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mRNAs involved in copper homeostasis are regulated by the nonsense-mediated mRNA decay pathway depending on environmental conditions

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ABSTRACT

The nonsense-mediated mRNA decay pathway (NMD) is an mRNA degradation pathway that degrades mRNAs that prematurely terminate translation. These mRNAs include mRNAs with premature termination codons as well as many natural mRNAs. In *Saccharomyces cerevisiae* a number of features have been shown to target natural mRNAs to NMD. However, the extent to which natural mRNAs from the same functional group are regulated by NMD and how environmental conditions influence this regulation is not known. Here, we examined mRNAs involved in copper homeostasis and are predicted to be sensitive to NMD. We found that the majority of these mRNAs have long 3′-UTRs that could target them for degradation by NMD. Analysis of one of these mRNAs, *COX19*, found that the long 3′-UTR contributes to regulation of this mRNA by NMD. Furthermore, we examined an additional mRNA, *MAC1* under low copper conditions. We found that low copper growth conditions affect NMD sensitivity of the *MAC1* mRNA demonstrating that sensitivity to NMD can be altered by environmental conditions. *MAC1* is a copper sensitive transcription factor that regulates genes involved with high affinity copper transport. Our results expand our understanding of how NMD regulates mRNAs from the same functional group and how the environment influences this regulation.

1. Introduction

The nonsense-mediated mRNA decay pathway (NMD) is an mRNA degradation pathway that degrades select mRNAs. Specifically, NMD degrades mRNAs that prematurely terminate translation due to premature termination codons (PTC) or other features. PTCs can arise as a result of improper pre-mRNA processing or random mutations. NMD also has other roles and has been shown to degrade many natural transcripts (Deliz-Aguirre et al., 2011; Guan et al., 2006; He et al., 2003; Johansson et al., 2007; Kebaara and Atkin, 2009; Lelivelt and Culbertson, 1999). The extent and reason for the targeting of most of these natural mRNAs is generally unknown.

The NMD pathway is highly conserved in all tested eukaryotes from yeast to humans, and serves as an important regulator of gene expression and cellular function. In *S. cerevisiae nmd* mutants, 5–10% of the transcriptome is affected (Guan et al., 2006; He et al., 2003; Johansson et al., 2007). Further research in the fruit fly *Drosophila melanogaster* and humans indicates that similar percentages of the respective transcriptomes are affected when NMD is inactivated (Mendell et al., 2004; Rehwinkel et al., 2005; Wittmann et al., 2006). Three core trans-acting factors are required for a functional NMD pathway in all eukaryotes. These are the up-frameshift proteins Upf1p, Upf2p and Upf3p. Inactivation of any one of these three proteins selectively stabilizes mRNAs that are regulated by the pathway (He et al., 1997).

NMD targets can be regulated by the pathway directly or indirectly. Direct NMD targets have significantly altered decay rates in cells with a functional NMD pathway compared to their decay rates in cells with a nonfunctional NMD pathway. On the other hand, indirect NMD targets have similar decay rates in cells with a functional or non-functional NMD pathway. Initially, it was perceived that all direct targets of the pathway contain a PTC. However, with the ever increasing number of natural mRNAs that are degraded by the pathway, it is apparent that other targeting mechanisms exist. Previous studies in *S. cerevisiae* have identified features that target natural mRNAs to the pathway. These features include mRNAs subject to leaky ribosomal scanning (Welch and Jacobson, 1999), a translated upstream open reading frame (uORF) (Gaba et al., 2005), −1 ribosomal frameshifts (into an alternative reading frame) (Belew et al., 2011, 2014), inefficiently spliced pre-mRNAs (He et al., 1993), and mRNAs with atypically long 3′-untranslated regions (UTRs) (Deliz-Aguirre et al., 2011; Kebaara and Atkin, 2009; Parker, 2012; Peccarelli and Kebaara, 2014b;

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Rebbapragada and Lykke-Andersen, 2009; Schweingruber et al., 2013). One specific targeting mechanism of interest is the presence of a long 3′-UTR on an mRNA (Deliz-Aguirre et al., 2011; Kebaara and Atkin, 2009; Rebbapragada and Lykke-Andersen, 2009). In *S. cerevisiae*, mRNA 3′-UTRs are fairly short and typically range in size from 50 to 200 nts with a median size of ~121 nts (Graber et al., 2002). In general, mRNAs with 3′-UTRs that are 350 nt or longer are considered atypically long and are likely to be regulated by NMD (Deliz-Aguirre et al., 2011; Kebaara and Atkin, 2009).

Interestingly enough, some mRNAs produce different isoforms of the same mRNA that vary in their 3′-UTR lengths. Transcripts that vary in the 3′-UTR length can be produced as a result of alternative 3′-end processing (Deliz-Aguirre et al., 2011; Guisbert et al., 2007; Kebaara and Atkin, 2009; Pelechano et al., 2013). Additionally, mRNA 3′-end processing is sometimes sensitive to growth conditions, and differing conditions can produce mRNA isoforms of different lengths (Kim Guisbert et al., 2007). Thus, genes that produce multiple transcripts that vary in their 3′-UTR lengths may generate one form of the transcript that is degraded by NMD while the other may not be regulated by the pathway.

NMD can regulate gene expression by controlling mRNA levels in response to environmental stimuli and stressors. Many different stressors exist including copper (Hodgins-Davis et al., 2012). Copper is a micro-nutrient that is essential for a number of cellular functions (Deliz-Aguirre et al., 2011; Hodgins-Davis et al., 2012). Specifically, copper serves as a cofactor in superoxide anion detoxification, iron metabolism, and mitochondrial oxidative phosphorylation. Even though copper is required for normal cellular function, copper in its free form is extremely toxic to the cell (Kim et al., 2013). Thus, the copper concentration inside of the cell must remain around one free copper molecule per individual cell to avoid toxicity (Hodgins-Davis et al., 2012). In order to avoid cell death due to copper induced toxicity, cells have developed a number of mechanisms to maintain copper homeostasis. These mechanisms include copper compartmentalization and sequestration. Specific proteins involved in these mechanisms have been identified; however, the actual mechanisms involved in copper homeostasis are still under investigation (Deliz-Aguirre et al., 2011; Kim et al., 2013). Interestingly enough, yeast cells with an inactive NMD pathway are more tolerant of high copper levels. Further analysis of this copper tolerance phenotype revealed that the enhanced copper tolerance of *nmd* mutants cells is partly due to the sequestration of copper into the vacuole (Deliz-Aguirre et al., 2011; Wang et al., 2013).

It is important to understand how NMD regulates natural mRNAs involved in specific cellular processes and how this regulation is altered in changing environmental conditions. Here, we used copper homeostasis in *S. cerevisiae* as a model. Global expression profiling studies identified *CTR2*, *CTR3*, *MAC1*, *COX23*, *CRS5*, *PCA1*, *FRE2*, and *COX19* mRNAs as potential NMD targets (Table 1) (Guan et al., 2006; Johansson et al., 2007). The proteins encoded by these eight mRNAs are involved in various aspects of copper homeostasis. Here, we examined the regulation of these mRNAs by NMD. The majority of these mRNAs (*MAC1*, *PCA1*, *CTR2*, *COX23*, *COX19*, and *FRE2*) were found to have atypically long 3′-UTRs that could contribute to their degradation by NMD (Deliz-Aguirre et al., 2011; Kebaara and Atkin, 2009; Peccarelli et al., 2014). This study shows how NMD regulates mRNAs from the same functional group, and also demonstrates that the presence of multiple NMD inducing features may increase the sensitivity of mRNAs to the pathway. Lastly, we found that low copper growth conditions lead to alternative forms of the *MAC1* mRNA that vary in the length of the 3′-UTR. These alternative forms of the mRNA have altered sensitivity to NMD. This result shows that environmental conditions can influence alternative 3′-end processing, and also NMD sensitivity of specific mRNAs to the pathway.

2. Materials and methods

2.1. Yeast strains

The strains and genotypes of *Saccharomyces cerevisiae* used in this study are listed in Table S1. Standard techniques were used to grow and maintain all of the yeast strains (Ausubel et al., 1998).

2.2. DNA methods

Plasmids used in this study were maintained in *E. coli* DH5α. *CYC1-COX19 3′UTR* fusion construct was generated using cloning free PCR by fusing PCR products containing the *CYC1* 5′-UTR and ORF to 700 nts from the *COX19 3′-UTR*. *COX19-CYC1 3′UTR* fusion construct was generated by fusing PCR products containing the *COX19 5′-UTR* and ORF to 350 nts from the *CYC1* 3′-UTR. The *CYC1-COX19 3′UTR* and *COX19-CYC1 3′UTR* fusion DNA products were inserted into TOPO-TA cloning vector according to the manufacturer's instructions. All plasmids were sequenced to verify sequences and ensure that the correct fusion constructs were generated. *CYC1-COX19* 3′-UTR was digested with *BamHI* and *NotI* before ligation to the yeast vector pRS425. *COX19-CYC1* 3′-UTR was digested with *BamHI* and *XhoI* before ligation into pRS425.

2.3. RNA methods

Yeast total RNA was used for accumulation and half-life northerns. The total RNA was extracted using the hot phenol method from yeast strains harvested at mid-log phase (Kebaara et al., 2003). Half-life experiments utilized yeast strains with the *rpb1-1* background. *rpb1-1* is a temperature sensitive allele of RNA polymerase II (Nonet et al., 1987). When yeast cells harboring the *rpb1-1* allele are subjected to the non-permissive temperature of 39 °C, mRNAs are no longer transcribed. After transcription inhibition, yeast cells were harvested at different time points, from 0 to 35 min. Total RNA was then extracted and equivalent concentrations of the total RNA (15 μg) were run on a 1.0% agarose-formaldehyde gel for both accumulation and half-life northerns (Peccarelli and Kebaara, 2014a). The RNA was then transferred to a GeneScreen Plus® (PerkinElmer, Boston, MA) nitrocellulose membrane. Transfers were done using the capillary blot transfer protocol from the NorthernMax™ Complete Northern Blotting kit (Life Technologies, Grand Island, NY). All northern blots were probed with oligolabeled DNA probes. Each probe was prepared by PCR or through the digestion of DNA fragments from plasmids. The oligolabeled DNA probe fragments were labeled with [α-^{32}P] dCTP using the RadPrime DNA Labeling System (Life Technologies, Grand Island, NY). The northern blots were phosphorImaged™ using a Typhoon Phosphorimager (Amersham Pharmacia Biotech, Inc.). *SCR1* RNA was used as a control to normalize all mRNA levels. *SCR1* is an RNA polymerase III transcript and is NMD insensitive. *CYH2* pre-mRNA was also used as a control. *CYH2* pre-mRNA is a known target for NMD-mediated degradation and was used to confirm the NMD phenotype of the yeast strains (He et al., 1993). Northerns were quantified using ImageQuant software. Sigmaplot 2000, Version 6.10 software was used to calculate half-lives by graphing $\log_{10}$ of the percent mRNA remaining versus time (SPSS Science, Chicago, IL).

2.4. 3′ RACE

3′ RACE was performed using the 3′ RACE System for Rapid Amplification of cDNA Ends kit (Life Technologies, Grand Island, NY) and was done as described in Kebaara et al., 2012 (Kebaara et al., 2012). In detail, 5 μg of yeast total RNA from wild-type or