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Copper Tolerance of *Saccharomyces cerevisiae* Nonsense-Mediated mRNA Decay Mutants

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Abstract
The eukaryotic nonsense-mediated mRNA (NMD) is a specialized pathway that leads to the recognition and rapid degradation of mRNAs with premature termination codons, and importantly some natural mRNAs as well. Natural mRNAs with atypically long 3′-untranslated regions (UTRs) are degraded by NMD in *Saccharomyces cerevisiae*. A number of *S. cerevisiae* mRNAs undergo alternative 3′-end processing producing mRNA isoforms that differ in their 3′-UTR lengths. Some of these alternatively 3′-end processed mRNA isoforms have atypically long 3′-UTRs and would be likely targets for NMD-mediated degradation. Here, we investigated the role NMD plays in the regulation of expression of *CTR2*, which encodes a vacuolar membrane copper transporter. *CTR2* pre-mRNA undergoes alternative 3′-end processing to produce two mRNA isoforms with 300-nt and 2-kb 3′-UTRs. We show that both *CTR2* mRNA isoforms are differentially regulated by NMD. The regulation of *CTR2* mRNA by NMD has physiological consequences, since *nmd* mutants are more tolerant to toxic levels of copper relative to wild-type yeast cells and the copper tolerance of *nmd* mutants is dependent on the presence of *CTR2*.

Keywords: copper tolerance, nonsense-mediated mRNA decay, *CTR2*, mRNA decay, alternative 3′-UTR processing

Introduction

The nonsense-mediated mRNA decay (NMD) pathway is an mRNA decay mechanism that is conserved in all eukaryotic organisms that have been examined so far. It triggers the rapid degradation of mRNAs with premature termination codons preventing the production of potentially harmful truncated proteins. NMD also regulates the expression of some natural mRNAs as well (reviewed in Culbertson and Leeds 2003; Amrani et al. 2006; Nicholson et al. 2009). Three core trans-acting factors are required for NMD in all eukaryotes. These are the Upf1p, Upf2p and Upf3p proteins. The requirement for these proteins was originally discovered in the yeast *Saccharomyces cerevisiae* and later found in multicellular eukaryotes. These proteins have distinct roles in the NMD pathway because loss of any one of these three proteins is sufficient to selectively stabilize mRNAs that are targeted for NMD-mediated degradation.

Cis-acting elements that trigger the decay of natural mRNAs by the NMD pathway have been described. Atypically long 3′-untanslated regions (UTRs) target natural mRNAs for rapid degradation by NMD (Kebaara and Atkin 2009). In *S. cerevisiae*, 3′-UTRs tend to be short. They typically range in size from 50 to 200 nucleotides, with a median length of 121 nucleotides (Graber et al. 1999). Natural mRNAs with 3′-UTRs of at least 350 nt tend to be degraded by the NMD pathway (Kebaara and Atkin 2009).

In *S. cerevisiae*, a number of mRNAs undergo alternative 3′-end processing producing mRNA isoforms with different 3′-UTR lengths. This alternative 3′-end pre-mRNA processing can be sensitive to growth conditions (Guisbert et al. 2007). A number of the alternatively processed *S. cerevisiae* mRNAs have atypically long 3′-UTRs and would be likely targets for NMD-mediated degradation. The *S. cerevisiae CTR2* mRNA is an example of a pre-mRNA that undergoes alternative 3′-end processing producing two mRNA isoforms with 300 nt and 2-kb long 3′-UTRs. *CTR2* encodes a copper transporter of the vacuolar membrane that controls the flux of copper into the vacuole (Rees et al. 2004). Copper is an essential micronutrient that is required as a cofactor for a variety of cellular processes. However, if copper is not carefully regulated, it can be toxic to cells. Hence, cells have evolved delicate mechanisms for homeostatic metabolism of copper. Proteins involved in copper uptake, distribution, compartmentalization, and sequestration have been identified. However, the mechanism of action and regulation of copper homeostasis remains to be determined (Lee et al. 2000).

The *CTR2* pre-mRNA alternative 3′-end processing is responsive to the presence of *HRP1/NAB4* (Guisbert et al. 2007). *HRP1/NAB4* is an essential heterogeneous nuclear ribonucleoprotein (hnRNP) that is part of the cleavage and polyadenylation complex. Hrp1p/Nab4p is involved in pre-mRNA cleavage site selection (Minvielle-Sebastia et al. 1998). The *CTR2* pre-mRNA alternative 3′-end processing produces *CTR2* mRNA isoforms differing in the length of their 3′-UTRs (Guisbert et al. 2007). Guisbert et al. (2007) showed that the *CTR2* mRNA with the longer 3′-UTR accumulates to higher levels in a temperature-sensitive *HRP1/NAB4* mutant, *nab4-7*, than in a wild-type yeast strain. They also demonstrated that *nab4-7* mutants tolerated toxic copper levels and that *CTR2* was required for the tolerance to excess copper by the *nab4-7* mutants. Based on this data, they proposed

that *Hrp1/nab4* regulates alternative 3′ pre-mRNA processing and that *CTR2* alternative 3′-end processing is important for copper tolerance in yeast.

Hrp1p/Nab4p has also been implicated in mRNA export and NMD (Gonzalez et al. 2000). Mutations in *HRP1/NAB4* stabilize nonsense-containing mRNAs. Additionally, Hrp1p/Nab4p was found to interact with Upf1p, a core component of the NMD pathway (Gonzalez et al. 2000). This suggests that the effect of Hrp1p/Nab4p on *CTR2* mRNA could also be due to NMD. The studies reported here were carried out to determine the extent to which the increased accumulation of the *CTR2* mRNA with the long 3′-UTR and the tolerance to toxic copper levels of the *nab4-7* mutant could be attributed to inhibition of NMD.

Here, we report that NMD differentially regulates the accumulation of the two *CTR2* mRNA isoforms. Both *CTR2* mRNA isoforms are regulated by NMD; however, only the longer *CTR2* mRNA isoform accumulates in NMD mutants. This NMD-dependent regulation of CTR2 has physiological consequences since *nmd* mutants are more tolerant to toxic levels of copper. We also show that *CTR2* is required for the copper tolerance of *nmd* mutants.

Materials and methods

Yeast strains

The *S. cerevisiae* strains used in this study and their genotypes are listed in Table 1. Yeast strains were grown and maintained using standard techniques (Ausubel et al. 1998).

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
W303a	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Wente et al. (1992)
AAY320	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1-Δ2 (URA3)*	Kebaara et al. (2003a, b)
AAY334	*MATa ura3Δ his3-11,15 trp1-1 leu2-3,112 rpb1-1*	Kebaara et al. (2003a, b)
AAY335	*MATa ura3Δ his3-11,15 trp1-1 leu2-3,112 rpb1-1 upf1-Δ2 (URA3)*	Kebaara et al. (2003a, b)
ctr1Δ/ctr2Δ/ctr3Δ	*MATa ctr1::ura3::KanR ctr2::HIS3 ctr3::TRP1 lys 2-801*	Rees et al. (2004) and Pena et al. (1998)
YAO417-1D	*MATa leu2Δ2 ura3-52 nab4 Δ2::LEU2[pNAB4.46] NAB4*	Minvielle-Sebastia et al. (1998)
YAO431-1C	*MATa leu2Δ2 ura3-52 nab4Δ2::LEU2[pNAB4.58] nab4-7*	Minvielle-Sebastia et al. (1998)
AAY513	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ctr2::HIS3*	This study
AAY514	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1-Δ2 (URA3) ctr2::HIS3*	This study
HFY1300	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 trp1-1can1-100 UPF1 nmd2::HIS3 UPF3*	He and Jacobson (1995)
HFY861	*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 trp1-1can1-100 UPF1 NMD2 upf3::HIS3*	He et al. (1997)

Determination of copper tolerance

Yeast cells were grown to mid-log phase in yeast extract, peptone, and dextrose (YPD) medium, complete minimal (CM) medium, or CM dropout medium lacking leucine. Ten-fold serial dilutions of the cells were spotted onto CM medium or CM medium containing 1 mM copper and incubated at room temperature for 3–4 days depending on the yeast strain used.

RNA methods

Yeast total RNA used for mRNA steady-state levels was extracted by the hot phenol method from yeast cells harvested at mid-log phase (Kebaara et al. 2003a, b). Total RNA, used to measure mRNA decay rates, was harvested as described in Kebaara et al. (2003a, b). For both steady-state levels and mRNA decay northern, equal amounts of RNA (15 μg) were resolved on 1.0% agarose-formaldehyde gels. The RNAs in these gels were transferred to GeneScreen Plus (PerkinElmer, Boston, Massachusetts) using the capillary blot transfer protocol recommended by the manufacturer or with the NorthernMax Complete Northern Blotting kit (Ambion, Inc., Austin, Texas). The NorthernMax Complete Northern Blotting kit was used for hybridization of the northern blots with oligolabeled DNA probes. DNA templates for probe synthesis were prepared by PCR. The probes were labeled with [α-^{32}P]dCTP (PerkinElmer Inc., Waltham, Massachusetts) using a RadPrime DNA Labeling System (Invitrogen Corp., Carlsbad, California) as described in Atkin et al. (1995). Northern blots were phosphorImaged using a Storm or Typhoon phosphorImager (Amersham Pharmacia Biotech, Inc.). mRNA levels were normalized using *SCR1* RNA, an RNA polymerase III transcript that is insensitive to NMD (Maderazo et al. 2000). *CYH2* pre-mRNA is a target for NMD and was used to confirm the NMD phenotype of our yeast cells (He et al. 1993). mRNA decay rates were determined by graphing $\log_{10}$ of the percent mRNA remaining versus time using SigmaPlot 2000, Version 6.10 (SPSS Science, Chicago, Illinois).

Results

The longer* CTR2 *mRNA isoform accumulates to higher levels in* nmd *mutants

To test the hypothesis that the alternatively processed *CTR2* mRNA isoforms with atypically long 3′-UTRs are degraded by NMD, steady-state *CTR2* mRNA levels and mRNA half-lives were measured using northern blots. If the *CTR2* mRNA isoforms are targets for NMD-mediated degradation, we expected the *CTR2* mRNAs to accumulate in *nmd* mutants compared to wild-type yeast cells. Two *CTR2* mRNA isoforms were detected on northern blots when yeast cells were grown on rich media (YPD, Fig. 1a). The longer *CTR2* mRNA was approximately 2,600 base pairs (bp) long and accumulated 3.9 ± 0.8 (n = 3)-fold higher in *nmd* mutant (*upf1Δ*) yeast compared to wild-type yeast cells (*UPF1*, Fig. 1a). The shorter *CTR2* mRNA isoform was approximately 900-bp long and accumulated 1.0 ± 0.04 (n = 3)-fold in *upf1Δ* yeast cells compared to *UPF1* (Fig. 1a). In wild-type yeast cells (*UPF1*), the short isoform accumulated 26.0 (±1.1)-fold higher than the longer isoform. In contrast, the short *CTR2* mRNA isoform accumulated only 6.7 (±0.9)-fold higher than the longer *CTR2* mRNA isoform in the *upf1Δ* yeast cells (Fig. 1a) because the longer *CTR2* mRNA isoform accumulates to higher levels in *upf1Δ* mutants than wild-type cells.

To test whether the change in accumulation of the longer, 2600nt *CTR2* mRNA isoform was due to the stabilization of the mRNA in *nmd* mutants, *CTR2* mRNA half-lives were measured in wild-type (*UPF1*) and *upf1Δ* mutant yeast cells. We used yeast cells that have the *rpb1-1* allele of RNA polymerase II (Nonet et al. 1987; Kebaara and Atkin 2009). The pattern of decay for the long *CTR2* mRNA isoform in both *upf1Δ* and *UPF1* yeast cells after arrest of transcription was complex in three independent experiments. An increase in mRNA levels was observed 3 min after inhibition of transcription, followed by a decrease, a slight increase and then gradual decrease of the long mRNA (Fig. 1b, c). This pattern of decay was not duplicated by the 900-bp (short) *CTR2* mRNA isoform. Furthermore, it is not due to ongoing transcription of the *CTR2* mRNA, because expression of the *CYH2* pre-mRNA and the short *CTR2* mRNA isoform is highest at time 0 followed by a steady decrease in mRNA abundance. This is consistent with a rapid and tight inhibition of RNA polymerase II activity. The short *CTR2* mRNA isoform was rapidly degraded in *UPF1* yeast cells with a half-life of 2.5 ± 1.3 (n = 3) min compared to 5.2 ± 0.8 (n = 3) min in *upf1Δ* yeast cells (Fig. 1b, c). The differences in the accumulation of the long *CTR2* isoform and the half-lives of the short *CTR2* mRNA isoform suggest that both *CTR2* mRNA isoforms are regulated by NMD, although this regulation is complex.

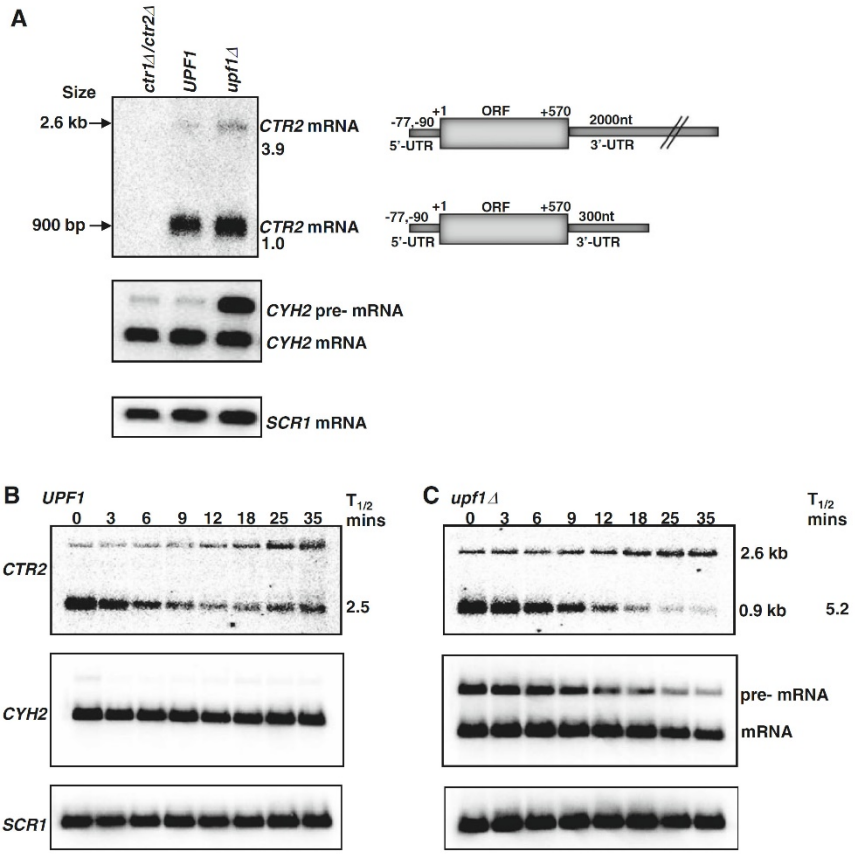

Figure 1. *Caption next page.*

Figure 1. Both *CTR2* mRNA isoforms are regulated by NMD, but only the longer *CTR2* mRNA isoform accumulates in *upf1Δ* mutant yeast cells. (**A**) Northern blot analysis of mRNAs encoded by *CTR2* in *ctr1Δ/ctr2Δ*, *UPF1* (W303a) and *upf1Δ* (AAY320) yeast cells. The relative accumulation in wild-type and *upf1Δ* mutant yeast cells and the schematics of the mRNA isoforms are shown to the right of the PhosphorImages. *CYH2* pre-mRNA is degraded by NMD and is used to confirm the NMD phenotype of the yeast cells. *SCR1* is a loading control because it is transcribed by RNA polymerase III and is not degraded by NMD, or involved in copper metabolism. (**B**) Northern blots of mRNA remaining versus time after transcription was inhibited using the temperature-sensitive allele of RNA polymerase II *rpb1-1* in *UPF1* (AAY334) yeast cells. mRNA half-lives for the short *CTR2* mRNA isoforms is shown to the right of the PhosphorImage. This mRNA isoform is degraded faster in wild-type, *UPF1* (AAY334) cells than *nmd* mutant, *upf1Δ* (AAY335) yeast cells (C). Middle PhosphorImage is of *CYH2* pre-mRNA and mRNA. *CYH2* pre-mRNA and mRNA are used as controls for the half-life experiments. The bottom PhosphorImage is of *SCR1*, which is used as a loading control. The mRNA half-life for the shorter *CTR2* mRNA isoform was calculated from three independent experiments. (**C**) Northern blot of mRNA remaining versus time as described in B, using *upf1Δ* (AAY335) yeast cells. mRNA half-life was also determined for the shorter (0.9 kb) *CTR2* mRNA isoform from three independent experiments and is shown to the right of the PhosphorImage.

CTR1 *and* **FRE1** *mRNAs, which also encode proteins involved in copper metabolism, do not accumulate in* **nmd** *mutants*

The observation that the differential accumulation and stability of the *CTR2* mRNA isoforms depends on NMD led us to examine the extent to which NMD regulates additional mRNAs involved in copper metabolism. If other mRNAs involved in copper metabolism are regulated by NMD, we expect these mRNAs to accumulate in *nmd* mutants. The mRNAs examined using northern blots were *CTR1* and *FRE1*. *CTR1* encodes a high-affinity copper transporter of the plasma membrane involved in cellular copper uptake. *FRE1* encodes both a ferric and cupric reductase of the plasma membrane. The *CTR1* mRNA showed no difference in accumulation in wild-type and nmd mutant yeast cells. In the conditions studied, the CTR1 gene encoded one mRNA of 1,700 bp that accumulated 1.0 ± 0.0 (n = 3)-fold in upf1D mutant compared to wildtype yeast cells (*UPF1*) (Fig. 2). Similarly, the *FRE1* mRNA showed no significant difference in accumulation in wild-type and *upf1Δ* mutant yeast cells as well. It accumulated 1.1 ± 0.1 ($n = 3$)-fold in *upf1Δ* yeast cells compared to *UPF1* yeast cells (Fig. 2).

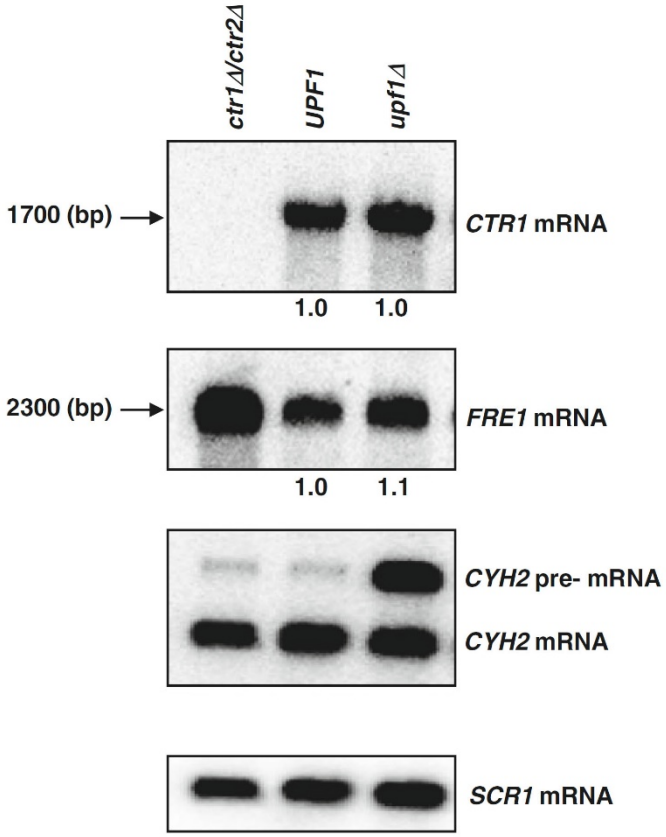

Figure 2. *CTR1* and *FRE1* mRNAs, which encode proteins involved in copper metabolism, do not accumulate in an NMD-dependent manner. Northern blot analysis of mRNAs encoded by *CTR1* and *FRE1* in *ctr1Δ/ctr2Δ*, *UPF1* (W303a) and *upf1Δ* (AAY320) yeast cells. The relative accumulation of the *CTR1* and *FRE1* mRNAs in wild-type (*UPF1*) and *nmd* (*upf1Δ*) mutant yeast cells are shown below the respective PhosphorImages. *CYH2* pre-mRNA and *SCR1* are used as controls as described in Figure 1.

In addition to examining the steady-state levels of *CTR1* and *FRE1* in wild-type and *nmd* mutant yeast strains using northern analysis, data generated from global expression studies identifying mRNAs regulated by the NMD pathway were examined. Johansson et al. (2007) identified potential direct substrates of the NMD pathway based on their association with Upf1p. Out of 41 mRNAs implicated in copper metabolism by the Saccharomyces Genome Database (SGD), 5 mRNAs were found by Johansson et al. (2007) to preferentially associate with Upf1p. These mRNAs include *CTR2*, *CTR3*, *MAC1*, *COX23*, and *CRS5*. The same study also found that *COX19*, *FRE2*, and *PCA1* mRNAs were down-regulated upon NMD reactivation (Table 2). In the NMD reactivation method, a factor required for NMD (*UPF2*) is under the control of a regulated promoter and yeast cells with this system have an inactive NMD pathway until placed in NMD-inducing conditions. When NMD is induced, mRNAs regulated by NMD are subsequently down-regulated (Johannsson et al.

2007). Guan et al. (2006) also identified *COX19* as a direct NMD substrate, while they identified *PCA1* and *FRE2* as indirect NMD substrates (Table 2). The potential regulation of eight mRNAs involved in copper metabolism in *S. cerevisiae* by NMD suggests that the NMD pathway is well involved in copper homeostasis in *S. cerevisiae*.

Table 2. Genes involved in copper utilization in *S. cerevisiae* and identified as NMD regulated by previous studies

Gene name	Protein function	Enriched through Upf1p-TAP[a]	Down-regulated on reactivation of NMD[b]	Fold change ratios (FCR)[c]
CRS5	Copper-binding metallothionein	3.13	—	—
MAC1	Copper-sensing transcription factor involved in regulation of genes required for high-affinity copper transport	2.04	—	—
CTR3	High-affinity copper transporter of the plasma membrane	2.50	—	—
CTR2	Copper transporter of the vacuolar membrane	2.70	—	—
COX23	Mitochondrial intermembrane space protein that functions in mitochondrial copper homeostasis, important for functional cytochrome oxidase expression	2.62	—	—
PCA1	Cadmium transporting P-type ATPase; may also have a role in copper and iron homeostasis	—	0.39	1.2 (indirect)
FRE2	Ferric reductase and cupric reductase reduce siderophore-bound iron and oxidized copper before uptake by transporters	—	0.41	0.9 (indirect)
COX19	Protein required for cytochrome *c* oxidase assembly	—	0.34	1.9 (Direct)

a. Average fold increase from Johansson et al. (2007).
b. Decrease in mRNA levels 60 min after NMD reactivation, from Johansson et al. (2007).
c. Direct and indirect targets as classified by Guan et al. (2006) based on their fold change ratios (FCR). The FCR was calculated by dividing the half-life of the mutant strain by the half-life in the wild-type strain. When the FCR was greater than or less than 1 the mRNA was categorized as a direct NMD target. Direct targets have significantly altered decay rates in yeast cells with a nonfunctional NMD pathway compared to yeast cells with a functional NMD pathway. Indirect targets have the same decay rates in yeast cells regardless of their NMD status.

Regulation of CTR2 *mRNA by NMD is physiologically significant*

The regulation of several natural mRNAs by the NMD pathway in yeast has physiological consequences (Dahlseid et al. 1998, 2003; Kebaara and Atkin 2009; Taylor et al. 2005). To determine the extent to which the NMD-mediated regulation of the *CTR2* mRNA isoforms is physiologically significant, the copper response of *nmd* mutants was tested on media with limiting or excess copper levels. Wild-type (*UPF1*) and *upf1Δ* yeast cells were spotted on medium containing excess or limiting amounts of copper and their growth was examined. There was no observable difference in growth rates between wild-type (*UPF1*) and

upf1Δ yeast cells grown with limiting copper levels. In contrast, *upf1Δ* yeast cells were more tolerant to excess copper (CM with 1 mM copper) compared to wild-type (*UPF1*) yeast cells (Fig. 3a). This phenotypic test implicates the NMD pathway in copper homeostasis. *nab4-7* yeast cells were used as a positive control because an *nab4-7* mutant has been shown to be more tolerant to 1 mM copper than wild-type yeast cells (Guisbert et al. 2007). The relative difference in growth between the *upf1Δ* or *nab4-7* and the isogenic wild-type cells (*UPF1* and *NAB4*, respectively) was similar in 1 mM copper.

Three core trans-acting factors are required for NMD-mediated degradation of mRNAs in yeast. These are the products of *UPF1*, *UPF2*, and *UPF3* genes. Mutation or deletions of any one, or combination of these genes have similar effects on mRNAs that are degraded by the NMD pathway (He et al. 1997). To test whether *upf2Δ* and *upf3Δ* mutant cells are similarly tolerant to copper, the phenotypic test was repeated with *upf2Δ* and *upf3Δ* yeast cells (Fig. 3b). All three *nmd* mutants were more tolerant to toxic copper levels compared to the wild-type (*UPF1*) yeast cells. These results demonstrate that the copper tolerance phenotype observed in *upf1Δ* strains was not limited to *upf1Δ* yeast cells but is a common property of *nmd* mutants in general (Fig. 3b).

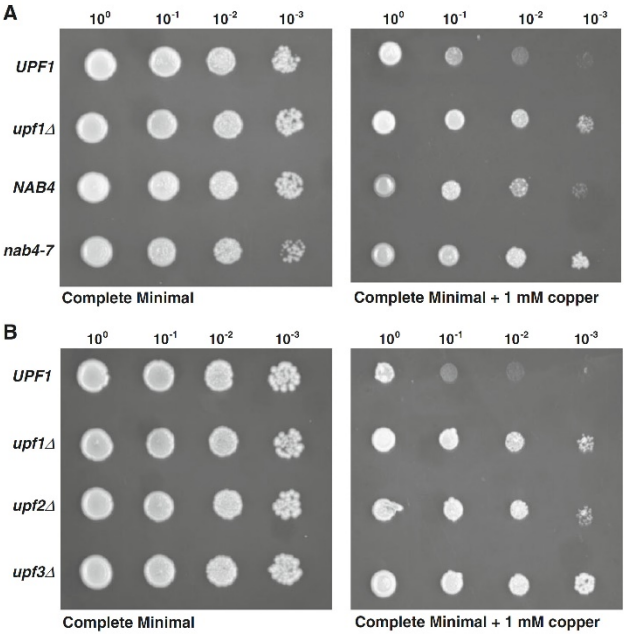

Figure 3. *nmd* mutant yeast cells are more tolerant to 1 mM copper than wild-type yeast cells. (A) *UPF1* (W303a), *upf1Δ* (AAY320), *HRP1/NAB4* (YAO417-1D), and *nab4-7* (YAO431-1C) yeast cells were grown to mid-log phase. Tenfold serial dilutions of the cells were spotted onto complete minimal medium (*left*) or complete minimal medium containing 1 mM copper (*right*) and incubated at room temperature for 4 days. *nab4-7* is a temperature-sensitive mutant that was previously shown to be resistant to toxic copper levels (Guisbert et al. 2007). (B) The experiment described in A was repeated with wildtype (*UPF1*, *UPF2*, *UPF3*; W303), upf1D (AAY320), *upf2Δ* (HFY1300), and *upf3Δ* (HFY861) yeast cells and incubated at 30°C for 3 days.

Copper tolerance of the* upf1Δ *mutant requires the presence of the* CTR2 *gene

The observations that *nmd* mutants produced *CTR2* mRNA isoforms that were regulated by NMD and *nmd* mutants were more tolerant to toxic copper levels led us to test the contribution of *CTR2* to the copper tolerance phenotype of *nmd* mutants. If *CTR2* plays a role in the copper tolerance observed in *nmd* mutants, we expect that copper tolerance will be lost in *nmd* mutants when *CTR2* is deleted from the yeast cells. To test the involvement of *CTR2* to the tolerance of 1 mM copper by *nmd* mutant cells, the *CTR2* gene was deleted in wild-type (*UPF1*) and *upf1Δ* mutant yeast cells. *UPF1*, *upf1Δ*, *UPF1ctr2Δ*, and *upf1Δctr2Δ* yeast cells were grown on either CM plates or CM plates containing 1 mM copper. As observed in Fig. 4a, the upf1D yeast cells were more tolerant to the excess levels of copper compared to wild-type (*UPF1*) yeast cells. Deletion of *CTR2* had no effect on the growth of wild-type yeast cells on 1 mM copper, consistent with previous observations (Guisbert et al. 2007). However, deletion of the *CTR2* gene rendered the *nmd* mutant yeast cells (*upf1Δ ctr2Δ*) as sensitive to 1 mM copper as the wild-type (*UPF1*) yeast cells (Fig. 4a).

Complementation of the *CTR2* gene in *nmd* mutant yeast cells (*upf1Δ ctr2Δ*) with an HA-epitope-tagged Ctr2p (Portnoy et al. 2001) recovered the copper tolerance phenotype of the *nmd* mutant yeast cells (*upf1Δ ctr2Δ*), although the copper tolerance phenotype was not to the same level as the *upf1Δ* mutant yeast cells (Fig. 4b). These results suggest that the resistance to toxic copper levels by the *upf1Δ* mutant is dependent on the presence of *CTR2*.

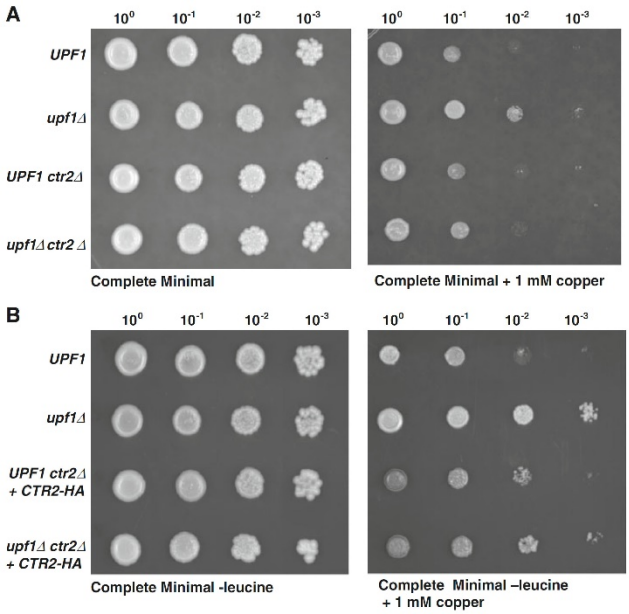

Figure 4. (A) *upf1Δ* mutant yeast cells tolerance to 1 mM copper is dependent on the presence of the *CTR2* gene. *UPF1* (W303), *upf1Δ* (AAY320), *UPF1 ctr2Δ* (AAY513), and *upf1Δ ctr2Δ* (AAY514) yeast cells were grown to mid-log phase, serially diluted, and spotted onto complete minimal medium (*left*) and complete minimal medium containing 1 mM copper (*right*) and incubated at 30°C for 3 days. (B) *UPF1* (W303), *upf1Δ* (AAY320) transformed with pRS315 (leucine vector), and *UPF1 ctr2Δ* (AAY513), and *upf1Δ ctr2Δ* (AAY514)

transformed with a HA-epitope-tagged *CTR2*. The transformants were grown as described in Figure 4a, spotted on selective medium lacking leucine (*left*) or selective medium lacking leucine containing 1 mM copper (*right*), and incubated for 3 days.

Discussion

Here, we tested the hypothesis that the alternatively 3′-end processed *CTR2* mRNA isoforms with atypically long 3′-UTRs could be potential NMD targets. We have demonstrated that, in the conditions tested, *CTR2* pre-mRNA produces two mRNA isoforms that are regulated by NMD; however, NMD mutants only accumulate the longer *CTR2* mRNA isoform (Fig. 1a). *CTR2* encodes for a copper transporter that controls the flux of copper into the vacuole. The NMD-dependent regulation of *CTR2* is important because *nmd* mutants are more tolerant of toxic copper levels than wild-type yeast cells (Fig. 3) and this copper tolerance by the *nmd* mutants requires the *CTR2* gene because deletion of the *CTR2* gene renders these mutants as sensitive to copper as wild-type yeast cells (Fig. 4). The regulation of *CTR2* mRNA by NMD may not be limited to *CTR2*. Other mRNAs involved in copper utilization may be regulated by NMD (Table 2), but this is not a universal occurrence among all mRNA involved in copper utilization, given that *CTR1* and *FRE1* mRNAs that are involved in copper metabolism are not regulated in an NMD-dependent manner (Fig. 2).

Both *CTR2* mRNA isoforms are regulated by the NMD pathway, but this regulation is complex. Only the long *CTR2* mRNA isoform accumulated to higher levels in *nmd* mutants. While the short *CTR2* mRNA isoform did not accumulate in *nmd* mutants, it was degraded more rapidly in wild-type yeast cells compared to *upf1Δ* mutants, which lack a functional NMD pathway (Fig. 1b, c). The regulation of the *CTR2* mRNA isoforms by the NMD pathway is not surprising because they both have relatively long 3′-UTRs (300 nt and 2.0 kb, respectively) and we have shown that atypically long 3′-UTRs target natural yeast mRNAs for decay by the NMD pathway (Kebaara and Atkin 2009). Long 3′-UTRs have also been shown to target natural mRNAs for NMD-mediated degradation in other organisms including humans (Eberle et al. 2008; Singh et al. 2008). This implies that NMD could similarly regulate alternatively 3′-end processed mRNAs in other eukaryotes.

The short *CTR2* mRNA should have accumulated to higher levels in *nmd* mutants than wild-type cells if NMD alone regulated its mRNA levels. Instead, this mRNA accumulated to the same level in wild-type and *nmd* mutant cells (Fig. 1a). This suggests that yeast cells have a mechanism to sense the short *CTR2* mRNA isoform levels. The short *CTR2* mRNA levels could be maintained by regulating either its transcription or processing. The involvement of Hrp1p/Nab4p in both 3′-end processing and NMD suggests that differential 3′-end processing could be responsible in part. However, differential 3′-end processing is unlikely to fully account for the effect observed because the increased levels of the longer *CTR2* in *upf1Δ* yeast cells are insufficient to account for both its stabilization and a change in 3′-end processing. *CTR2* mRNA levels may also be coordinated with transcription. Transcription has been linked to polyadenylation by previous studies (Richard and Manley 2009). Future studies are needed to determine the mechanisms responsible for maintaining the short *CTR2* mRNA isoform levels.

The long *CTR2* mRNA isoform had a complex mRNA decay pattern (Fig. 1b, c) that was similar to the one we observed previously for an mRNA encoded by a gene involved in stress response (Taylor et al. 2005). Inhibition of transcription using the temperature-sensitive *rpb1-1* allele of RNA polymerase II induces a general stress response. A study on the global heat stress response in *S. cerevisiae* determined that *CTR2* transcripts respond to heat stress (Castells-Roca et al. 2011). Following heat stress, some mRNAs, like the long *CTR2* mRNA isoform, may be stabilized and protected from NMD. Future studies are required to determine why the heat stress response is distinct between the two *CTR2* mRNA isoforms and the factors required for the response.

The *S. cerevisiae* NMD pathway is involved in copper homeostasis because *nmd* mutants are tolerant to toxic copper levels. *CTR2* is important for this tolerance to copper, since deletion of *CTR2* restores copper tolerance of the *nmd* mutants to wild-type levels in the conditions we tested. NMD may also regulate the expression of other genes involved in copper homeostasis. *CTR3*, *MAC1*, *COX23*, *PCA1*, *FRE2*, *COX19*, and *CRS5* are mRNAs that encode proteins involved in copper homeostasis and are potentially regulated in an NMD-dependent manner (Johansson et al. 2007; Guan et al. 2006; Table 2). Although other mRNAs involved in copper metabolism may be NMD targets, deletion of *CTR2* eliminated the copper resistance phenotype of *nmd* mutants. Therefore, up-regulation of *CTR2* in the *nmd* mutants is critical for copper resistance. However, we also showed that not all mRNAs involved in copper utilization are regulated by NMD. *CTR1* and *FRE1* mRNAs, encoding proteins involved in copper homeostasis, are not regulated in an NMD-dependent manner (Fig. 2). NMD also regulates genes involved in the homeostatic regulation of other metals. In fact, NMD is involved in the homeostatic regulation of magnesium (Johansson and Jacobson 2010).

The physiological consequences resulting from the degradation of natural mRNAs by NMD in yeast and other organisms are beginning to be recognized. The degradation of natural *CTR2* mRNA by NMD causes yeast cells defective in the NMD pathway to tolerate toxic copper levels. This tolerance to copper by *nmd* mutants requires the presence of the *CTR2* gene. The underlying physiology as to why *nmd* mutants are more tolerant to toxic copper levels compared to wild-type yeast cells needs to be further examined. One hypothetical explanation is that *nmd* mutants tolerate toxic copper due to sequestration of excess intracellular copper.

Additional physiological consequences that result from the deficiency of the NMD pathway have been reported. In yeast, *nmd* mutants have altered chromosome structure and grow at reduced rates on some nonfermentable carbon sources (Altamura et al. 1992; Dahlseid et al. 1998, 2003; Lew et al. 1998). We have shown that *nmd* mutants are sensitive to Calcofluor White (Kebaara and Atkin 2009). *CPA1* mRNA degradation by NMD is regulated by arginine, where addition of arginine causes ribosomal stalling at the termination codon of an upstream open reading grame (uORF), leading to the destabilization and NMD-mediated degradation of *CPA1* mRNA (Gaba et al. 2005). In addition, thiamine starvation has been shown to induce alternative transcripts involved in thiamine metabolism and some of these transcripts are degraded in an NMD-dependent manner (Johansson et al. 2007).

The regulation of natural mRNAs by the NMD pathway is widespread. Degradation of natural mRNAs has been observed in organisms ranging from yeast to humans. NMD affects 5–10% of the *S. cerevisiae* transcriptome (Lelivelt and Culbertson 1999; He et al. 2003; Guan et al. 2006; Johansson et al. 2007) *D. melanogaster* (Rehwinkel et al. 2005) and humans (Mendell et al. 2004; Whittmann et al. 2006).

NMD-mediated regulation of alternatively 3′-end processed transcripts with atypically long 3′-UTRs may occur in other pre-mRNAs besides *CTR2*. This regulation has the potential to be responsive to growth environment if the growth conditions affect alternative 3′ cleavage and polyadenylation utilization. Determining the alternative mRNA isoforms produced in different growth conditions will further our understanding of the role NMD plays in regulating the expression of alternatively 3′-end processed mRNAs with atypically long 3′-UTRs.

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