysis. Yeast cultures expressing chromosomally encoded, UPF1-HA, HAP2-RLI1, or plasmid-encoded, UPF1-HA, alleles were grown to mid-log phase and flash frozen on dry ice. Cell pellets were heated in 5 M urea for 2 min at 95 °C, and lysed by mechanical disruption with glass beads by vortexing for 5 min. Solution A was added to lysates (125 mM Tris-HCl, pH 6.8, 2% SDS) and samples vortexed for 1 min, followed by heating to 95 °C for 2 min. Glass beads and cellular debris were cleared from lysates by centrifugation at 13,200 r.p.m. for 4 min. Equivalent optical density units (A260) of lysate in 1 × SDS sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM DTT (dithiothreitol), 10% glycerol, 0.05% bromphenol blue) were separated on 7.5% Bis-Tris polyacrylamide gels by electrophoresis in 1 × SDS running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). Proteins were transferred to nitrocellulose (polyvinylidene difluoride) transfer membrane (Thermo Scientific; 88,518) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) by electroblotting at 4 °C for 2 h at 250 mA. Membranes were blocked (5% milk powder in 1 × TBS (Tris-buffered saline)) for 1 h at room temperature. Between incubations, membranes were washed with 1 × TBS (0.1% Tween-20) three times each for 15 min. Signals were detected by chemiluminescence using Blue Ultra Autorad film (GeneMate; F-9029).

Conditional deletion of RLI1. Chromosomally encoded RLI1 was placed under control of the GAL1 promoter using standard methods15. Briefly, the HisMX6-PULF1-HA insertion cassette was PCR amplified using Phusion High Fidelity DNA polymerase (NEB; M0350S) and oKB1123/oKB1124. PCR products were run on 1% agarose gels, purified using Zymoclean Gel DNA Recovery Kits (Zymo Research; D4002), and transformed into wild-type yeast (yKB154). For deletion experiments, PULF1-SCH::RLI1 cells expressing plasmid-encoded G2P, PGAL1-3HA-RLI1 were made isogenic by initial density of OD600 = 0.1. Cells were grown for 20 h at 30 °C, and aliquots were removed and collected by centrifugation every 2 h. Cell aliquots were flash frozen on dry ice for downstream analysis of RLI1 protein and reporter mRNA expression by western and northern blotting, respectively.

Data availability. The data that support the findings of this study are available from the corresponding author on request.

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Author contributions

L.D.S. and K.E.B. conceived and designed the study; L.D.S. and D.L.W. performed the experiments; and L.D.S., D.L.W. and K.E.B. wrote the manuscript.

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