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# Nonsense-mediated mRNA decay of the ferric and cupric reductase mRNAs *FRE1* and *FRE2* in *Saccharomyces cerevisiae*

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## Abstract

The nonsense-mediated mRNA decay (NMD) pathway regulates mRNAs that aberrantly terminate translation. This includes aberrant mRNAs and functional natural mRNAs. Natural mRNA degradation by NMD is triggered by mRNA features and environmental cues. *Saccharomyces cerevisiae* encodes multiple proteins with ferric and cupric reductase activity. Here, we examined the regulation by NMD of two mRNAs, *FRE1* and *FRE2,* encoding ferric and cupric reductases in *S. cerevisiae*. We found that *FRE2* mRNAs are regulated by NMD under non-inducing conditions and that the *FRE2* 3'-UTR contributes to the degradation of the mRNAs by NMD. Conversely, *FRE1* mRNAs are not regulated by NMD under comparable conditions. These findings suggest that regulation of functionally related mRNAs by NMD can be differential and conditional.

### Keywords

*Saccharomyces cerevisiae*; *FRE1–2* mRNA; mRNA decay; biometal homeostasis; Nonsense-mediated mRNA decay

## INTRODUCTION

The nonsense-mediated mRNA decay (NMD) pathway is a highly conserved mRNA degradation pathway. NMD is conserved in all tested eukaryotes from yeast to humans and functions as an important regulator of gene expression. In *Saccharomyces cerevisiae*, ~5–10% of the transcriptome is affected when NMD is non-functional [1, 2]. Similar results were observed in studies involving *Drosophila* and humans [3–5]. Upf1p, Upf2p and Upf3p are three core NMD factors required for the pathway to function.

NMD was first identified as a pathway that degrades premature termination codon (PTC) containing mRNAs thus preventing the synthesis of truncated proteins. NMD is now also recognized as a pathway that degrades natural mRNAs that typically encode fully functional proteins. Thus, NMD plays dual roles, one in mRNA surveillance and a second in regulation of gene expression. NMD-mediated degradation of natural mRNAs has been observed in diverse organisms including *S. cerevisiae*, *D. melanogaster*, *Caenorhabditis elegans*, and humans.

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Natural mRNAs are targeted by the pathway through a variety of recognized features in eukaryotes. NMD targeting features have been previously described [6, 7]. Nevertheless, it is important to note that the presence of any NMD targeting feature on an mRNA does not always elicit NMD [8, 9]. These observations suggest that NMD targeting can function in specific cellular context and environmental conditions [10, 11].

Previous studies have shown that NMD can differentially regulate genes in response to environmental changes. For example, our studies in *S. cerevisiae* showed that cells with an inactive NMD pathway are more tolerant of high copper [12]. This finding prompted us to investigate mRNAs regulated by NMD and involved in copper homeostasis with the goal of elucidating their regulation by NMD. Understanding the role NMD plays in copper homeostasis is important because copper is essential for cellular function but can be toxic in excess. Furthermore, understanding copper and other nutritional metal homeostatic mechanisms in yeast will provide understanding into the regulatory mechanisms used by other organisms as these mechanisms have been conserved throughout evolution [13].

Analysis of mRNAs involved in copper homeostasis and regulated by NMD identified one mRNA encoding a protein with ferric and cupric reductase activity. This mRNA, *FRE2* encodes two low abundance mRNA isoforms under normal growth conditions. The *FRE2* transcripts have 3'-UTRs that differ in length. *FRE2* also has a upstream open reading frame (uORF) [14] and is predicted to be subject to −1 PRF (Programmed Ribosomal Frameshifting) [15](Fig 1B). Collectively these features are known to target mRNAs to the NMD pathway [2, 7]. The *S. cerevisiae* genome contains additional genes that are categorized as iron/copper reductases. Examination of genome wide studies investigating changes in mRNA level in response to NMD shows that additional mRNAs in this group may be regulated by NMD [1, 16]. We previously found that under normal growth conditions *FRE1* mRNA does not accumulate to higher levels in NMD mutants [12].

Here, we examined genome wide studies investigating changes in mRNA expression levels in yeast cells with a functional and non-functional NMD pathway for changes in the expression of *FRE1, FRE2, FRE3, FRE4, FRE5, FRE6, FRE7* and *FRE8* mRNAs. We then examined the regulation of *FRE1* and *FRE2* mRNAs by NMD under inducing and non-inducing growth conditions. Specifically, the response of *FRE1* and *FRE2* mRNAs to iron and copper availability. Fre1p and Fre2p are the major ferric and cupric reductases in *S. cerevisiae*. In addition, we examined the functionality of the NMD targeting features within *FRE2* by determining the extent to which the long 3'-UTR is sufficient to target *FRE2* mRNAs to NMD.

## MATERIALS AND METHODS

### 2.1 Yeast Strains

All *Saccharomyces cerevisiae* strains and genotypes used in this study are listed in Table 1 and Table 1 supporting material. Yeast strains were maintained and grown using standard techniques [17]. Yeast strains used to measure mRNA half-lives have the temperature sensitive allele of RNA polymerase II, *rpb1–1*. *rpb1–1* yeast strains grow at 28°C (permissive temperature) and growth is inhibited at 39°C (the non-permissive temperature). Subsequently,

mRNA decay is monitored at different time points after transcription has been inhibited at the non-permissive temperature [18]. Typical mRNA half-lives are measured over a 35 minute time period.

### 2.2 Growth of Yeast Strains

For analysis of the mRNAs under low copper conditions, wild-type and NMD mutant strains were grown in low copper complete minimal (CM) media. This media contained yeast nitrogen base without copper (YNB-$CuSO_4$-$FeCl_3$) and 100 uM of bathocuproinedisulfonic acid (BCS) (Sigma-Aldrich, St. Louis, MO, USA). For analysis of the mRNAs under low iron conditions, wild-type and NMD mutant strains were grown in low iron CM media containing 100μM Bathophenanthrolinedisulfonic acid (Sigma-Aldrich). Glassware used in these experiments was soaked in 10% nitric acid overnight to remove trace amounts of copper and iron. All yeast cells used for low copper and low iron northerns were initially grown to saturation in CM media and then sub-cultured into copper or iron deficient media in acid washed glassware.

To analyze the mRNAs under high copper conditions, Wild-type and NMD mutant yeast cells were grown in CM media supplemented with 100μM copper (high copper media). As with the low copper and iron conditions, the yeast cells were first grown to saturation in CM media then sub-cultured into media supplemented with 100μM copper.

### 2.3 RNA Methods

*S. cerevisiae* total RNA was used for all mRNA accumulation, half-life experiments as described in *Peccarelli and Kebaara, 2014* [19]. Northern blots were probed with oligolabeled DNA probes that were labeled with [$\alpha-^{32}$P] dCTP using the RadPrime DNA Labeling System (Thermo Fisher Scientific, Carlsbad, CA). All probes were generated by PCR. Northern blots were phosphorImaged™ using a Typhoon Phosphorimager (Amersham Pharmacia Biotech, Inc.). All northerns were probed with *CYH2* as an NMD control to confirm the NMD phenotype of the yeast strains. *CYH2* pre-mRNA is a known NMD target, while *CYH2* mRNA is not [20]. *SCR1* RNA was used as a loading control to normalize RNA levels. *SCR1* is an RNA polymerase III transcript that is insensitive to NMD. All northerns were quantified using ImageQuant software. Sigmaplot 2000, Version 6.10 software was used to calculate half-lives as described in *Peccarelli and Kebaara, 2014* [19].

### 2.4 DNA Methods

To create a fusion constructs containing the long 3'-UTRs from *FRE2*, 1450 nt DNA from the 3′-UTR were PCR amplified. Subsequently, DNA containing the 5'-UTR and ORF of a second gene, *CYC1*, was amplified by PCR. *CYC1* mRNA is not an NMD target and we and others have used it previously to characterize NMD targeting features [10, 21]. Third, ligation mediated PCR fused the two PCR fragments. To create *CYC1FRE2* 3'-UTR, DNA comprising the *CYC1* 5'-UTR and ORF were fused to 1450 nt from the *FRE2* 3'-UTR. This construct contained the *CYC1* promoter and *FRE2* terminator sequences. Alternatively, to create *FRE2CYC1* 3'-UTR, DNA comprising the 5'-UTR and ORF of *FRE2* were fused to 350 nt DNA from the *CYC1* 3'-UTR. The *FRE2CYC1* 3'-UTR construct contained the *FRE2* promoter and *CYC1* terminator sequences.

The fusion constructs were then inserted into the TOPO-TA cloning vector according to manufacturer's instructions, and sent for sequencing to verify sequences and confirm that the correct fusion construct was generated. Next, *CYC1FRE2* 3'-UTR and *FRE2CYC1* 3'-UTR in TOPO-TA were digested with *BamHI* and *NotI* prior to ligation into the yeast vector pRS425.

## 3. RESULTS

### 3.1 Regulation of mRNAs encoding proteins with ferric and cupric reductase activity by NMD.

The *S. cerevisiae* genome encodes *FRE1-FRE8* homologous metalloreductases. Several genome-wide studies examining regulation of mRNAs by NMD reported changes in expression of a subset of these mRNAs in wild-type and NMD mutants. *FRE2* and *FRE3* were reported to be upregulated upon NMD reactivation [16]. Guan et al., [2] categorized *FRE3* and *FRE6* as direct NMD targets with altered decay rates in wild-type and NMD mutants, while *FRE2* was categorized as an indirect target by the same study. Because *FRE1* and *FRE2* are the major ferric and cupric reductases in *S. cerevisiae*, we examined the regulation of these mRNAs under normal growth conditions, low copper, high copper and low iron.

### 3.2 *FRE2* mRNAs isoforms are regulated by NMD under non-inducing conditions, whereas *FRE1* mRNAs are immune to NMD under inducing and non-inducing conditions.

We previously reported *FRE2* mRNA steady-state levels and half-lives in wild-type and NMD mutant yeast strains grown in rich media (Table 2). We found that *FRE2* mRNAs have very low expression levels in wild-type yeast cells [10]. Additional previous studies have reported very low *FRE2* mRNA expression in wild-type yeast strains in non-inducing conditions [22]. To confirm that we were detecting the *FRE2* mRNAs, we used an *FRE2* deletion strain on northern blots. We found that the major *FRE2* mRNA isoform was missing from the deletion strain (Supporting Fig 1A, 1B). Comparing wild-type and NMD mutants, the expression levels of the mRNA isoform were much higher in NMD mutants (Fig 1C). We quantified the two major *FRE2* mRNA isoforms and found that both isoforms accumulated to higher levels in the NMD mutant (Fig. 1C).

Georgatsou et al [22] and others have used iron deplete conditions to induce *FRE2* expression. In response to low iron, the *AFT1* transcription factor induces the expression of *FRE1*, *FRE2*, *FRE3*, and *FRE5* mRNAs. In addition, *FRE1* expression has been reported to be controlled by copper availability unlike *FRE2*. When yeast cells were grown under copper deplete conditions by adding 100 μM Bathocuproinedisulfonic acid (BCS), we detected three *FRE2* mRNA isoforms. Two of the mRNA isoforms accumulated to higher levels in the NMD mutants relative to the wild-type strains (Fig. 1D). The longest mRNA isoform accumulated to similar levels as in rich media and CM media (Fig. 1D, Table 2). Interestingly, under iron deplete conditions only one major highly expressed *FRE2* mRNA isoform of ~3800nt was detected. A minor band of ~3500 nt was also detected (Figure 1F). Under *FRE2* inducing conditions the *FRE2* mRNA was expressed higher in wild-type strains (*UPF1*) and in the NMD mutants (*upf1Δ*) relative to low copper conditions (Figure 1F).

Notably, this *FRE2* mRNA isoform did not accumulate to higher levels in NMD mutants (Fig 1F). This was not only observed with *upf1Δ* yeast strain but also *upf2Δ* and *upf3Δ* indicating that this is a general NMD phenotype (Fig 1F). Moreover, this observation is distinct from what we observed in CM and low copper conditions demonstrating that under low iron conditions *FRE2* mRNAs are not regulated by NMD.

Under low iron conditions NMD is functional. We previously reported that *COX19* mRNA is regulated by the NMD pathway under distinct conditions, including low copper and high copper. *COX19* encodes a protein that is found in the cytosol and the mitochondrial intermembrane space and is required for the assembly of cytochrome c oxidase. We found that under low iron conditions *COX19* mRNA accumulated 5.2 (± 1.3) fold higher in the *UPF1* deletion strain relative to the wild-type strain, demonstrating that under this conditions *COX19* mRNA is regulated by NMD (Fig 1F). Furthermore, *CYH2* pre-mRNA is usually used as an NMD control and it does not accumulate to higher levels in wild-type cells (*UPF1*) relative to NMD mutants in these conditions.

Because *FRE1* is the major ferric/cupric reductase and *FRE1* is regulated by copper and iron availability, we also examined the regulation of *FRE1* mRNA under comparable conditions. Previously, we detected one *FRE1* transcript of 2300 nt that did not accumulate to higher levels in NMD mutants under normal growth conditions [12]. Under low copper conditions one major *FRE1* mRNA was detected. A shorter low abundance transcript of 2000 nt was also present. The major *FRE1* mRNA isoform accumulated to higher levels under low copper relative to CM, indicating that *FRE1* mRNA levels are induced by copper depletion (Fig 1E). Furthermore, *FRE1* mRNA did not accumulate to higher levels in NMD mutants relative to the wild-type strains in CM or under low copper conditions (Fig 1E,). Likewise, under low iron conditions the *FRE1* mRNA was expressed at higher levels relative to CM but did not accumulate to higher levels in any NMD mutant (*upf1Δ*, *upf2Δ* or *upf3Δ*) strains, demonstrating that the *FRE1* mRNA is not regulated by NMD under these conditions either (Fig 1F).

Because Mac1p is a copper sensing transcription factor that activates the expression of genes under low copper conditions, we examined the levels of *FRE2* and *FRE1* in a wild-type *mac1Δ* strain and NMD mutant *mac1Δ* strain. Using yeast strains with a different genetic background (Supporting table 1), the *FRE2* mRNA accumulated to higher levels in wild-type than an NMD mutant *mac1Δ* strain (supporting Fig 1B), supporting the notion that *FRE2* increase maybe an indirect effect due to the lack of iron. However, similar results were not observed for *FRE1* mRNA which accumulated to the highest levels in the NMD mutant *mac1Δ* strain relative to the wild-type and *mac1Δ*. These observations suggest that *FRE1* and *FRE2* mRNAs are differentially regulated by NMD and that in specific conditions *FRE1* mRNAs can be regulated by NMD (supporting Fig 1B).

Altogether, these data supports the conclusion that *FRE1* mRNA is immune to NMD-mediated degradation under both inducing and non-inducing conditions examined here, but can be sensitive to NMD in a *mac1Δ* stain. However, *FRE2* mRNAs are regulated by NMD under non-inducing conditions but not under inducing conditions (including in a *mac1Δ* strain)

### 3.3 Decay of *FRE1* and *FRE2* mRNA in wild-type and NMD mutant strains in response to copper availability.

Because *FRE2* mRNA steady-state accumulation levels increase in NMD mutants relative to wild-type yeast strains in rich media, CM and low copper conditions, we examined the stability of the mRNAs in response to copper availability. Under low copper conditions the two lowly expressed *FRE2* mRNA isoforms detected were degraded faster in NMD mutants relative to wild-type strains (Fig 2A, B). The half-life of the longer *FRE2* mRNA isoform in the wild-type strain (*UPF1*) was 43.5± 6 mins relative to 15 ± 4 mins in the NMD mutant strain. Similarly, the half-life of the shorter *FRE2* mRNA in the wild-type strain (*UPF1*) was 17.5 ± 4 mins relative to 10 ± 6 mins in the NMD mutant strain (Table 2). Notably, under these conditions, the NMD pathway is functional because the *CYH2* pre-mRNA is degraded rapidly in wild-type cells relative to the NMD mutants (Fig 2A, B). Under copper supplemented conditions (100 μM Cu), *FRE2* mRNAs were undetectable in either wild-type or NMD mutant strains (Fig 2C, D). This observation suggests that high copper conditions suppress expression of *FRE2*.

To compare *FRE1* and *FRE2* regulation, the decay of *FRE1* mRNA was examined under low and high copper conditions. As observed with the steady-state accumulations, copper deplete conditions induce *FRE1* expression (Fig 1E). Under copper deplete conditions two *FRE1* mRNA isoforms of 2300 nt and 2000 nt were detected. Both *FRE1* mRNA isoforms were degraded faster in the NMD mutant relative to the wild-type strain similar to *FRE2* (Fig 3A, B). The half-life of the 2300 nt *FRE1* mRNA isoform in the wild-type strain (*UPF1*) was 12.5± 3 mins relative to 7.3 ± 1 mins in the NMD mutant strain. Similarly, the half-life of the 2000 nt *FRE1* mRNA in the wild-type strain (*UPF1*) was 13 ± 5 mins relative to 6 ± 3 mins in the NMD mutant strain. Because the *FRE1* mRNA is induced under these conditions relative to the *FRE2* mRNA, the faster decay of the *FRE1* mRNAs in NMD mutants can be noticeably observed on the northern blot (Fig 3A, B).

Under copper supplemented conditions (100 μM Cu), two lowly expressed *FRE1* mRNAs were detectable in both the wild-type and NMD mutant strains (Fig 3C, D). The half-life of longer *FRE1* mRNA isoform in the wild-type strain (*UPF1*) was 7.3± 2 mins relative to 12 ± 3 mins in the NMD mutant strain. Conversely, the half-life of shorter *FRE1* mRNA in the wild-type strain (*UPF1*) was 13 ± 4 mins relative to 10 ± 1 mins in the NMD mutant strain. Thus, the longer *FRE1* mRNA isoform was degraded slightly faster in the wild-type strain relative to the NMD mutant, while the shorter *FRE1* mRNA isoform appears to be degraded at comparable rates in both strains (Fig 3C, D). Interestingly, the more stable isoform under copper supplemented conditions was the shorter *FRE1* isoform compared to the longer isoform under low copper conditions. Altogether, we find that under low copper conditions *FRE1* and *FRE2* mRNAs are degraded faster in the NMD mutants relative to the wild-type yeast strains. Under high copper conditions both *FRE1* and *FRE2* mRNAs are expressed at low levels.

### 3.4 The *FRE2* 3'-UTR is not sufficient to target an NMD insensitive mRNA to the pathway but it contributes to the degradation of the mRNAs by the pathway

Because *FRE2* mRNAs are differentially regulated by NMD under inducing and non-inducing conditions we examined the functionality of the NMD targeting features within the *FRE2* mRNAs. *FRE2* mRNAs have multiple potential NMD targeting features (Fig 1A[10]). We investigated the role the atypically long 3′-UTRs plays in the NMD-mediated degradation of the mRNAs.

We hypothesized that the atypically long 3'-UTRs (700 and 1300 nts, respectively) of the *FRE2* mRNAs contribute to the degradation of the mRNAs by NMD (Fig. 1B). This hypothesis was based on our previous observations that atypically long 3'-UTRs on other mRNAs affect their regulation by NMD [10, 21]. Furthermore, previous studies have demonstrated that replacement of an NMD-insensitive mRNA's 3'-UTR with an atypically long 3'-UTR can cause the mRNA to be targeted by NMD [8, 21]. We generated the *CYC1FRE2* 3'-UTR fusion mRNA by replacing the 3'-UTR of *CYC1* with 1450nt downstream of the *FRE2* ORF containing the *FRE2* 3'-UTR (Fig. 4A). The *CYC1FRE2* 3'-UTR fusion construct encoded one mRNA of ~ 1.7 kb, which is consistent with an mRNA containing a 3'-UTR of 1300 nt. No mRNA of ~1100 nt was detected, which would be consistent with an mRNA containing a 3'-UTR of 700 nt. Expression of the *CYC1FRE2* 3'-UTR was driven by the *CYC1* promoter. Promoter elements have been shown to affect both processing and NMD-mediated degradation of mRNAs. Unlike the *FRE2* mRNAs, the *CYC1FRE2* 3'-UTR mRNA did not accumulate to substantially higher levels in NMD mutants (Table 3). Additionally, no statistically significant difference in decay rate between the wild-type (25 $\pm$8.2 min) and the NMD mutant strain (11 $\pm$5.0 min) was detected (Fig.4C and E, Table 3). In both the wild-type and NMD mutant strains, the *CYC1FRE2* 3'-UTR mRNA levels are low at the 0-time point, the levels increase before gradually decreasing (Figs. 4C and 4E). Determination of the mRNA half-lives from 6–35 minutes due to the atypical pattern of decay showed that the *CYC1FRE2 3′-UTR* mRNA was still degraded at a faster rate in the NMD mutant (Fig 4C and 4E and Table 3). This pattern of decay differs from the *FRE2* mRNAs and demonstrates that the *CYC1FRE2* 3'-UTR mRNA is not regulated by NMD like the *FRE2* mRNAs. In addition the atypical decay pattern of the *CYC1FRE2* 3'-UTR mRNA is not due to inefficient transcription turnoff because the expression of the *CYH2* pre-mRNA decreases from time 0 to 35 minutes (Figs. 4C and 4E).

Subsequently, we determined whether the *FRE2* mRNAs could be regulated by NMD in the absence of the long 3'-UTR. We expected that *FRE2* mRNAs would still be regulated by NMD after the removal of the 3'-UTR because the mRNAs have additional recognizable NMD targeting features (Fig 1A). To test this hypothesis, we generated the *FRE2CYC1* 3'-UTR fusion construct, which contains the 5'-UTR and ORF of *FRE2* fused to 250 nt *CYC1* 3'-UTR (Fig. 4B).

The plasmid encoded *FRE2CYC1* 3'-UTR fusion construct produced one highly expressed transcript, unlike the endogenous *FRE2* which generates two low abundance transcripts in wild-type yeast strains. Additionally, the *FRE2CYC1* 3'-UTR mRNA did not accumulate to substantially higher levels in NMD mutants (Table 3). Furthermore, no substantial difference in decay rate was observed between the *FRE2CYC1* 3'-UTR fusion mRNA in the wild-type

strain (20 ±10.40 mins) relative to the NMD mutant (23 ± 15.50 mins) (Figs. 4D, 4F and Table 3). These results demonstrate that the *FRE2CYC1* 3'-UTR mRNA is not regulated by NMD under these conditions. In addition, these results suggest that the *FRE2* 3'-UTR has a general instability element that is required for *FRE2* mRNAs to be regulated by NMD. Removal of the *FRE2* 3'-UTR causes the mRNAs to escape degradation by NMD; however, this 3'-UTR is not sufficient to cause degradation of the *CYC1* mRNA by NMD. Altogether, these observations suggest that the NMD targeting features within the *FRE2* mRNA function cooperatively.

## 4.0 DISCUSSION

The results presented here demonstrate that mRNAs encoding proteins involved in ferric and cupric reductase activity are differentially regulated by NMD. This kind of regulation would allow yeast cells to regulate expression of specific mRNAs in response to copper and iron availability. We show that the concentrations of the metals in the environment can result in differential regulation of two cupric/ferric reductases in yeast, *FRE1* and *FRE2*. In addition, we show that the *FRE2* mRNAs have a functional NMD targeting feature that could target the mRNAs to NMD.

The expression of *FRE1* mRNA increases under copper and iron deplete conditions, but the steady-state levels of the *FRE1* mRNAs are not regulated by NMD under either condition. Furthermore, under high copper, *FRE1* mRNA expression is reduced. Conversely, expression of *FRE2* mRNA increases under iron deplete conditions. Under these conditions *FRE2* mRNAs are not regulated by the NMD pathway, unlike in complete minimal and low copper conditions. These results suggest that under conditions requiring higher levels of Fre2p, the mRNAs are stabilized, and immune to NMD-mediated degradation. The higher expression levels of *FRE2* and *FRE1* mRNAs under low iron conditions could also be due to increased transcription or retention of the mRNAs in the nucleus. Future experiments will determine the role transcription or retention of the mRNAs in the nucleus influence the response of *FRE1* and 2 mRNAs to low iron conditions.

The expression of *FRE1* and *FRE2* mRNAs in response to iron and copper availability is regulated by transcriptional activators. Mac1p is a copper sensing transcription factor that activates the expression of genes under low copper conditions. Mac1p induces the expression of *CTR1* and *CTR3*, plasma membrane copper transporters and *FRE1*/ *FRE7* metalloreductases under low copper conditions. Conversely, in a *mac1Δ* yeast strain, *FRE2* mRNA was substantially induced under copper and iron deplete media [22]. Their data suggest that under copper limiting conditions Mac1p indirectly functions in a negative role on *FRE2* gene expression, while it positively regulates *FRE1*. Indirect effect of Mac1p on *FRE2* mRNA expression is because the Fet3p iron oxidase needs copper to function with the iron transporter (Ftr1p). We previously found that *MAC1* mRNA is differentially regulated by NMD [10]. Under normal growth conditions *MAC1* mRNAs were directly regulated by NMD while under low copper conditions, the mRNAs were not regulated by the pathway. Taken together, these observations suggest that the increased expression of *FRE1* mRNAs under low copper conditions and the lower expression levels of the *FRE2* mRNAs is due to stabilization of *MAC1* under these conditions. Enhanced decay of both *FRE1* and *FRE2*

mRNAs in NMD mutants under low copper conditions may be due to stabilization of additional factors that are involved in the decay of the mRNAs. Interestingly, enhanced decay of both *FRE1* and *FRE2* mRNAs in NMD mutants under low copper conditions do not result in detectable changes in steady-state mRNA accumulations (Fig. 1D, E). This could be due to transcript buffering whereby comparable mRNA steady-state levels are maintained by adjustment in mRNA synthesis or degradation [23]

Furthermore, in response to low iron, the *AFT1* transcription factor induces the expression of *FRE1*, *FRE2*, *FRE3*, and *FRE5* mRNAs. *AFT1* mRNA has been reported to be directly regulated by the NMD pathway under normal growth conditions [2]. Under low iron conditions, both *FRE1* and *FRE2* mRNAs did not accumulate to higher levels in NMD mutants, indicating that the mRNAs are not regulated by the pathway under this conditions, and suggesting that *AFT1* may also be differentially regulated by NMD based on iron availability.

*FRE2* mRNAs have a functional NMD targeting feature. This observation suggests that functionally related mRNAs may be regulated by the pathway through similar NMD targeting features. Specifically, we found that the *CTR2*, *FRE2* and *COX19* mRNAs have atypically long 3'-UTRs that affect their regulation by NMD. We showed that removal of the *FRE2* 3'-UTR rendered the mRNA insensitive to NMD. This suggests that the 3'-UTR is required to target the *FRE2* mRNA to the pathway. Conversely, our previous studies found that removal of either the *CTR2* or *COX19* mRNAs 3'-UTR resulted in mRNAs that were still sensitive to NMD-mediated degradation [10, 21].

We expected that the *FRE2* 3'-UTR would be sufficient to target other NMD insensitive mRNAs to pathway; however, this was not the case and was also in contrast with what we found with the long 3'-UTRs from *CTR2*, *COX19* and *COX23* mRNAs. Both the *CTR2*, *COX19* and *COX23* long 3'-UTRs were sufficient to cause degradation of *CYC1* by NMD. Alternatively, the *CYC1FRE2* 3'-UTR fusion mRNA was not regulated by NMD. This suggests that the *FRE2* 3'-UTR is not sufficient to target an NMD insensitive mRNA to the pathway. Although the *FRE2* 3'-UTR used in this study was atypically long, there are a few possibilities as to why it does not cause the rapid decay of the *CYC1* mRNA. First, it is possible that elements within the *FRE2* 5'-UTR or ORF are required for NMD to occur and that the long 3'-UTR itself is not sufficient to trigger degradation by NMD. Additionally, *FRE2* and *CYC1FRE2* 3'-UTR utilize different promoters. Promoters driving expression of genes can influence decay of mRNAs. As mentioned above, removal of the *FRE2* 3'-UTR resulted in an mRNA that was insensitive to the pathway. Overall, these results indicate that another feature or features present within the *FRE2* 5'-UTR and/or ORF functions cooperatively with the 3'-UTR to target *FRE2* mRNAs to NMD. Since these observations are distinct from our previous observations with the *CTR2*, *COX19*, and *COX23* mRNA 3'-UTRs, they suggest that the key NMD targeting feature within these functionally related mRNAs is the 3'-UTR, and the other features enhance this targeting mechanism. This idea is supported by the fact that this mechanism of regulation is extensively found in this group of mRNAs; however, the other potential NMD targeting features are distinct [10, 21]. Moreover, in other studies using mammalian cell lines, a long 3′-UTR was identified as an important NMD –inducing feature of translated natural NMD targets [24].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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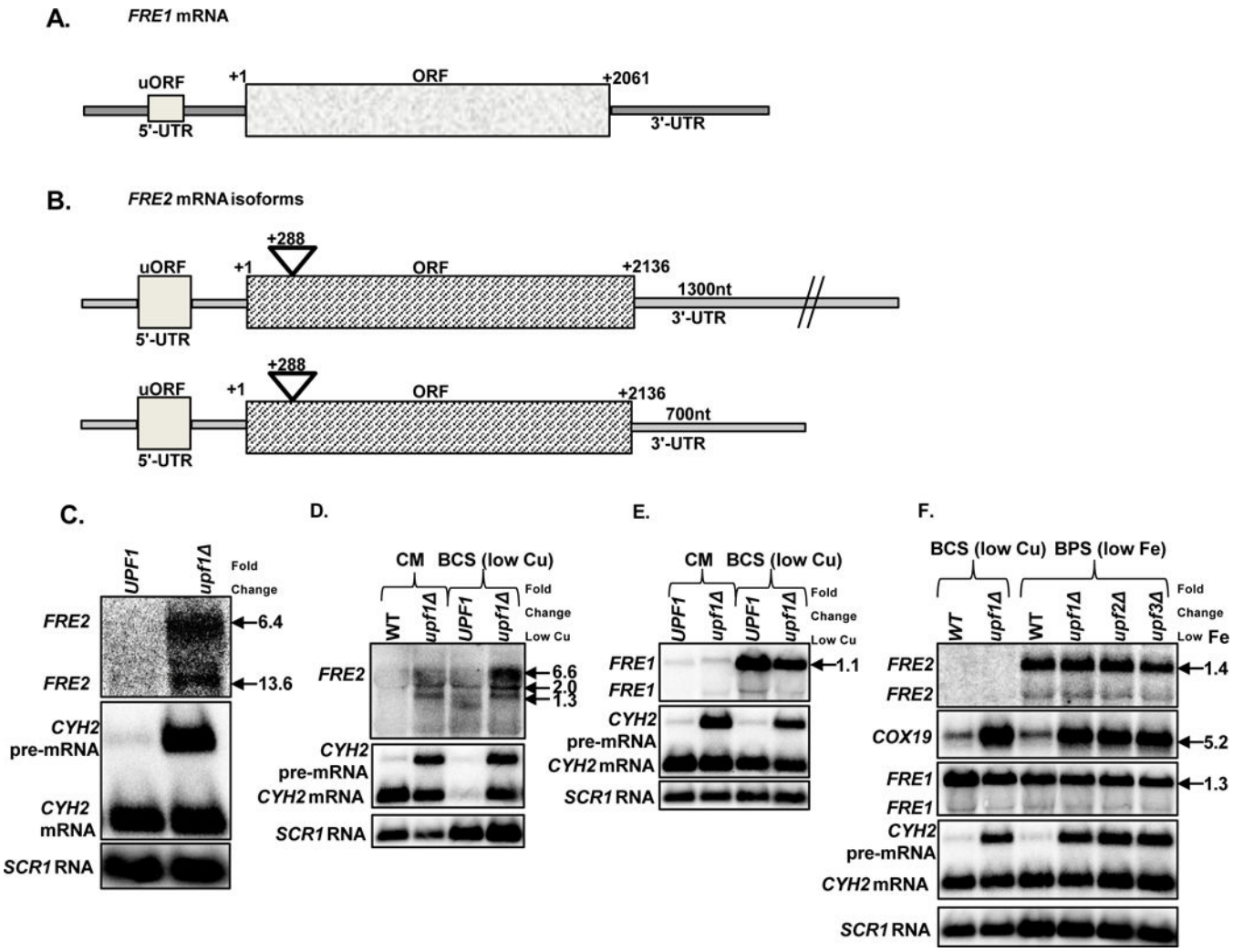


**Figure 1. *FRE1* mRNA is immune to NMD mediated degradation whereas, *FRE2* mRNAs isoforms are differentially regulated by NMD under inducing and non-inducing conditions**

Schematic representations of *FRE1* (A) and the *FRE2* mRNA isoforms (B) showing the Upstream open reading frame (uORF), the open triangle indicates the potential −1 programmed ribosomal frameshift (−1PRF) and the atypically long 3′-UTRs. Representative mRNA steady-state accumulation levels (C, D, E and F). Steady-state accumulation levels were measured with total RNA from wild-type strain W303 (*UPF1*) [25], and NMD mutants (*upf1Δ*) [18], HFY1300 (*upf2Δ*) [26], HFY861 (*upf3Δ*)[27] [18]. The northern blots were probed with DNA specific to the *FRE1, FRE2* and *COX19* (1F) open reading frames (ORFs). The major *FRE2* mRNA isoform fold change (*upf1Δ/UPF1*) are shown to the right of the northern blots (C, D, and F). *FRE1* mRNA fold change (*upf1Δ/UPF1*) are shown to the right of northern blots (E and F) and *COX19* mRNA fold change (*upf1Δ/UPF1*) is shown to the right of northern blot (F). The fold change shown to the right of 1D and 1E is for low copper conditions, while the fold changes to the right of 1F are for low iron conditions. *CYH2* and *SCR1* were used as controls. *CYH2* pre-mRNA was used as an NMD control because *CYH2* pre-mRNA is degraded by NMD. *SCR1* was used as a loading control for all northern blots. *SCR1* is an RNA polymerase III transcript that is not regulated by NMD.

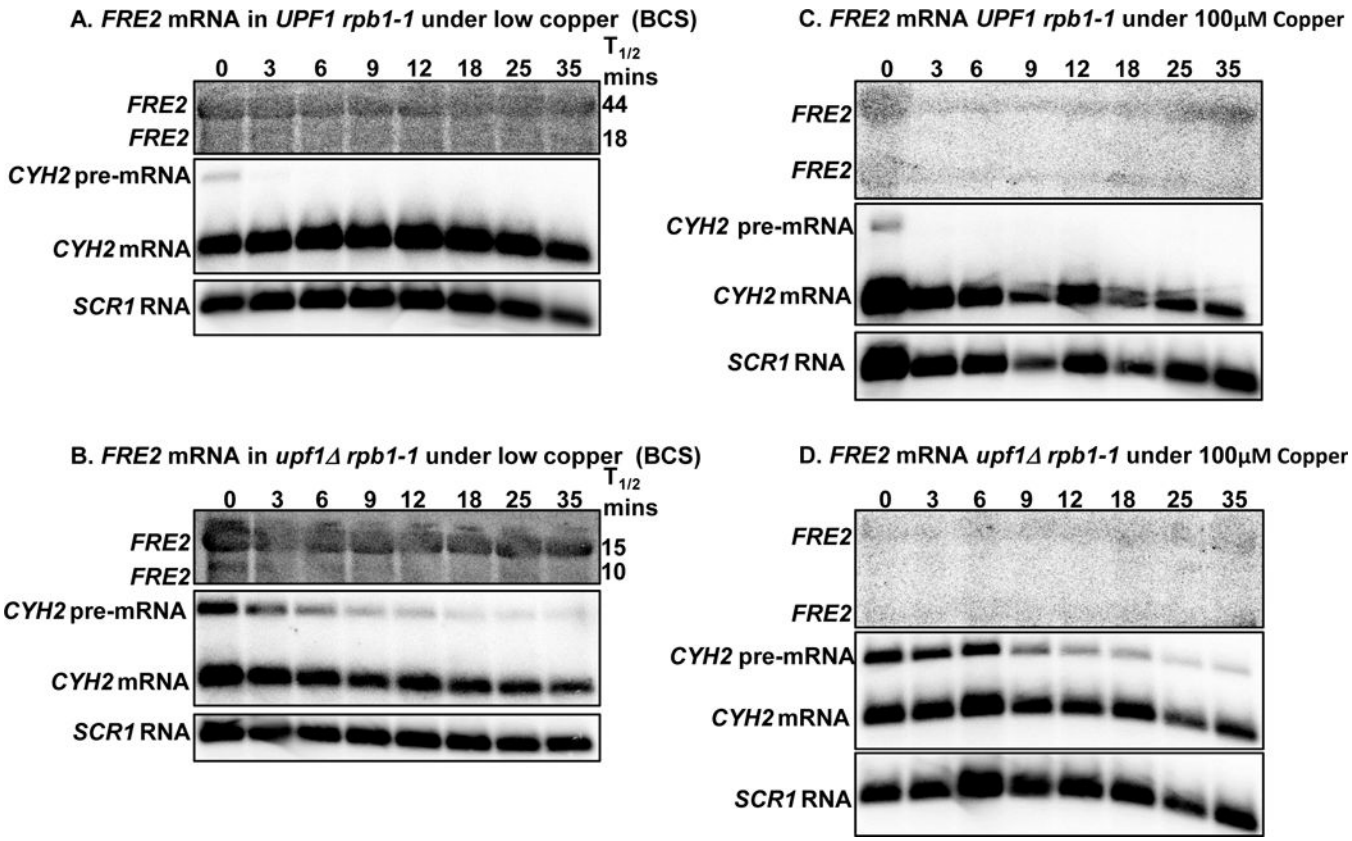


**Figure 2. Decay of *FRE2* mRNAs in wild-type and NMD mutant strains is responsive to copper availability.**

Representative half-life northerns of *FRE2* mRNA. The mRNA half-lives were measured with total RNA extracted from wild-type strain AAY334 (*UPF1 rpb1–1*)[18] and NMD mutant strain AAY335 (*upf1Δ rpb1–1*)[18] grown under 100 μM Bathocuproinedisulfonic acid (BCS), low copper (A, B) and in 100 μM copper (C, D). Yeast cells were harvested at eight time points over 35 minutes. Individual time points are indicated above the half-life northerns. The half-lives were determined using SigmaPlot and are shown to the right of the northerns. All half-life measurements are an average of at least two independent experiments. *CYH2* and *SCR1* were used as controls as described in Figure 1.

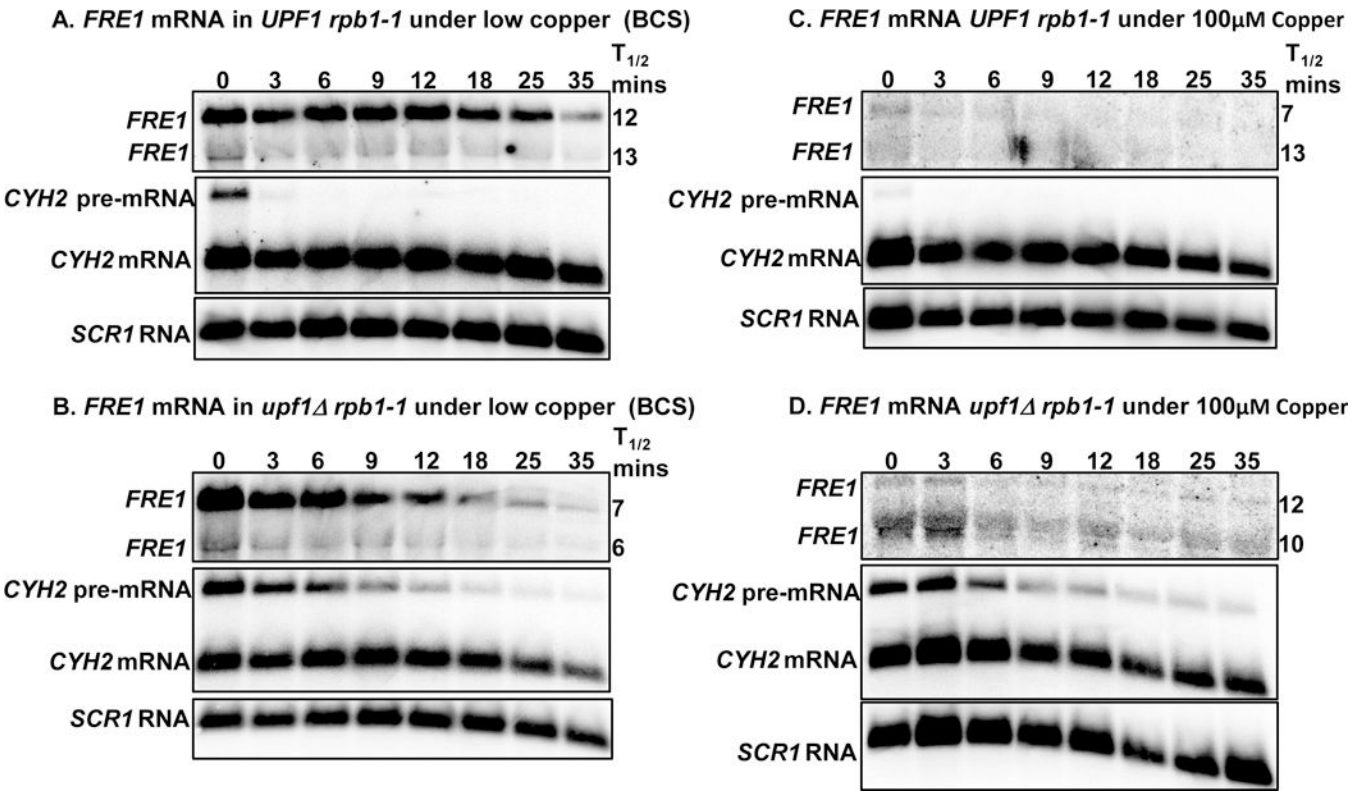


**Figure 3. Decay of *FRE1* mRNA in wild-type and NMD mutant strains is responsive to copper availability.**

Representative half-life northerns of *FRE1* mRNA. The mRNA half-lives were measured with total RNA extracted from wild-type strain AAY334 (*UPF1 rpb1–1*)[18] and NMD mutant strain AAY335 (*upf1Δ rpb1–1*)[18] grown under low copper (A, B) and in 100 μM copper (C, D). Yeast cells were harvested at eight time points over 35 minutes. Individual time points are indicated above the half-life northerns. The half-lives were determined using SigmaPlot and are shown to the right of the northerns. All half-life measurements are an average of at least two independent experiments. *CYH2* and *SCR1* were used as controls as described in Figure 1.

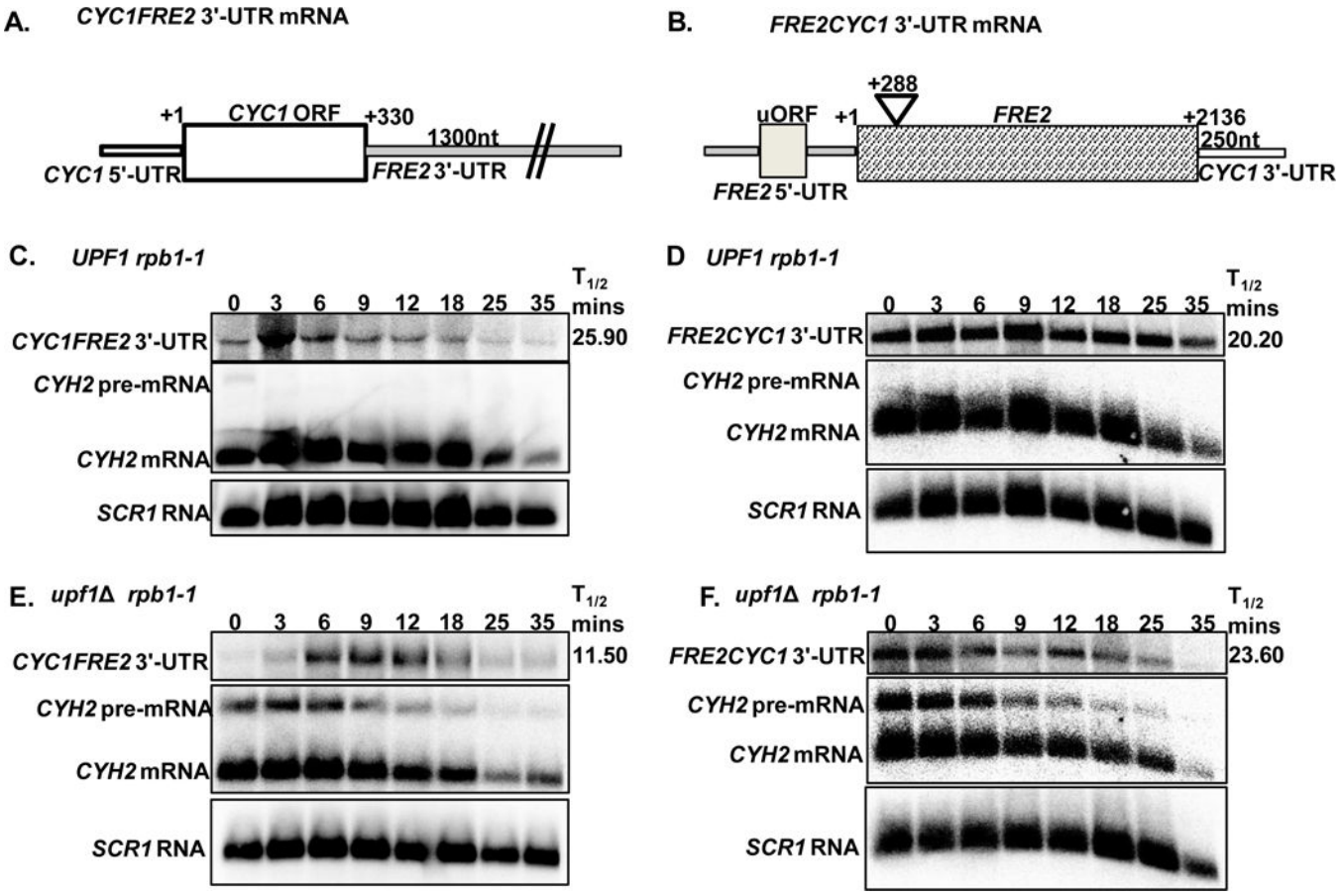


**Figure 4. The *FRE2* 3'-UTR is not sufficient to target an NMD insensitive mRNA to the pathway but it contributes to the degradation of the mRNAs by the pathway**

Schematic representation of *CYC1FRE2* 3'-UTR (A) and *FRE2CYC1* 3'-UTR fusion mRNAs (B). Representative mRNA half-life northern blots of *CYC1FRE2* 3'-UTR (C and E, left panels) in wild-type (*UPF1 rpb1–1*), and NMD mutants (*upf1Δ rpb1–1*) respectively. The northerns were probed with DNA specific to the *FRE2* 3'-UTR. Representative mRNA half-life northern blots of *FRE2CYC1* 3'-UTR mRNA (D and F right panels) in wild-type (*UPF1 rpb1–1*), and NMD mutants (*upf1Δ rpb1–1*) respectively. The northern blots were probed with DNA specific to the *FRE2* 5'-UTR and ORF. All yeast cells were grown in complete minimal media lacking leucine, and were harvested at eight time points over thirty-five minutes after transcription inhibition. Specific time points are indicated above the half-life northern blots and the half-lives are shown to the right of the northern blots. The half-lives were determined using SigmaPlot. *CYH2* and *SCR1* are used as controls as described in Figure 1.

**Table 1.**

*Saccharomyces cerevisiae* strains used in this study.

| Yeast Strain | Genotype | Source |
|---|---|---|
| **W303** | *a, ade2–1, ura3–1, his3–11,15, trp1–1, leu2–3,112, can1–101* | [25] |
| **AAY320** | *a, ade2–1, ura3–1, his3–11,15, trp1–1, leu2–3,112, can1–100, UPF1::URA3 (upf-Δ2)* | [18] |
| **AAY334** | *a, ADE2, ura3–1 or ura3–52, his3–52, his3–11,15, trp1–1, leu2–3,112, rpb1–1* | [18] |
| **AAY335** | *a, ADE2, ura3–1 or ura3–52, his3–52, his3–11,15, trp1–1, leu2–3,112, rpb1–1, upf1-Δ2, (URA3)* | [18] |
| **HFY1300** | *MATα ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 trp1–1can1–100 UPF1 nmd2::HIS3 UPF3* | [26] |
| **HFY861** | *MATa ade2–1 ura3–1 his3– 11,15 trp1–1 leu2–3,112 trp1–1can1–100 UPF1 NMD2 upf3::HIS3* | [27] |

**Table 2.**

*FRE1* and *FRE2* mRNA steady-state accumulations and half-lives

| Standard Name | Relative mRNA accumulation (*upf1Δ*/*UPF1*) | mRNA half-life (mins) *UPF1* | mRNA half-life (mins) *upf1Δ* |
|---|---|---|---|
| ***FRE2*** * **(long)** | 6.4 ± 0.5 | 7.6 ± 3.2 | 84.2 ± 21 |
| ***FRE2*** * **(short)** | 13.6 ± 3.5 | 10.8 ± 2.7 | 31.2 ± 10.3 |
| ***FRE2*** **(long low Cu)** | 6.6 ± 3.3 | 43.5 ± 6.3 | 15 ± 4.3 |
| ***FRE2*** **(short low Cu)** | 1.3 ± 1.2 | 17.5 ± 3.5 | 10 ± 5.6 |
| ***FRE1*** **(long low Cu)** | ND | 12.5 ± 3.5 | 7.3 ± 1.5 |
| ***FRE1*** **(short low Cu)** | 1.1 ± 0.7 | 13 ± 5.7 | 6 ± 3.0 |

ND- Not determined

* The *FRE2* mRNA steady-state levels and half-lives under normal growth conditions were previously reported [10]

Wild-type (W303) and NMD mutants (AAY320) were used in mRNA steady-state accumulation measurements, while AAY334 and AAY335 were used to determine half-lives. All yeast strains used were grown under standard conditions in complete minimal media, or under low copper (low Cu) conditions. The steady-state and half-life experiments were done at least twice and are reported as averages ± standard deviation.

**Table 3.**

mRNA steady-state accumulation levels and half-lives of the *CYC1FRE2 3′-UTR* and *FRE2CYC1 3′-UTR* fusion mRNAs.

| Name | Relative mRNA accumulation (*upf1Δ*/*UPF1*) | mRNA half-life (mins) *UPF1* | mRNA half-life (mins) *upf1Δ* |
|---|---|---|---|
| ***CYC1FRE2* 3'-UTR** | 3.0 ±2.1 | 25.9±8.2 (0–35mins) | 11.5 ±5.0 (0–35mins) |
| | | 27.7±6.5 (6–35mins) | 19.7 ± 6.8 (6–35mins) |
| ***FRE2CYC1* 3'-UTR** | 1.0±0.3 | 20.2±10.4 | 23.6 ±15.5 |

The steady-state mRNA accumulation and half-lives of the *CYC1FRE2 3′-UTR* and *FRE2CYC1 3′-UTR* mRNA fusions. Wild-type (W303) and NMD mutants (AAY320) were used in mRNA steady-state accumulation measurements, while AAY334 and AAY335 transformed with the respective fusion constructs were used to determine half-lives. All yeast strains used were grown under standard conditions in complete minimal media lacking leucine. The steady-state and half-life experiments were done in triplicate and are reported as averages ± standard deviation. The half-lives of the *CYC1FRE2 3′-UTR* fusion mRNA where determined from 0–35 and 6–35 minutes because of the pattern of decay (Fig 4C and 4E)