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Supplemental Data

Nonsense-Mediated mRNA Decay in Yeast Does Not Require PAB1 or a Poly(A) Tail

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GFP-RZ mRNA are Efficiently Cleaved and Translated *in vivo*

The steady state abundance of *GFP-RZ* and *GFP(PTC)-RZ* mRNAs was evaluated in the wild-type, *ski7Δ* and *ski7Δupf1Δ* strains used in this study. Cells harboring a reporter plasmid were grown in minimal media lacking uracil and containing galactose as carbon source (SC-URA + 2% galactose) to mid-log phase. Total cell RNA was isolated and separated on formaldehyde agarose gels. Northern blotting and detection of *GFP* mRNA revealed several observations. First, two RNA species could be observed representing ribozyme-cleaved *GFP-RZ* mRNA and a larger species, presumably generated from 3' end formation significantly downstream of the ribozyme cleavage site. Importantly, the higher molecular weight species was equally abundant in all three strains analyzed, indicating that ribozyme cleavage efficiency is similar in wildtype, *ski7Δ* and *ski7Δufp1Δ* cells (Supplementary Figure 4A). Second, and consistent with our determined half-life values, deletion of *SKI7* or both *SKI7* and *UPF1* resulted in an increased steady-state level of *GFP-RZ* mRNA, while only in the double mutant were levels of *GFP(PTC)-RZ* mRNA seen to increase (Supplemental Figure 4A).

Our observation that *GFP-RZ* mRNA can be destabilized by a PTC suggests that the mRNA is present in the cytoplasm and, as is required for NMD, is a substrate for translation. Consistent with this, the mRNA is rapidly degraded by the cytoplasmic exosome (Meaux and van Hoof, 2006; Figure 1). It has been reported previously, however, that GFP protein is not detectable from cells harboring the *GFP-RZ* reporter plasmid, leading to the conclusion that the mRNA is not efficiently translated (Dower et al, 2004). We re-evaluated GFP protein levels in wild-type and a *ski7Δ* strain using Western blot analysis. As shown in Supplemental Figure 4B, GFP protein was readily detectable in a lysate of wild-type yeast cells (lane 3). It is difficult to pinpoint why GFP protein was previously not detected, however, differences in yeast strains, growth conditions, antibody concentrations, chemiluminescent reagents, and exposure times may contribute to the differences in protein expression or detection. It may also be relevant that the previous analyses was done using a slightly different plasmid (*TDH3-GFP-RZ* on a 2μ TRP1 plasmid *vs. GAL-GFP-RZ* on a CEN URA3 plasmid). Noteworthy is the observation that GFP protein levels were increased in *ski7Δ* cells (Supplemental Figure 4B, lane 4). This latter observation is consistent with the higher steady-state RNA level (Supplemental Figure 4A) and half-life value (Figure 1) for *GFP-RZ* mRNA in *ski7Δ* cells versus wild type. Our findings indicate that *GFP-RZ* mRNA is, in fact, translated, with protein levels correlating with mRNA levels in the cell.

Ribozyme-cleaved *GFP* and *HIS3* Reporter mRNAs are Unadenylated

The 5' product generated by RNA cleavage catalyzed by hammerhead ribozymes in yeast have been shown to be fairly uniform in size and not to have any detectable poly(A) tail *in vivo* (Baker and Parker, 2006; Dower et al, 2004; Duvel et al, 2002; Meaux and van Hoof, 2006). In particular, the *GFP-RZ* reporter used in this study has been characterized in a number of previous studies, and several lines of evidence demonstrate that the cleaved mRNA is unadenylated. First, *GFP-RZ* mRNA fails to associate with oligo dT-coated polystyrene–latex beads, while two control *GFP* reporter mRNAs harboring either a poly(A) tail or a DNA-encoded stretch of adenylate residues bind oligo dT efficiently (Dower et al, 2004). Second, the mobility of *GFP-RZ* mRNA does not change upon treatment with RNase H and oligo dT as assessed by formaldehyde agarose gels (Dower et al, 2004). In contrast, mobility shifts were evident for the same control *GFP* reporters described above. This latter observation was confirmed independently using high resolution polyacrylamide gels (Baker and Parker, 2006). Third, after internal cleavage of the RNA to generate a shortened ribozyme-cleavage product, *GFP-RZ* mRNA runs at the expected size for an unadenylated mRNA when compared to size markers (K.E.B. and R. Parker, unpublished results).

To test whether the *HIS3-RZ* and *HIS3(PTC)-RZ* mRNAs were also unadenylated we used the RNase H/oligo(dT) assay for the presence of poly(A) tails on RNA. Supplemental Figure 4C (left panel) shows that the mobility of *HIS3-RZ* and *HIS3(PTC)-RZ* mRNAs do not change upon RNase H/dT treatment, indicating that these

RNAs are unadenylated. As an important control, the blot was rehybridized with a probe specific for *RPL41A* mRNA, which shows a clear shift in mobility upon treatment with RNase H and oligo(dT) (supplemental Figure 4C, middle panel). This indicates that the RNase H assay worked as designed. The *RPL41A* mRNA has been reported to be approximately 300 nts with the 3 bands in the +dT lanes probably representing alternative polyadenylation sites (Suzuki et al, 2001; Tharun and Parker, 2001; Yu and Warner, 2001).

RNA cleavage catalyzed by the hammerhead ribozyme results in a 5' product terminating in a 2'-3' cyclic phosphate (Hutchins et al, 1986). To the best of our knowledge, no enzymatic activity able to catalyze the addition of an adenylate (or any other) residue to a cyclic phosphate or resolve a cyclic phosphate on an mRNA *in vivo* has been described. These observations support our experimental data that the *GFP-RZ* and *HIS3-RZ* mRNAs are unadenylated in yeast cells.

NMD of *PGK1* Demonstrates Polarity in the Absence of PAB1

Destabilization of PTC-containing *PGK1* mRNA exhibits polarity, such that premature termination codons proximal to the 5' end of the ORF trigger faster decay of the mRNA by NMD compared to PTCs located distally, which result in less dramatic effects on mRNA stability (Cao and Parker, 2003; Losson and Lacroute, 1979; Peltz et al, 1993). Destabilization of *PGK1* mRNA by nonsense codons within the first three-quarters of the *PGK1* mRNA at positions 22, 125, 215 and 319 have been previously

shown (Hagan et al, 1995; Peltz et al, 1993). We demonstrate here, however, that nonsense codons within the final quarter of the *PGK1* ORF, up to and including, a PTC only 21 codons upstream of the native stop codon, also resulted in a significant, albeit less dramatic, reduction in mRNA levels (Supplementary Figure 2). The observation that very late PTCs trigger modest mRNA destabilization has also been observed in flies (Behm-Ansmant et al, 2007). The mechanism underlying the polarity of NMD substrate destabilization is unclear.

Our data demonstrate that the polarity of NMD for *PGK1* mRNA is maintained in cells lacking Pab1p (Figure 3). The observation that RNA levels increased as the position of the PTC became more distal from the 5' end of the *PGK1* ORF also indicates that in the absence of Pab1p, the relative position of the nonsense codon still elicits distinct downstream effects on mRNA abundance. PTCs at positions 319, 344, 370, and 395 of the *PGK1* ORF are downstream of the previously characterized DSE (Peltz et al, 1993; Hagan et al, 1995). How the relative position of a PTC differentially destabilizes the mRNA therefore remains unclear; however, our findings indicate that polarity is not simply an effect of the DSE or the relative distance between the terminating ribosome and PAB1.

It is noteworthy that steady-state levels of nonsense-containing *PGK1* mRNA in the *upf1Δ* cells all approach the abundance of 'normal' *PGK1* reporter mRNA, with the exception of *PGK1* mRNA containing a PTC at codon position 22. The reduced level for *PGK1* mRNA with the 22 codon ORF compared to 'normal' *PGK1* reporter mRNA in

the absence of NMD has been previously observed (Cao and Parker, 2003), and may be attributable to the particularly short reading frame (KEB and R Parker, unpublished observations; Heaton et al, 1992). In *pab1*Δ*spb2*Δ cells, *PGK1* mRNA with a 22 codon ORF (i.e. PTC at codon 22) was also observed to be less stable than wild-type *PGK1* mRNA; whether the cause of the mRNA destabilization was due to NMD or the short ORF, was not, however, evaluated (Caponigro and Parker, 1995).

SUPPLEMENAL EXPERIMENTAL PROCEDURES

Protein Analysis

GFP and Pab1p protein levels were analyzed by Western blot analysis of cell lysates separated by SDS-PAGE and transferred to nitrocellulose membranes. Protein detection was achieved using a polyclonal antibody to GFP (Open Biosystems) or a monoclonal to Pab1p (EnCor Biotech. Inc.), compared to loading controls detected by a monoclonal antibody to Pgk1p (Invitrogen) or polyclonal antibody to Rpl5p (Deshmukh et al, 1993) using the ECL Chemiluminescent system (GE Healthcare).

PAB1 Plasmids

To generate pKB253 and pKB269, *PAB1* was amplified from yeast by PCR using oKB150 (5' CAGTAGCTCTAGAGCGTGTAAGTGTGTGTACTATAGGGCACCG 3') and oKB151 (5' CCACTTAAAACTGTCTAGAGTTGCGTATTACGATGACCTTCGG 3') and cloned into the *Xba*I site of yCpLac33 (URA^+; Gietz and Sugino, 1988) and yCpLac111 (LEU^+; Gietz and Sugino, 1988), respectively.

DNA-directed Cleavage of RNA by RNase H

RNase H cleavage and polyacrylamide Northern blots were performed as described by Meaux and van Hoof (2006). RNA was isolated from wildtype strains containing *HIS3-RZ* or *HIS3(PTC)-RZ* reporter plasmids, incubated in the presence of

RNase H and an oligo (5’ CGCAAGAGAGATCTCCTACTTTCTCCC 3’) complementary to *HIS3* mRNA 256 nts upstream of the ribozyme cleavage site. In some samples mRNA poly(A) tails were removed by also including $(dT)_{18}$. RNA was separated by electrophoresis through 6% polyacrylamide and transferred to nylon membrane. The 3’ cleavage fragment of *HIS3* was detected by probing with 5’ radio-labeled oligonucleotide 5’ GGCAACCGCAAGAGCCTTGAACGCACTCTCAC 3’. Blots were also probed for *RPL41A* mRNA using oligonucleotide 5’ GACATTACGATACTCTTGAAAGAA 3’ to confirm RNA cleavage by RNase H and $(dT)_{18}$.

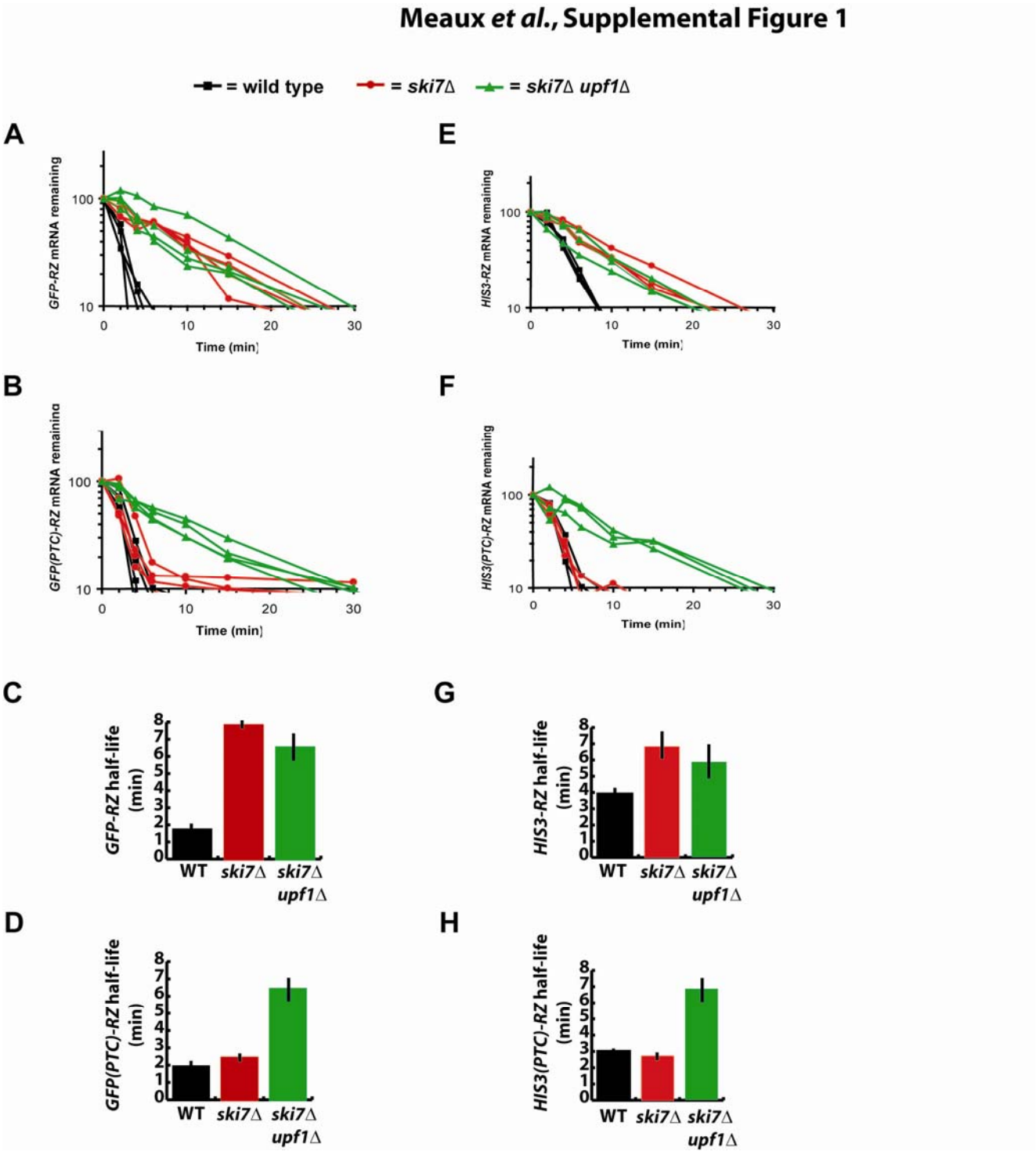

Figure S1. Stability of unadenylated *GFP* and *HIS3* mRNA.

mRNA stability was determined for *GFP-RZ* (**A** and **C**), *GFP(PTC)-RZ* (**B** and **D**), *HIS3-RZ* (**E** and **G**), and *HIS3(PTC)-RZ* (**F** and **H**) mRNA from 4 independent

experiments. Panels **A**-**D** depict the quantitation of each individual experiment. Black squares are values for mRNA in wild-type cells, red circles are values from *ski7Δ* mutants, and green triangles are values from *ski7Δupf1Δ* cells. Values on the y-axis represent percent RNA remaining relative to levels present when reporter gene transcription was inhibited (t=0). Panels **E**-**H** represent bar graphs depicting average half lives and standard deviation values determined in panels A-D.

Meaux *et al.*, Supplemental Figure 2

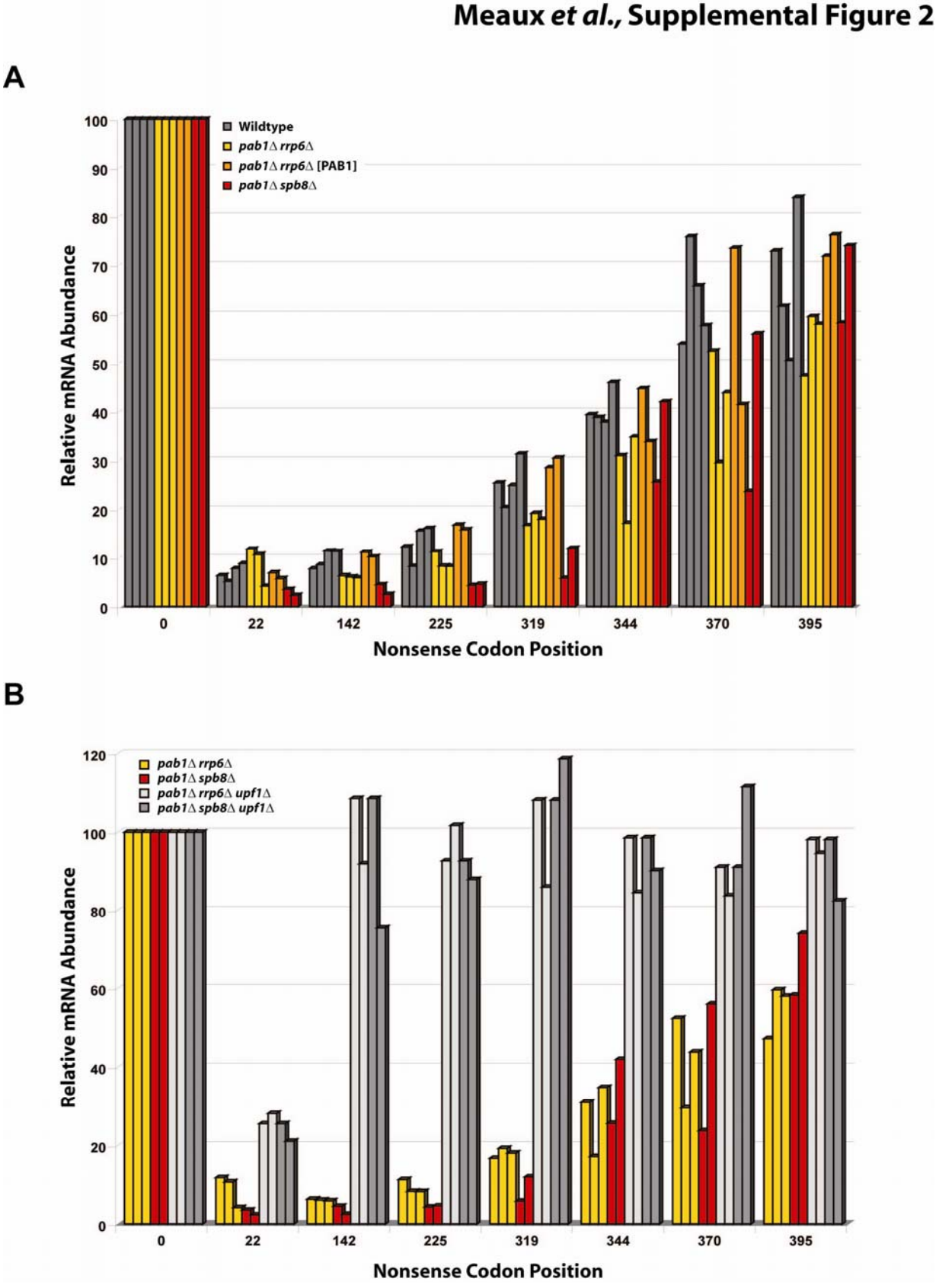

Figure S2. Degradation of Nonsense-containing mRNA by NMD does not Require PAB1.

A. Relative percent levels of 'normal' and nonsense-containing *PGK1* mRNA in various yeast strains. X-axis represents codon position of PTC within the *PGK1* ORF, and y-axis is the relative percent mRNA level compared to the level of 'normal' *PGK1* reporter mRNA. Each bar represents an individual experiment. **B.** Relative reporter mRNA levels in the absence of a functional NMD pathway (i.e. *upf1*Δ).

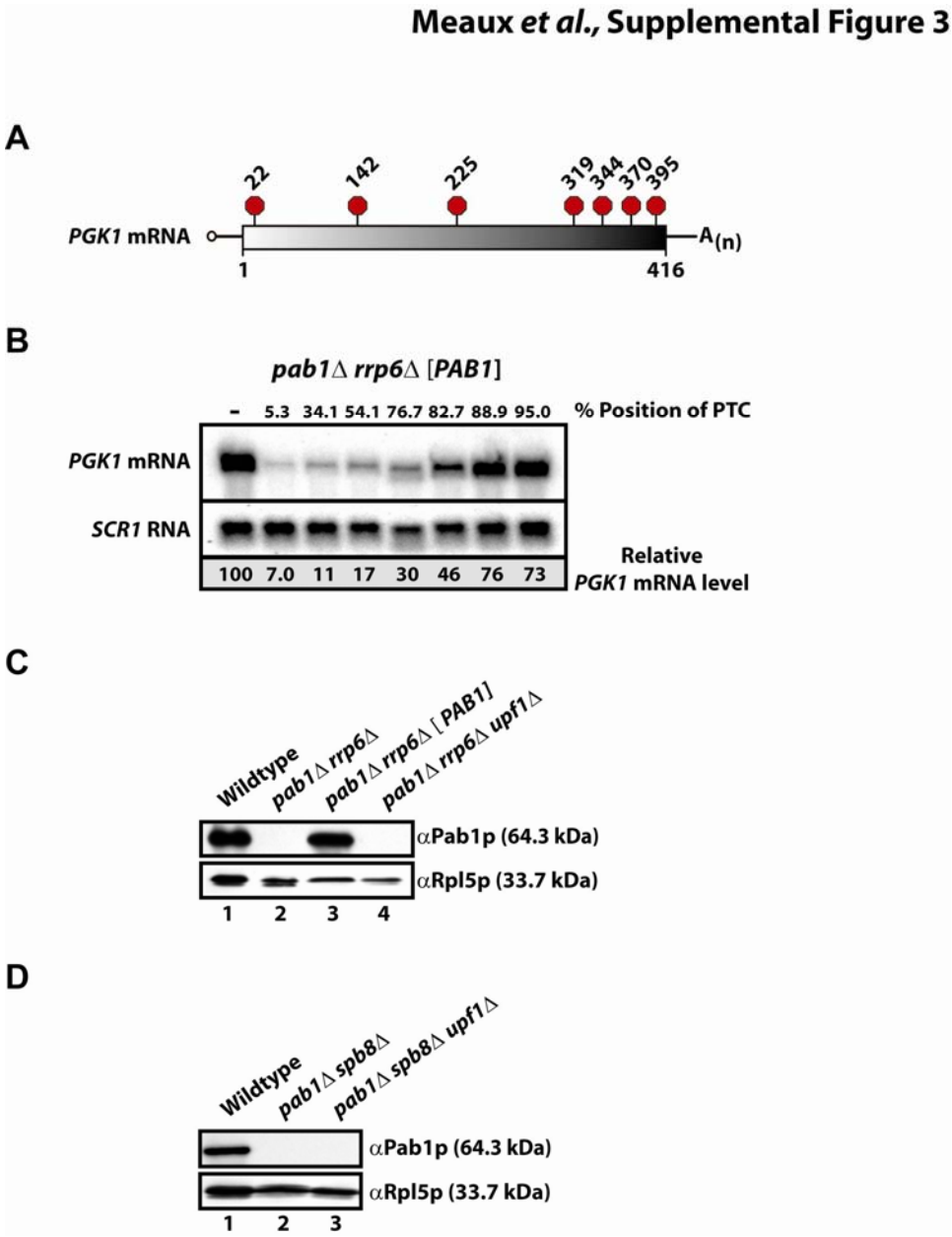

Figure S3. Degradation of Nonsense-containing mRNA is unaffected in cells deleted for *RRP6.*

A. Schematic of reporter mRNA highlighting positions within the *PGK1* ORF (box) of premature nonsense codons (stop signs). **B**. Northern blot analysis of steady state *PGK1* reporter mRNA in *pab1Δrrp6Δ* cells complemented with plasmid-encoded PAB1. Location of the PTC is 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 the experiment shown; results for multiple analyses are shown in Supplemental Figure 2. **C&D**. Western blot analysis of Pab1p levels to confirm the phenotype of *pab1Δ* strains and expression of Pab1p in cells harboring plasmid-encoded Pab1p. Large ribosomal protein L5 levels serve as a loading control.

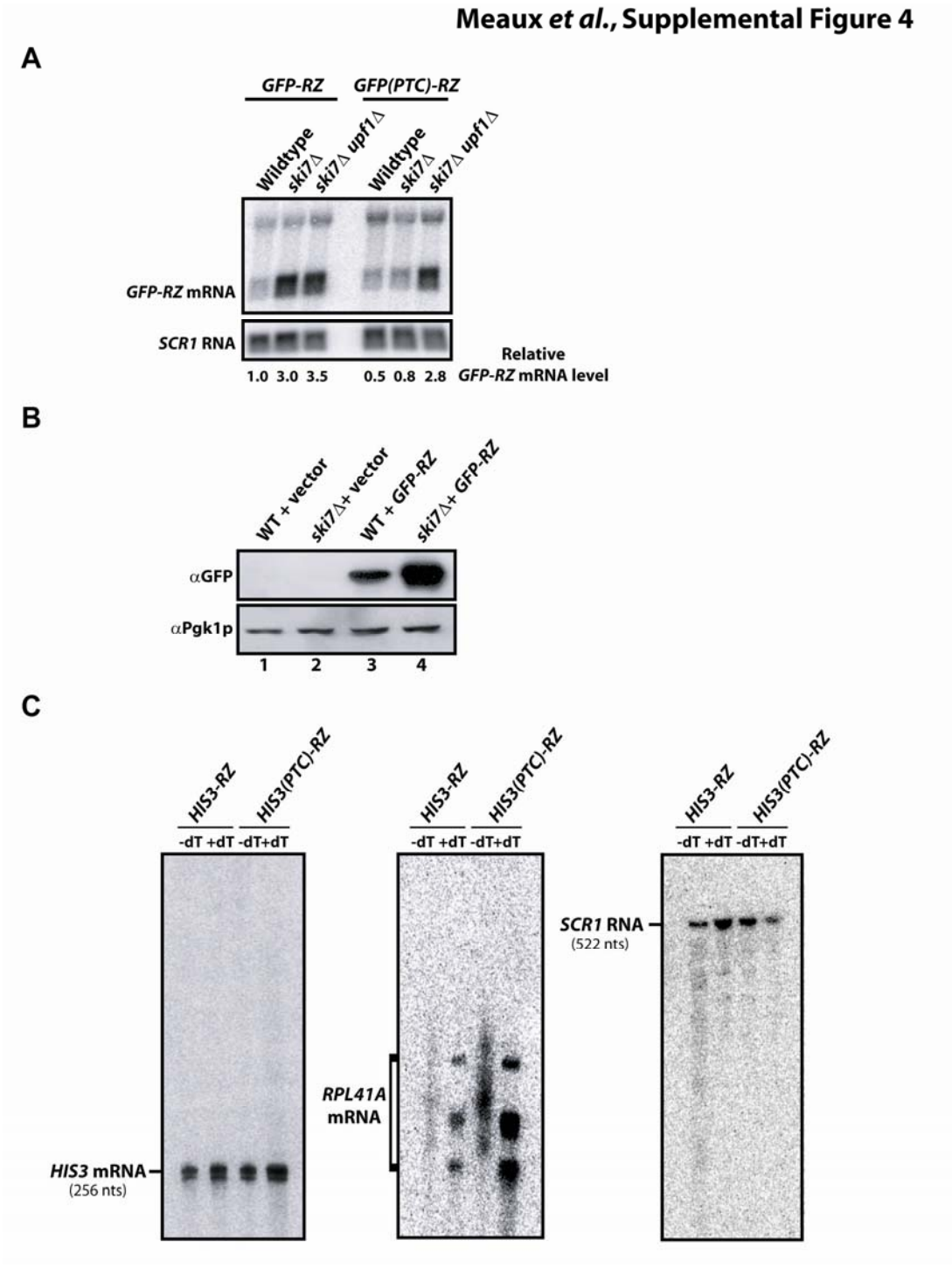

Figure S4. Ribozyme-cleaved mRNA is unadenylated and translated *in vivo*.

A. Northern blot analysis of steady-state *GFP-RZ* and *GFP(PTC)-RZ* reporter mRNA levels. Relative *GFP* mRNA levels represent levels in comparison to cleaved

GFP-RZ mRNA in wild-type cells after normalization (to *SCR1* RNA). **B**. Western blot analysis of GFP in cell lysates from wild-type and *ski7Δ* mutants harboring *GFP-RZ* reporter plasmid or vector alone. Detection of Pgk1p levels serves as a loading control. **C.** Northern blot analysis of *HIS3*, *RPL41A*, and *SCR1* RNA isolated from wild-type cells after treatment with RNase H. RNA was incubated with RNaseH and an oligo internal to *HIS3* either in the presence (+dT) or absence (-dT) of oligo dT, separated by electrophoresis through a 6% polyacrylamide gel and blotted. *HIS3* (left panel), *RPL41A* (middle panel) and *SCR1* (right panel) RNA signals are shown.

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