Title: . The role of Upf1p phosphorylation on nonsense suppression, and the interaction of Upf1p with other factors in *Saccharomyces cerevisiae*.

A. Introduction

One third of inherited human genetic diseases are caused by frame shift or nonsense mutations that generate premature termination codons (PTCs) in messenger RNAs (mRNAs) (Gonzalez, Wang and Peltz 2001). Transcripts containing PTC's are targeted for rapid turnover by a RNA surveillance mechanism known as nonsense-mediated mRNA decay (NMD). Recent studies have demonstrated an intimate relationship between the NMD pathway and cancer. The long-term goal of our work is to understand the mechanism of NMD and how it relates to cancer.

Many of the genes essential for NMD were first identified in *Saccharomyces cerevisiae*. Orthologues of most of these yeast genes have since been identified in *Caenorhabditis elegans, Drosophila melanogaster,* and mammals (Conti and Izaurralde 2005), demonstrating that the NMD pathway is a highly conserved pathway. Two genes crucial for NMD are UPF1 and UPF2. UPF1 (a.k.a. Upf1p in yeast) possesses RNA binding and RNA-dependent ATPase/helicase activities. In *C. elegans* and humans, UPF1 has been shown to undergo a cycle of phosphorylation and dephosphorylation essential for NMD (Conti and Izaurralde 2005); (Yamashita, Kashima and Ohno 2005); (Pal, Ishigaki, Nagy 2001); (Yamashita, Ohnishi, Kashima 2001). UPF2 encodes a central adaptor protein (called Upf2p in yeast) that appears to nucleate UPF1 and other NMD factors in a super-complex that triggers mRNA decay (Chang, Imam and Wilkinson 2007). We have recently shown that both yeast Upf1p and Upf2p are phosphoproteins (Wang, Cajigas, Peltz 2006).

In addition to degrading aberrant mRNAs, UPF1 regulate the levels of a large number of normal endogenous mRNAs (Culbertson and Neeno-Eckwall 2005); (Guan, Zheng, Tang 2006); (Lelivelt and Culbertson 1999); (He and Jacobson 2001); (Chan, Huang, Gudikote 2007). While the physiological consequences of this regulation is not yet clear, it is likely to be responsible for some of the other known functions of these factors. For example, human UPF1 has been shown to provide protection against genotoxic stress (Azzalin and Lingner 2006). In addition, UPF1 have been shown to be necessary for oxidative-stress responses in fission yeast (Rodriguez-Gabriel, Watt, Bahler 2006). The emerging roles of UPF1 in oxidative and genotoxic stress responses suggest they may also regulate tumor progression.

This has major implications, as it means that we can use *S. cerevisiae* as a model system to study the role of phosphorylation and dephosphorylation in regulating the NMD surveillance pathway. In particular, we intend to **elucidate the role of Upf1p phosphorylation in the NMD pathway**. **This RNA surveillance pathway is important, as it greatly reduces the synthesis of truncated proteins, some of which will have deleterious gain-of-function or dominant negative effects, including promotion of tumor growth (Chang, Imam and Wilkinson 2007; Holbrook, Neu-Yilik, Hentze 2004; Perrin-Vidoz, Sinilnikova, Stoppa-Lyonnet 2002).** We will use a combination of molecular, biochemical, and genetic approaches to accomplish these goals. These studies will provide insights into the regulation of the NMD pathway, both in yeast and in more complex eukaryotes. Overall, a better understanding of the NMD pathway should allow us to modulate mRNA stability and translation, and thereby regulate gene expression in the future to ameliorate cancer and other human genetic disorders caused by nonsense

B. Background and Significance

NMD is a conserved RNA surveillance pathway found in a wide variety of organisms, including *S. cerevisiae*, *C. elegans*, *Drosophila melanogaster*, and mammals (Conti and Izaurralde 2005; Culbertson and Leeds 2003). Nonsense mutations can cause many genetic disorders, including cystic fibrosis, hemophilia, β-thalassemias, Duchenne muscular dystrophy, Ulrich's disease and Marfan syndrome (Frischmeyer and Dietz 1999; Holbrook, Neu-Yilik, Hentze 2004; McKusick and Amberger 1994; Usuki, Yamashita, Higuchi 2004). For some diseases where only a small proportion of the functional protein is produced, patients suffer serious disease symptoms. In other cases, NMD insufficiently down-regulates a PTC-bearing mRNA that encodes a deleterious truncated protein. So, modestly increasing the expression of the functional protein can greatly reduce the severity or

eliminate the earlier mentioned disorders (Usuki, Yamashita, Higuchi 2004), while a means to boost NMD would provide therapeutic benefit in the later cases. Thus, modulation of the NMD pathway and translation termination clearly has the potential to be a very powerful approach to treat diseases caused by nonsense or frame-shift mutations.

The NMD RNA surveillance pathway. The NMD pathway operates in all eukaryotic cells that have been examined to date. Evidence suggests that NMD evolved as a quality-control mechanism to ensure that translation termination occurs at the appropriate location within the mRNA, preventing the synthesis of potentially deleterious proteins. NMD also regulates transcripts from wild-type genes using different sets of factors to regulate different subsets of transcripts. Substrates for this pathway include: (a) transcripts with premature termination codons (PTCs), (b) unspliced or aberrantly spliced mRNAs, (c) pre-mRNAs containing introns that have reached the cytoplasm, (d) mRNAs with upstream open reading frames (uORFs) and (e) mRNAs with extended 3' UTRs.

Two models have been proposed for the NMD pathway in eukaryotes (reviewed in Jacobson and Izaurralde 2006). The surveillance complex model posits that the degradation machinery discriminates between premature and normal termination codons using a downstream sequence element (DSE) located 3' of the premature termination codon (Zhang, Ruiz-Echevarria, Quan 1995). An alternative model proposes that mRNA decay is activated by the intrinsically aberrant nature of premature termination (Hilleren and Parker 2003). According to this *faux* 3'-UTR model, the DSE is a defective (*faux*) 3'-untranslated region (3'-UTR) that promotes mRNA decay because it lacks a termination regulatory factor(s) normally present on a legitimate 3'-UTR.

Various components essential for the activity of NMD have been identified. Mutation in any of these genes results in a stabilization of PTC-containing transcripts without affecting the decay rates of wild-type mRNAs. In yeast, the Upf1p, Upf2p, and Upf3p proteins have been the most extensively investigated. Upf1p possesses RNA binding and RNA-dependent ATPase/helicase activities. Upf3p is a basic protein harboring several nuclear localization signals and nuclear export sequences (Shirley, Ford, Richards 2002). Single or multiple deletions of the *UPF* genes encoding these three proteins have virtually identical effects on NMD, consistent with the notion that these proteins function in a complex.

The role of phosphorylation in NMD. **In higher eukaryotes, phosphorylation of UPF1 is crucial for NMD (reviewed in Wilkinson, 2003).** This was initially revealed by genetic and biochemical studies in *C. elegans*, suggesting that its UPF1 orthologue, SMG2, must undergo cycles of phosphorylation and dephosphorylation to function in NMD (Page, Carr, Anders 1999). **Subsequent studies have indicated that phosphorylation of the nematode UPF1 is mediated by SMG-1, a novel PI3-K-related protein kinase (Grimson et al., 2004).** The dephosphorylation of **UPF1** is carried out by a protein complex formed by the NMD proteins SMG-5 and SMG-7 and protein phosphatase 2A (PP2A) (Anders, Grimson and Anderson 2003).

In humans, UPF1 is phosphorylated to its highest extent when polysome-associated and to its lowest extent when in the ribosome-free state (Pal et al., 2001). As in *C. elegans*, mammals use the PI3-K-related protein kinase, SMG-1, to phosphorylate UPF1 (Yamashita, Kashima and Ohno 2005; Yamashita, Ohnishi, Kashima 2001) and a complex containing SMG-5 and SMG-7 to dephosphorylate UPF1 (Chiu, Serin, Ohara 2003). Depletion or inhibition of SMG-1, SMG-5, or SMG-7 inhibits NMD, suggesting that a cycle of UPF1 phosphorylation and dephosphorylation mediated by these molecules is essential for NMD (Ohnishi, Yamashita, Kashima 2003).

C. Specific Aim

While it is becoming clear that NMD is a physiological relevant pathway, we still know little about how NMD is regulated. This proposal addresses this issue, focusing on the role of phosphorylation and dephosphorylation in regulating the NMD pathway in *S. cerevisiae*. **In particular, we hypothesize that the Upf1p protein undergoes a cycle of phosphorylation and dephosphorylation that is necessary to**

activate the NMD surveillance pathway. In support of our hypothesis, a recent study conducted by our groups demonstrated that yeast Upf1p is a phosphoproteins (Wang, Cajigas, Peltz 2006).

We propose that *S. cerevisiae* will be an efficient system to elucidate the role of phosphorylation in NMD because of (i) its powerful genetics, (ii) the sophisticated biochemical approaches that have been worked out in this organism, and (iii) the large amount already known about the *cis*- and *trans*-acting factors involved in NMD in yeast (for review see: (Beelman and Parker 1995) and (Mendell, Sharifi, Meyers 2004); (Decker and Parker 2002); (Baker and Parker 2006); (Gonzalez, Wang and Peltz 2001). We believe that these advantages of *S. cerevisiae* NMD, should allow us to make significant progress in understanding the role of UPF1 and phosphorylation in NMD. We propose that using yeast to understand fundamental aspects of NMD will allow much more rapid progress than could be achieved in mammalian cells. **We plan to use this knowledge to elucidate the molecular pathways involved in NMD in higher eukaryotes.** The long-term goal of our work is to identify means to upregulate the NMD RNA surveillance pathway to treat cancer and other human genetic disorders caused by nonsense and frame shift mutations. In addition, because UPF1 is also involved in genotoxic and oxidative stresses, our results may bring to light approaches to modulate these stress responses as a means to ameliorate inflammatory diseases and cancer.

The specific goal of this research proposal is to elucidate the role of Upf1p and phosphorylation in the NMD pathway.

D. Research Designs and Methods

The purpose of this proposal is to understand the role of phosphorylation in NMD. One goal is to address a major unanswered question in the field: why is phosphorylation and dephosphorylation of the Upf proteins required to trigger NMD. Importantly, we found that phosphorylation of Upf2p is essential for interaction with NMD proteins and for the NMD pathway itself. We plan to now study this issue in more depth being our aim to elucidate the functional role of Upf1p phosphorylation in NMD.

Aim I. Elucidate the role of Upf1p phosphorylation in NMD. The goal of this Aim is to understand the functional significance of UPF1 phosphorylation. We will determine whether Upf1p phosphorylation is essential for NMD and translation termination. These studies are critical in order to understand both the functional role of UPF1 phosphorylation and why a cycle of UPF1 phosphorylation and dephosphorylation drives NMD.

***Effect of Upf1p phosphorylation on the stability of PTC-containing mRNAs*.**
We already have identified phosphorylation sites on Upf1p. We will proceed to examine the function of Upf1p mutants that lack these specific phosphorylation sites. These experiments, will involve mutating the phosphorylated residues to Ala and Asp, which mimic non-phosphorylated and constitutively phosphorylated forms of Ser/Thr, respectively. We propose to further dissect their role in the activity of NMD by analyzing mutants that we have constructed.

We will examine the ability of these mutants to function in NMD by measuring steady-state mRNA levels and mRNA turnover rates of PTC-containing reporter transcripts, as previously described (Wang et al., 2006). To do this, we will transform *upf1Δ* strains with the different *upf1* mutant alleles of interest. To assay for NMD activity, a PTC-containing mini-*PGK1* allele will also be transformed into these isogenic strains. This mini-*PGK1* transcript is a very sensitive substrate of the NMD pathway; we previously showed it is rapidly degraded in wild-type cells but stabilized in strains defective in this turnover mechanism (Gonzalez et al., 2000). Cells will be grown to mid-logarithmic phase and transcription will be inhibited by the addition of the inhibitor thiolutin (a gift from Pfizer, Groton, CT). Cell aliquots will be removed at different times following the addition of the inhibitor. Total RNA will be isolated and mRNA half-lives will be determined by Northern-blot analysis as previously described (Gonzalez et al., 2000).

We predict that there will be at least two classes of Ala-substitution mutants: (i) those that rescue NMD and (ii) those that do not (or poorly) rescue NMD. Those in the latter category can be tested for defects in other biochemical activities. We predict that some Asp-substitution mutants will also have enhanced NMD. If so, we

will interpret this as indicating that the phosphorylation of the mutated residue promotes NMD, as Asp mimics constitutively phosphorylated Ser or Thr residues. Alternatively, if an Asp-substitution mutant has reduced NMD activity (as we previously showed for Ser 32 in Upf2p (Wang et al., 2006), we will interpret this result as suggesting that phosphorylation of that particular residue has a negative (regulatory) function or that the residue must undergo a cycle of phosphorylation and dephosphorylation to elicit NMD. We will perform further biochemical analysis of Asp-substitution mutants with either enhanced or reduced NMD activity, as described below.

E. Preliminary Studies

Yeast Upf1p is a phosphoprotein. The discovery that yeast Upf2p is a phosphoprotein prompted us to assess whether the same is also true of yeast Upf1p. Our data indicates Upf1p is a phosphoprotein. Because *C. elegans* and mammalian UPF1 are also phosphorylated (Page, Carr, Anders 1999; Pal, Ishigaki, Nagy 2001; Wilkinson 2003; Yamashita, Ohnishi, Kashima 2001), this indicates that UPF1 phosphorylation is a conserved event in eukaryotic organisms.

Identification of specific phosphorylation sites in yeast Upf1p using mass spectrometry (MS) analysis. Human UPF1 has been shown to be phosphorylated at specific sites (Yamashita, Ohnishi, Kashima 2001). To assess if these sites are conserved in yeast Upf1p, sequence alignments were performed with other known UPF1 orthologues using Biology Workbench (http://workbench.sdsc.edu/). Therefore, we used the NetPhos Internet tool (http://www.cbs.dtu.dk/services/NetPhos/) to predict targets of phosphorylation within Upf1p. These results showed various potential phosphorylation sites in Upf1p that can act as substrates for different types of kinase families. Twenty (20) different serine, threonine and tyrosine residues in Upf1p were predicted as targets for more than one protein kinase family, and five (5) of these amino acids were highly conserved across species when compared to Upf1p orthologues in *D. melanogaster, C. elegans* and humans.

To determine whether the putative phosphorylation sites obtained from the bioinformatics analysis are indeed phosphorylated, we purified Upf1p from *upf1-delta* yeast strains transformed with a plasmid encoding a Flag-tagged *UPF1* allele and then subjected it to in-gel digestion and mass spectrometry analysis. The Mass Spectrometry analysis identified various phosphorylation sites in yeast Upf1p. Interestingly, some of the phosphorylated Upf1p peptides obtained from this analysis are conserved in yeast, *D. melanogaster, C. elegans* and humans.

F. Future Experiments

Once we determine the effect of phosphorylation in the stability of the PTC containing mRNA we will be moving forward to other steps on the dissection of the NMD pathway. **We will be determining the effects of Upf1p phosphorylation on Upf1's interactions with other NMD components like eukaryotic release factor (eRF3) and Hrp1p, and the other Upf proteins. We also want to determine the role of Upf1p phosphorylation in translation termination and in nonsense suppression.**

All together, these studies will help us understand how phosphorylation of Upf1p regulates the assembly of the surveillance complex and why a cycle of Upf1p phosphorylation and dephosphorylation is required for NMD. We consider that these studies are very important in order to understand the functional role of Upf1p phosphorylation in yeast and higher eukaryotes. Our recent results obtained from the mass spectrometry analysis indicating that several phosphorylated sites in yeast Upf1p are conserved across different species make our studies even more significant. We are convinced that the rapid elucidation of these fundamental issues in yeast will allow us to formulate specific hypotheses regarding NMD in higher eukaryotes that we can rapidly investigate in the future.

G. Time Table

This project will be developed during the two months of summer 2008. The time plan that summarizes the expected development of this research is summarized in the next table.

Detailed Time Table

Week	1	2	3	4	5	6	7	8
Yeast cultures	X	X	X	X	X			
PCR mutations		X	X	X	X			
Sequencing				X	X			
Transformation					X	X		
Northern and Western Blots					X	X	X	
Writing/Presentation							X	X

H. Budget

This research project is part of an effort for the development of committed young college professor that want to learn by practicing the basics of competitive research. The facilities that the researcher will be using are part of the Center for Biological Studies Building (Julio Garcia Diaz) at the University of Puerto Rico, Rio Piedras Campus. Specific research materials and associated cost for the project are summarized in the following table.
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Detailed Budget Description

Item	**Company**	**Cost**	**Observation**
PCR Mutation Kit	Stratagene	350	Include Pfu Enzyme
Primers for DNA sequencing	Primer One Corp	300	2 pairs
Sequencing	Gene Sp Corp	35/ sequence	18 sequences
ECL Western Blot kit	Amersham	400	Chemioluminiscence
P32 for Northern Blot	Amersham	225	Probes for Northern
Micropipettes	Raining	175	3