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Nonsense-mediated mRNA decay in yeast does not require PAB1 or a poly(A) tail

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SUMMARY

Eukaryotic mRNAs harboring premature translation termination codons are recognized and rapidly degraded by the nonsense-mediated mRNA decay (NMD) pathway. The mechanism for discriminating between mRNAs that terminate translation prematurely and those subject to termination at natural stop codons remains unclear. Studies in multiple organisms indicate that proximity of the termination codon to the 3′ poly(A) tail and the poly(A) RNA binding protein, PAB1, constitute the critical determinant in NMD substrate recognition. We demonstrate that mRNA in yeast lacking a poly(A) tail can be destabilized by introduction of a premature termination codon and importantly, this mRNA is a substrate of the NMD machinery. We further show that in cells lacking Pab1p, mRNA substrate recognition and destabilization by NMD is intact. These results establish that neither the poly(A) tail, nor PAB1, is required in yeast for discrimination of nonsense codon-containing mRNA from normal by NMD.

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) is a conserved quality control mechanism in eukaryotic cells that recognizes and eliminates mRNA encoding premature translational termination signals in their protein coding region (Baker and Parker, 2004; Maquat, 2004). The rapid degradation of mRNA lacking a complete open reading frame (ORF) protects cells from accumulation of truncated polypeptides that can confer deleterious dominant-negative or gain-of-function phenotypes (Pulak and Anderson, 1993). Mutations in components of the NMD machinery lead to mental retardation in humans or embryonic lethality in mice, underscoring the functional importance of NMD (Medghalchi et al, 2001; Tarpey et al, 2007). Furthermore, NMD is a potent modulator of phenotypic outcome for many human genetic diseases (Frischmeyer and Dietz, 1999; Kuzmiak and Maquat, 2006).

NMD substrates encompass not only mRNAs harboring premature termination codons (PTCs) introduced by mutation, but also a significant population of the yeast, *Drosophila*, and human transcriptomes (He et al, 2003; Lelivelt and Culbertson, 1999; Mendell et al, 2004; Rehwinkel et al, 2005). Many endogenous mRNA targets that undergo NMD contain PTCs as a consequence of inefficient pre-mRNA splicing (He et al, 1993), upstream ORFs (Oliveira and McCarthy, 1995), or use of out-of-frame AUG start codons (Welch and Jacobson, 1999).

Interestingly, mRNAs terminating translation at a natural stop codon but harboring an extension of their 3′ UTR are also substrates for NMD (Muhlrad and Parker, 1999; Pulak and Anderson, 1993). For all NMD substrates, therefore, translation termination occurs in an inappropriate context and NMD appears to result from an alteration in the spatial relationship between the termination codon and other features of the mRNA (Baker and Parker, 2004).

Translation termination is considered aberrant as a consequence of its occurrence upstream of a second signal consisting of mRNA sequence elements and associated protein markers. In one model, prematurely terminating ribosomes fail to displace a downstream mRNA-protein complex (mRNP) able to trigger decay of the mRNA by recruiting proteins required for NMD (Maquat, 2004). Ribosomes that elongate through the entire ORF and terminate at a natural stop codon, in contrast, displace the downstream mRNP to prevent NMD. Proteins deposited at exon-exon junctions (exon junction complexes, EJCs) during pre-mRNA splicing serve to mark upstream stop codons as premature in mammalian cells (Zhang et al, 1998a; 1998b). Although recognition of mRNAs harboring PTCs is highly dependent upon a downstream exon-exon junction in vertebrates, exceptions have been described, demonstrating that a downstream EJC is not universally required for mammalian NMD (Buhler et al, 2006; Wang et al, 2002; Weil and Beemon, 2006).

In yeast, flies, worms, and plants, PTC definition can occur independently of a downstream exon boundary, and EJC protein components are either predominantly absent or not required for NMD (Culbertson and Leeds, 2003; Gatfield et al, 2003; Longman et al, 2007; Muhlrad and Parker, 1994; van Hoof and Green, 1996). Therefore, other determinants must present the downstream signal required for NMD substrate recognition. Yeast mRNAs are proposed to harbor downstream sequence elements (DSEs) within the coding region which bind protein factors (*e.g.* Hrp1) that trigger NMD if not removed during translation elongation (Gonzalez et al, 2000; Peltz et al, 1993). The few characterized DSEs, however, share loose sequence consensus making it difficult to estimate their ubiquity within ORFs and their importance in NMD. Moreover, analogous elements have not been identified in other organisms (Behm-Ansmant et al, 2007; Maquat, 2004).

An alternative paradigm for recognition of nonsense-containing mRNAs is the 'faux UTR' model which posits that translation termination at a PTC is intrinsically aberrant because it occurs upstream of elements unable to function as an authentic 3′ UTR (Amrani et al, 2004, 2006). Ribosomes at a normal stop codon, in contrast, terminate proximal to the 3′ UTR and engage in interactions necessary for efficient termination and stabilization of the mRNA. According to this model, an appropriate mRNP context includes the mRNA poly(A) tail and poly(A) binding protein, PAB1. Importantly, tethering of PAB1 downstream and proximal to a PTC stabilizes NMD substrates in both yeast and *Drosophila* (Amrani et al, 2004; Behm-Ansmant et al, 2007). In addition, targeting of mRNAs with extended 3′ UTRs by NMD is consistent with PAB1 and the poly(A) tail providing important positional information for substrate recognition by NMD.

We evaluated the requirement for an mRNA poly(A) tail and PAB1 in recognition of NMD substrates. We show that introduction of a PTC into a yeast mRNA lacking a poly(A) tail can result in destabilization of the mRNA, and that rapid decay of the mRNA requires the NMD factor Upf1p, confirming it as a *bona fide* NMD substrate. Further, we reveal that in the absence of Pab1p, mRNAs harboring a PTC are recognized and destabilized in an Upf1p-dependent manner. Our findings clearly demonstrate that in yeast, neither an mRNA poly(A) tail nor Pab1p is required for NMD. These findings illustrate that the proximity of the terminating ribosome to PAB1 either plays no role in substrate discrimination by NMD, or is just one of several redundant features that distinguish a termination event as premature.

RESULTS

Unadenylated *GFP-RZ* mRNA is Targeted for Degradation by the Cytoplasmic Exosome

To evaluate the relevance of the poly(A) tail in NMD in yeast, we utilized a reporter gene containing the *GFP* ORF followed immediately by a hammerhead ribozyme (*GAL1-GFP-RZ*; Figure 1A; Dower et al, 2004). Inclusion of the ribozyme sequence results in RNA cleavage that generates a 6 nucleotide 3′ UTR that fails to undergo polyadenylation (see Supplemental Material).

Meaux and van Hoof (2006) reported that unadenylated mRNAs are rapidly degraded by the cytoplasmic exosome. To evaluate if *GFP-RZ* mRNA is also a substrate for the exosome, transcript stability was determined in wild-type cells and a strain lacking cytoplasmic exosome activity (*ski7Δ*). This analysis revealed that *GFP-RZ* mRNA was unstable in wild-type cells decaying with a half-life of approx. 2 min (Figure 1B; Supplemental Figure 1). In contrast, *GFP-RZ* mRNA was stable in *ski7Δ* cells, with a half-life of 8 min (Figure 1C). Unadenylated *GFP-RZ* mRNA is therefore present in the cytoplasm where it is degraded by the cytoplasmic exosome.

Nonsense Codon-containing Unadenylated mRNA is Targeted for Rapid Degradation

It has been shown that *GFP-RZ* mRNA levels are low, and not further decreased by the introduction of a PTC (Baker and Parker, 2006; Dower et al, 2004). Our above results indicate that in wild-type cells, *GFP-RZ* mRNA is indeed unstable, and that further destabilization of the mRNA by the presence of a PTC might have escaped detection. We therefore reasoned that destabilization of an unadenylated mRNA by NMD might be observed when the contribution of the cytoplasmic exosome to the decay of the mRNA is removed.

We measured decay rates of *GFP(PTC)-RZ* mRNA containing a PTC at codon 67 (Baker and Parker, 2006) in wild-type and *ski7Δ* cells. In contrast to the stabilization of *GFP-RZ* mRNA in *ski7Δ* cells, *GFP(PTC)-RZ* mRNA was unstable in both wild type and *ski7Δ* strains (Figure 1 E&F). Importantly, in *ski7Δ* cells *GFP(PTC)-RZ* mRNA stability was reduced compared to *GFP-RZ* mRNA (2 min versus 8 min; Figure 1 C&E), indicating that normal and premature translation termination events are distinguishable in the absence of a poly(A) tail.

Unadenylated mRNAs Harboring a PTC are Targets for NMD

One explanation for *GFP(PTC)-RZ* mRNA instability in *ski7Δ* cells is that it is degraded by the NMD pathway, while *GFP-RZ* mRNA is not. We therefore, tested if rapid degradation of *GFP(PTC)-RZ* mRNA required the NMD machinery. Specifically, we measured decay of *GFP (PTC)-RZ* mRNA in a mutant yeast strain lacking both Ski7p and an essential component of the NMD machinery, Upf1p (Leeds et al, 1991).

Analysis of *GFP(PTC)-RZ* RNA revealed significant stabilization of the mRNA in *ski7Δupf1Δ* cells (half-life=6.4 min; Figure 1G). In contrast, turnover of *GFP-RZ* mRNA was essentially unaffected by deletion of *UPF1* (Figure 1 C&D). Thus, mRNA destabilization caused by the introduction of a PTC observed in *ski7Δ* cells was reversed by inactivation of the NMD pathway. The presence of an mRNA poly(A) tail is therefore not required for distinguishing a normal stop codon from a PTC by the NMD surveillance machinery.

The experiments described above utilize the well characterized *GFP-RZ* reporter to evaluate decay of unadenylated mRNA (Baker and Parker, 2006; Dower et al, 2004). To ensure our results were not restricted to this heterologous reporter, we analyzed yeast *HIS3* harboring a hammerhead ribozyme 55 nt downstream of the natural stop codon. As shown in Figure 2, *HIS3-RZ* mRNA in *ski7Δ* cells was destabilized by introduction of a PTC (compare C&F;

Supplemental Figure 1), and rapid turnover of the PTC-containing mRNA required a functional NMD pathway (compare F&G). Thus, targeting of an unadenylated nonsense mRNA to NMD is not limited to the *GFP* reporter, and also can occur for yeast genes lacking a poly(A) tail. While detection of the destabilization of unadenylated mRNA by a PTC required that cytoplasmic exosome activity be eliminated, these results demonstrate that the NMD machinery is capable of substrate recognition independent of a poly(A) tail.

Pab1p is Not Required for Destabilization of PTC-containing mRNA

Our findings with unadenylated mRNAs indicate that an mRNA poly(A) tail is not required for NMD in yeast. An association between PAB1 and an mRNA may, however, occur independently of the poly(A) tail. Indeed, interactions between PAB1 and eIF4G and eRF3 are well characterized (reviewed in Mangus et al, 2003). Therefore, to determine whether PAB1 itself is necessary for recognition of an mRNA by NMD, we evaluated levels of *PGK1* reporter mRNA in yeast cells lacking Pab1p. Deletion of *PAB1* is lethal, but inviability can be suppressed by a variety of secondary mutations, including, deletion of *RRP6* (Dunn et al, 2005).

Levels of *PGK1* reporter mRNA harboring a PTC at several positions within the ORF (Figure 3A) were evaluated in wild-type and *pab1Δrrp6Δ* cells. In wild-type, mRNAs harboring a nonsense codon were less abundant relative to wild-type *PGK1* mRNA, and the decrease in nonsense mRNA was successively less dramatic as the PTC was positioned further 3′ within the *PGK1* ORF, as previously reported (Figure 3B; Supplemental Figure 2; Cao and Parker, 2003; Peltz et al, 1993). Importantly, destabilization of the PTC-containing *PGK1* mRNA in wild-type cells was dependent upon a functional NMD pathway (KEB and R Parker unpublished data). Analysis of reporter mRNAs in *pab1Δrrp6Δ* cells revealed the relative abundance of *PGK1* mRNAs to be essentially identical to levels observed for wildtype (Figure 3C). Specifically, RNA levels were decreased for *PGK1* mRNAs harboring PTCs, and the decrease correlated with the position of the PTC within *PGK1*. Our findings indicate that PTC-containing *PGK1* mRNA is distinguished from wild-type mRNA in the absence of Pab1p.

We ruled out an independent effect of the *rrp6Δ* allele on NMD through analysis of *PGK1* reporter mRNA in *pab1Δrrp6Δ* cells complemented with *PAB1*. We demonstrate that destabilization of PTC-containing *PGK1* mRNA was maintained in these cells (Supplemental Figure 3). We further analyzed NMD in a second yeast suppressor strain deleted for *PAB1*. In *pab1Δspb8Δ* cells (Boeck et al, 1998), nonsense and wild-type *PGK1* mRNA levels resembled those observed in both wild-type and *pab1Δrrp6Δ* cells (Figure 3D). Thus, PTC-containing mRNAs are detected and destabilized in yeast cells in the absence of Pab1p.

Reduction of PTC-containing mRNA in the Absence of Pab1p Requires the NMD Pathway

To determine if decreased RNA abundance of nonsense-containing *PGK1* mRNAs in *pab1Δ* mutants was a consequence of NMD, we measured RNA levels in *pab1Δrrp6Δ* and *pab1Δspb8Δ* strains that also harbored a lesion in *UPF1*. In *pab1Δrrp6Δupf1Δ* and *pab1Δspb8Δupf1Δ* cells, nonsense *PGK1* mRNA levels were similar to wild-type *PGK1* mRNA (Figure 4 A&B; see Supplemental Material). These data demonstrate that the reduction in PTC-containing mRNA levels observed in the *pab1Δ* cells requires a functional NMD pathway and that substrates for NMD are efficiently recognized in the absence of Pab1p.

As a second measure of NMD in cells lacking PAB1, we analyzed levels of *CYH2* pre-mRNA, an endogenous NMD target in yeast (He et al, 1993). We observed low levels of *CYH2* pre-mRNA in wild-type and *pab1Δrrp6Δ* cells (Figure 4C). In contrast, *CYH2* pre-mRNA in *pab1Δrrp6Δupf1Δ* mutants was elevated. We conclude that *CYH2* pre-mRNA is targeted for NMD in the absence of Pab1p. Importantly, *CYH2* mRNA and wild-type *PGK1* mRNAs were

similar despite the absence of Pab1p or a functional NMD pathway (Figure 4C). Therefore, in the absence of PAB1, the NMD machinery effectively discriminates between normal and nonsense-containing mRNAs.

DISCUSSION

Here we used ribozyme-cleaved reporter mRNA to evaluate the requirement of an mRNA poly (A) tail for NMD in yeast (Dower et al, 2004; Baker and Parker, 2006; Meaux and van Hoof, 2006). We show that unadenylated mRNA can be destabilized by introduction of a PTC, and that mRNA instability requires the NMD factor Upf1p (Figures 1&2). We further demonstrate that in yeast lacking the poly(A) tail binding protein, PAB1, mRNAs harboring a PTC are recognized and their levels reduced by the NMD machinery (Figures 3&4). Our findings show that the ability to discriminate between a normal and premature translation termination event can occur independently of an mRNA poly(A) tail or PAB1.

The faux UTR model for recognition of nonsense-containing mRNAs posits that translation termination at a PTC is intrinsically aberrant due to the failure of the terminating ribosome to engage in stabilizing interactions with the mRNA 3′ UTR (Amrani et al, 2006). According to the model, stabilizing signals are communicated to the ribosome by the proximal mRNP that includes the poly(A) tail and PAB1. An important tenet of this model is that a stop codon will be recognized as normal only if translation termination occurs sufficiently close to the poly(A) tail and its bound PAB1, and, therefore, in the absence of these elements, ribosomes terminating at a natural stop codon would fail to engage in the stabilizing interactions and be targeted for NMD. We find that unadenylated mRNA terminating at a natural stop codon is not stabilized by inactivation of the NMD pathway in either wild-type or *ski7Δ* cells (Baker and Parker, 2006; Figure 1&2, C versus D). Moreover, normal *PGK1* and *CYH2* mRNA are not targeted to NMD in the absence of PAB1 (Figure 4C). Therefore, transmission of a positive signal from PAB1 to the terminating ribosome is neither required for the stability of a 'normal' mRNA nor its ability to escape NMD.

Our findings are in conflict with interpretations of tethered function analysis used to support the faux UTR model. Tethering of PAB1 to mRNA downstream and proximal to a PTC is sufficient to stabilize an NMD substrate in both yeast and *Drosophila* (Amrani et al, 2004; Behm-Ansmant et al, 2007). Based on these findings, it has been interpreted that proximity of PAB1 to the PTC leads to redefinition of the premature termination event as normal and that PAB1 is critical for signaling proper translation termination. Our findings, however, demonstrate that PAB1 is dispensable for normal and aberrant translation termination to be distinguished, and necessitates reevaluation of this interpretation. Interestingly, mRNA stabilization by tethered PAB1 is not specific to NMD substrates and was first demonstrated for a 'normal' mRNA (Coller et al, 1998), illustrating that enhanced mRNA stability observed when PAB1 is tethered can occur independently of a role for PAB1 in NMD.

In *Drosophila* cells, nonsense-containing mRNA is stabilized by depletion of cytoplasmic PAB1 (PABPC1) by RNA interference (Behm-Ansmant et al, 2007). These data are in opposition to what is anticipated according to the faux UTR model (see above). These findings were, however, interpreted to indicate that PABPC1 was in some way required for NMD. The inconsistency between this conclusion and our findings may indicate a divergence in mechanism for NMD between flies and yeast. However, it is likely that depletion of PABPC1 impacts multiple steps in mRNA metabolism (*e.g.* 3′ end formation, nucleocytoplasmic transport, translation, stability) that might lead to stabilization of a PTC-containing mRNA indirectly. *Saccharomyces cerevisiae* strains suppressed for the essential function of PAB1 greatly facilitated analysis of PAB1 function in NMD by limiting these complications.

Our results establish that an interaction between the terminating ribosome and PAB1 does not determine whether a stop codon is perceived as normal or premature. Moreover, DSE elements previously defined as critical for NMD of PTC-containing *PGK1* also appear immaterial in our studies, as PTCs downstream of the characterized elements in *PGK1* mRNA still elicit NMD. While mRNAs with extended 3′ UTRs in yeast, *Drosophila* and mammals can be NMD substrates (Muhlrad and Parker, 1999, Behn-Ansmant et al, 2007; Buhler et al, 2006), not all mRNA with long 3′ UTRs are destabilized by the NMD pathway (Behn-Ansmant et al, 2007). The 6 nucleotides remaining downstream of the natural stop codon after cleavage of *GFP-RZ* mRNA further supports that NMD is not dependent on a 3′ UTR generated by conventional 3′ end formation (Figure 1; Baker and Parker, 2006). Finally, EJC complexes are not required for NMD in yeast, *C. elegans*, and *Drosophila*. Given the various second signals implicated in NMD substrate recognition, we predict that redundancy exists in the ability of aberrant mRNA to trigger NMD, although we can not exclude the possibility that additional elements necessary for NMD substrate discrimination remain to be uncovered. Future research evaluating the importance of coding region sequence and length, and features of the elongating polypeptide or ribosome itself in NMD are ongoing.

EXPERIMENTAL PROCEDURES

Strains

ski7Δ, *upf1Δ*, and *ski7Δupf1Δ* yeast are isogenic to wild-type BY4741 (Open Biosystems). The *ski7Δupf1Δ* mutant was constructed by crossing *ski7Δ::HYG* (Wilson et al, 2007) to *upf1*Δ::NEO and sporulating.

Wild-type, *pab1Δrrp6Δ* and *pab1Δspb8Δ* yeast strains have been described (Cao and Parker, 2003; Dunn et al, 2005). yKB300 (*pab1Δrrp6Δupf1Δ [PAB1])* was generated by deleting *UPF1* in *pab1Δrrp6Δ* cells complemented with pKB269 (*PAB1*+) by the method of Longtine *et al* (1998). yKB301 (*pab1Δrrp6Δupf1Δ*) was made by plating yKB300 on media containing 5′ fluoro-orotic acid to select for loss of *PAB1*+*URA3*+ plasmid. Loss of the *PAB1* plasmid was confirmed by Western blotting. *pab1Δspb8Δupf1Δ* (yKB310) cells were generated by deleting *UPF1* (Longtine *et al,* 1998).

Plasmids

GFP-RZ plasmid pAV298 contains 1.3 kbp *Spe*I and *Xho*I fragment of *GAL-GFP-RZ* (Dower et al, 2004) in pRS416 (Sikorski and Hieter, 1989). *GFP(PTC)-RZ* plasmid pAV339 is similarly derived from pKB214 (Baker and Parker, 2006). *HIS3-RZ* has been described (Meaux and van Hoof, 2006). To generate *HIS3(PTC)-RZ* (pAV462), the sequence 5′ T ATC **TAA** GAA TTC **TAA** CA (PTC in bold) was inserted in the *Nde*I site of *HIS3-RZ.*

Plasmids for wild-type *PGK1* (pRP469) and PTCs at codon positions 22, 142, 225, 319 have been described (Cao and Parker, 2003), while PTCs at codon positions 344, 370 and 395 were created by site-directed mutagenesis of pRP469 (KEB and R Parker, manuscript in prep).

RNA Analysis

Steady state RNA levels were measured from cells grown in SC-URA+ 2% galactose/1% sucrose at 24 °C and harvested at $OD_{600} = 0.4–0.5$. For RNA decay measurements, yeast strains harboring *GFP-RZ* reporter plasmids were grown at 30 °C in SC-URA + 2% galactose to mid log phase. Media was replaced with SC-URA + 4% glucose and culture aliquots removed over time. RNA was extracted and blotted by standard methods. Northern blots were probed with [32]P 5′ radio-labeled oligonucleotides specific for *GFP* (5′ GCTGTTACAAACTCAAGAAGGACCATGTGG 3′), *HIS3* (5′ CTACCACCGCTCTGGAAAGTGCCTCATCCA 3′), *PGK1* (oRP121; Decker and Parker,

1993), *CYH2* (5′ CCATACCTCTACCACCGGGGTGCTTTCTGTGCTTACCG), or the RNA of the signal recognition particle (*SCR1* RNA; 5′GTCTAGCCGCGAGGAAGG 3′). RNA levels were quantified using a STORM phosphoimager (GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Zhang J, Sun X, Qian Y, Maquat LE. Intron function in the nonsense-mediated decay of β-globin mRNA: indications that pre-mRNA splicing in the nucleus can influence mRNA translation in the cytoplasm. RNA 1998b;4:801–815. [PubMed: 9671053]
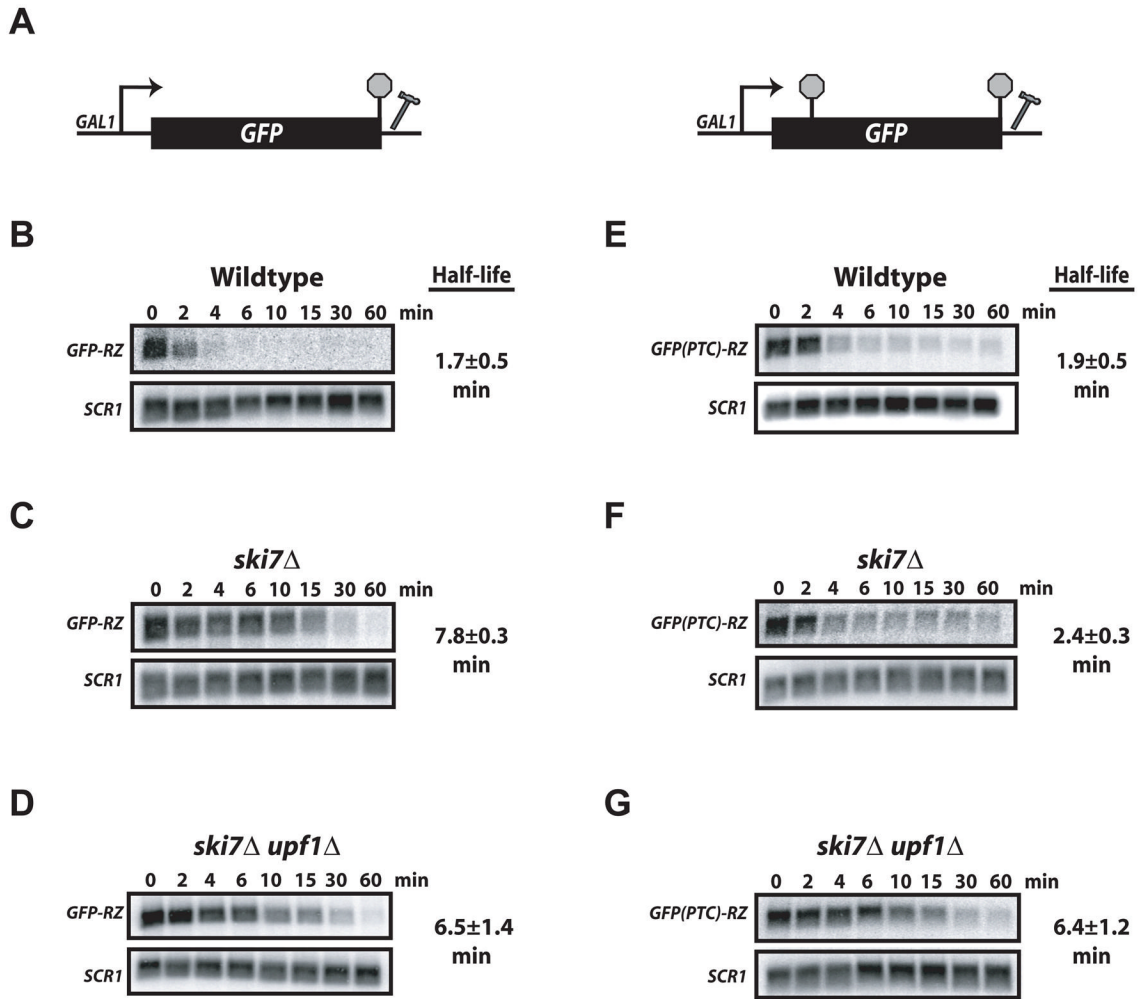
Figure 1. Unadenylated *GFP(PTC)-RZ* mRNA is Targeted for NMD
A. Schematic of *GFP-RZ* and *GFP(PTC)-RZ* reporter genes. Natural and premature stop codons (stop signs) and location of the hammerhead ribozyme directly 3′ of the *GFP* ORF are depicted. *GFP-RZ and GFP(PTC)-RZ* mRNA stability was determined by transcriptional shut-off analysis in wild-type cells (**B&E**), a mutant lacking cytoplasmic exosome activity, *ski7Δ* (**C&F**), and cells lacking both Ski7p and Upf1p (**D&G**). RNA isolated at various times after inhibition of reporter gene transcription (min) was analyzed by Northern blot and probed for *GFP* mRNA and *SCR1* RNA (loading control). mRNA half-lives are the average ± standard deviation of four independent experiments.
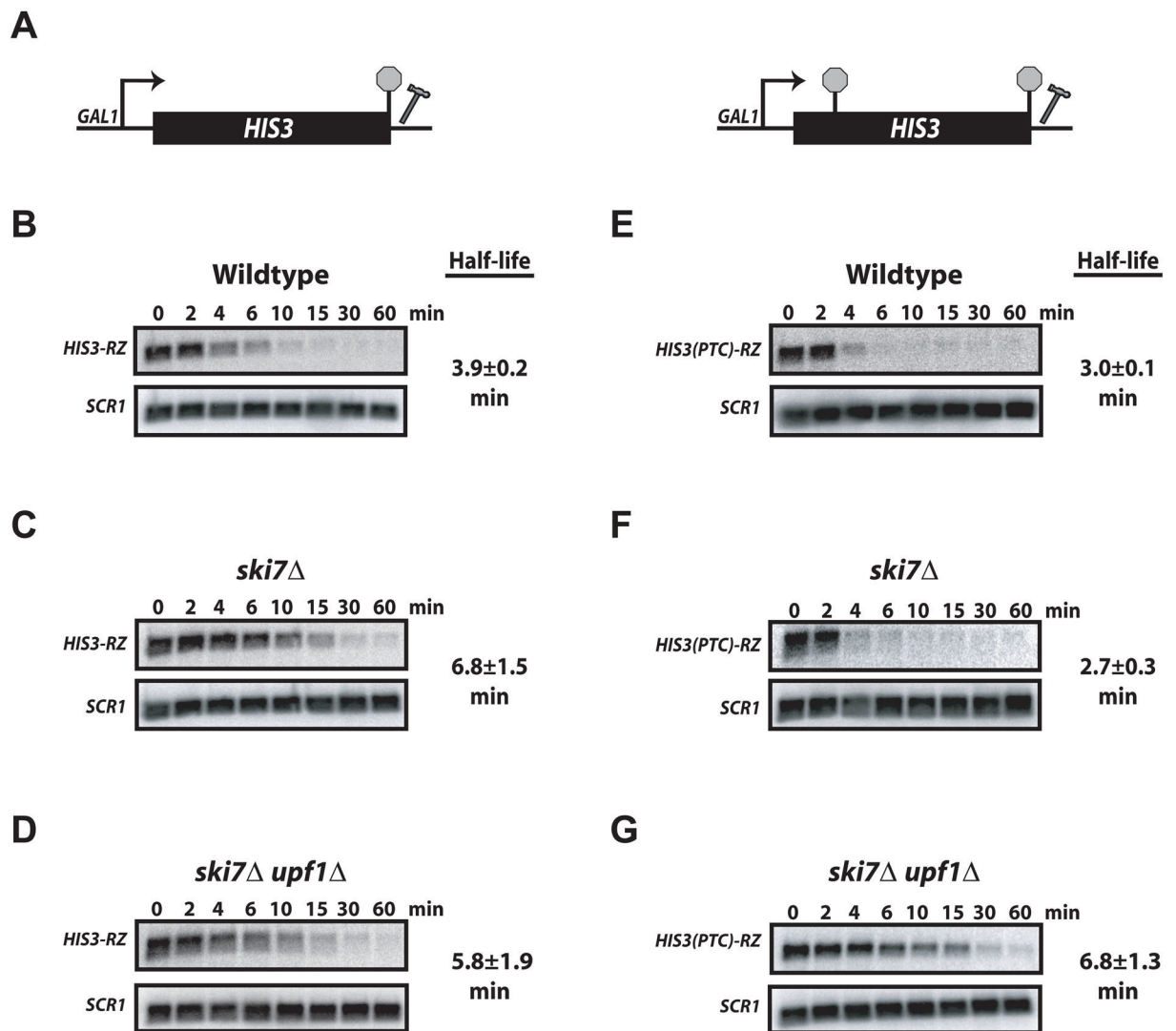
A

B

Wildtype Half-life

0 2 4 6 10 15 30 60 min

HIS3-RZ

SCR1

3.9±0.2 min

C

ski7Δ

0 2 4 6 10 15 30 60 min

HIS3-RZ

SCR1

6.8±1.5 min

D

ski7Δ upf1Δ

0 2 4 6 10 15 30 60 min

HIS3-RZ

SCR1

5.8±1.9 min

E

Wildtype Half-life

0 2 4 6 10 15 30 60 min

HIS3(PTC)-RZ

SCR1

3.0±0.1 min

F

ski7Δ

0 2 4 6 10 15 30 60 min

HIS3(PTC)-RZ

SCR1

2.7±0.3 min

G

ski7Δ upf1Δ

0 2 4 6 10 15 30 60 min

HIS3(PTC)-RZ

SCR1

6.8±1.3 min

Figure 2. Unadenylated *HIS3(PTC)-RZ* mRNA is Targeted for NMD
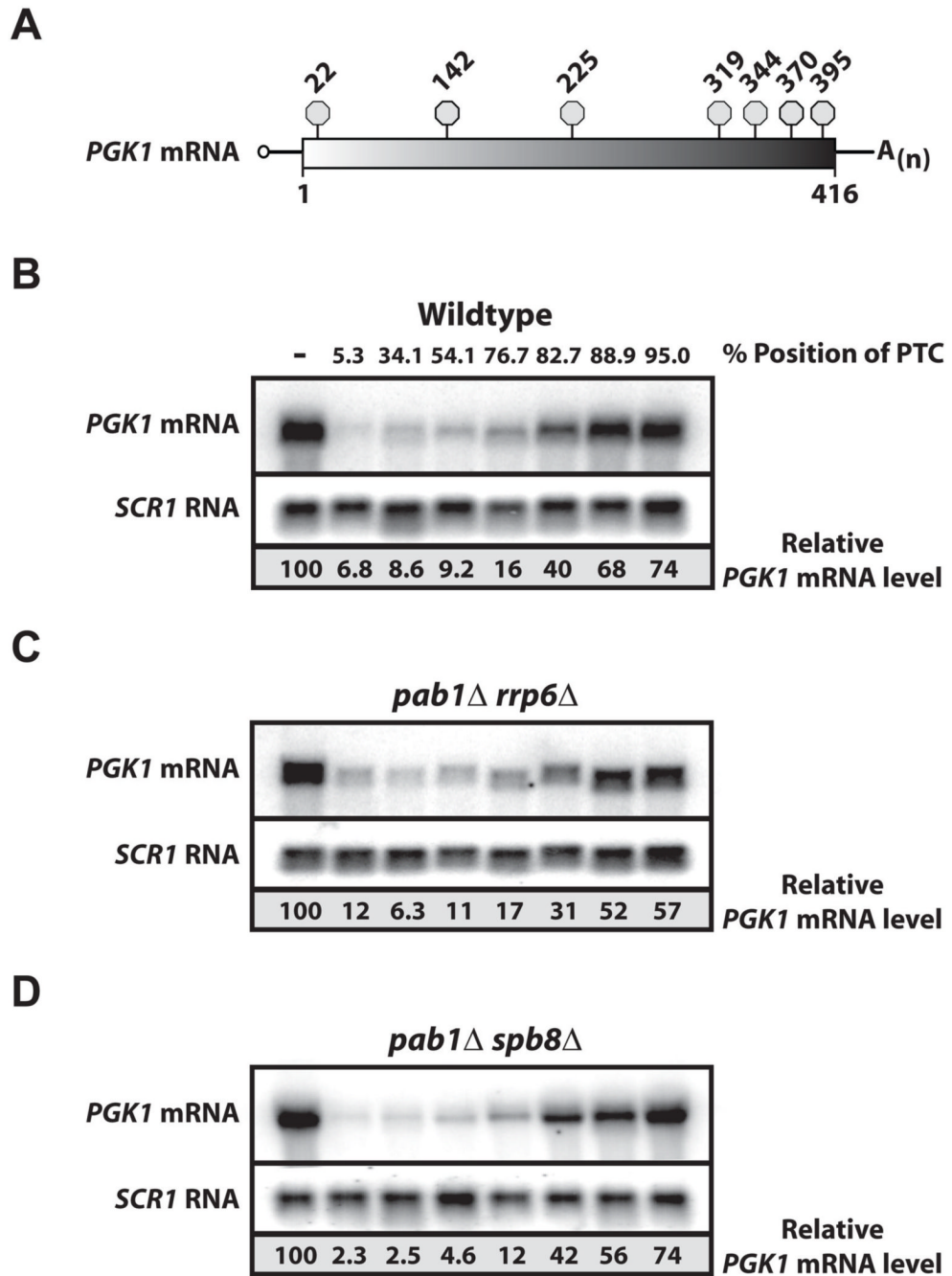Stability of *HIS3-RZ* and *HIS3(PTC)-RZ* mRNA was determined as described in Figure 1.

Figure 3. Nonsense-containing *PGK1* mRNA is Destabilized in the Absence of PAB1
A. Schematic of reporter mRNA highlighting positions within the *PGK1* ORF (box) of
premature nonsense codons (stop signs). Northern blot analysis of steady-state *PGK1* reporter
mRNA in wildtype (**B**), *pab1Δrrp6Δ* (**C**) or *pab1Δspb8Δ* (**D**) cells. PTC location indicated as
percent position into *PGK1* ORF. RNA levels were normalized to *SCR1* RNA and shown as
percent level relative to *PGK1* mRNA terminating at the natural stop codon for experiment
shown; results for multiple analyses are shown in Supplemental Figure 2.
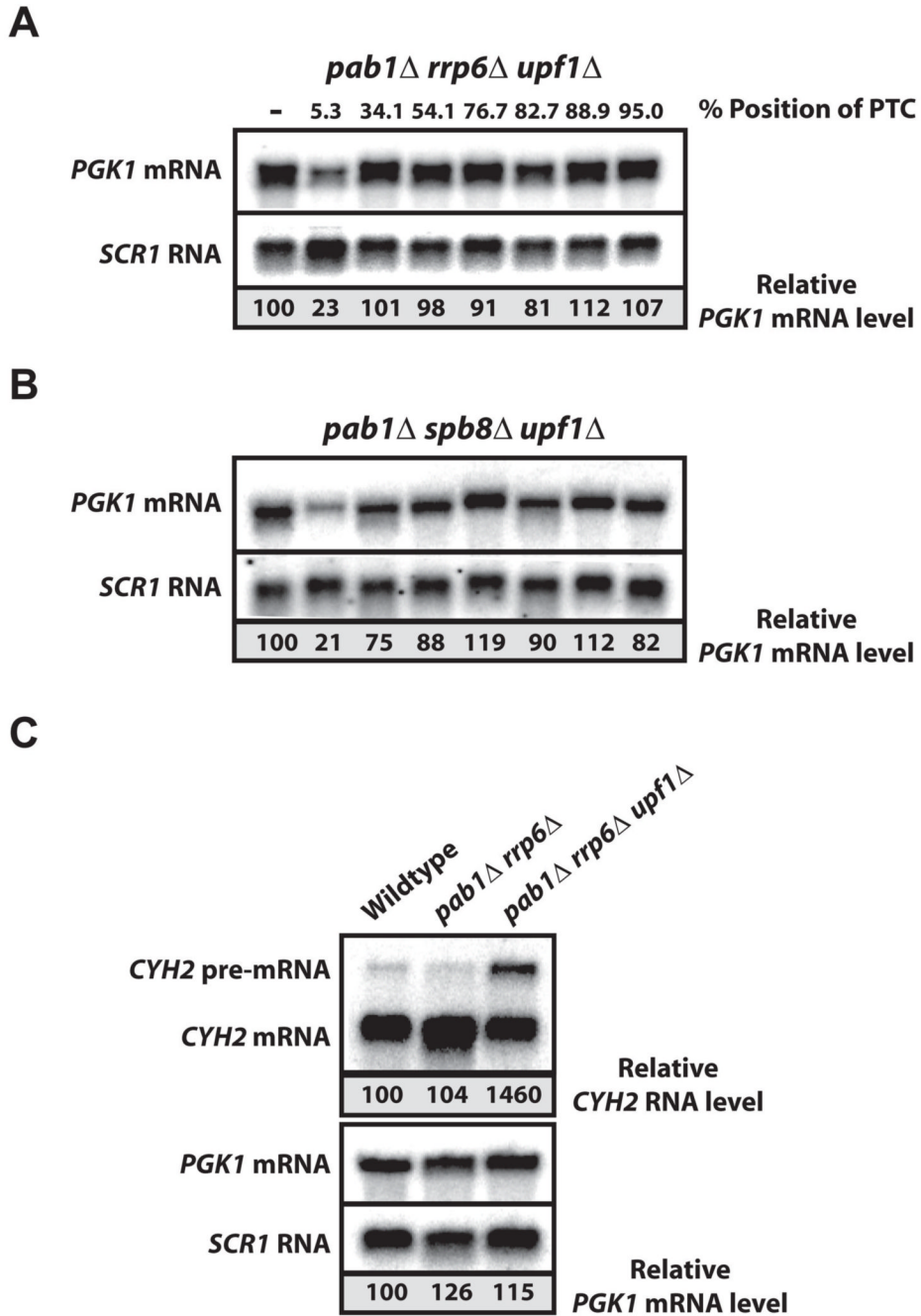
Figure 4. Degradation of Nonsense-containing mRNA in the Absence of Pab1p Requires a Functional NMD Pathway

PGK1 reporter levels were determined by Northern blot analysis of RNA from *pab1Δrrp6Δupf1Δ* (**A**) and *pab1Δspb8Δupf1Δ* (**B**). RNA levels were normalized to *SCR1* RNA loading control and shown as percent level relative to *PGK1* mRNA void of a PTC for the experiment shown; results for multiple analyses are shown in Supplemental Figure 2. **C.** Northern blot analysis of endogenous *CYH2* RNA and *PGK1* reporter mRNA levels in wild-type, *pab1Δrrp6Δ*, and *pab1Δrrp6Δupf1Δ* cells. *CYH2* pre-mRNA levels are normalized to *CYH2* mRNA, while *PGK1* mRNA levels are normalized to *SCR1* RNA. Both are indicated as percent level relative to abundance in wild-type.