Role of Ime4 Protein in PHO Regulon of S. cerevisiae.

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Role of Ime4 Protein in PHO Regulon of S.cerevisiae.

Jenisha Ghimire
Role of Ime4 Protein in PHO Regulon of S.cerevisiae.

A Thesis

Submitted to the Graduate Faculty of the University of New Orleans in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

by

Jenisha Ghimire

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Abstract

In the yeast Saccharomyces cerevisiae, the IME4 methyltransferase, interacts genetically with methyl binding protein, Pho92, to affect the expression of PHO regulon target genes. Cells mutant in IME4 or PHO92 show increases in the RNA abundance of PHO regulon target genes. The increase in the RNA abundance of the PHO regulon target genes is not additive in the cells double mutant in IME4 and PHO92. Hence, Ime4 and Pho92 interact in a single pathway in PHO regulon. Surprisingly, cells overexpressing IME4 and MUM2 shows increase in some PHO regulon target genes, indicating that IME4 affects the PHO regulon target genes through multiple mechanisms in different conditions. A promoter swap experiment revealed that one of the PHO regulon mRNAs that codes for phosphatase, PHO5, is a direct target of Ime4. Further experiments are required to examine whether the same is true for all PHO regulon mRNAs.

N6-methyladenosine, m6A, Methyltransferase, Ime4, Pho regulon, Pho92
Introduction

Post Transcriptional Modification of mRNA

In eukaryotes, transcription and translation occur in separate compartments, the nucleus and cytoplasm respectively. This separation allows the pre-mRNA in eukaryotes to go through extensive post-transcriptional modification, including 5’ capping of the pre mRNA by 7-methylguanosine, 3’ polyadenylation, and removal of introns and ligation of exons to give rise to final transcripts. These processing reactions occur during or shortly after transcription. The second most common form of such post transcriptional modification in eukaryotic RNA is adenosine methylation by methyltransferase proteins to produce N6-methyladenosine (m6A) (Fig 1).

The methylation of mRNA occurs in the mRNA of some viruses and virtually all eukaryotes, including yeast, humans, Drosophila and Arabidopsis thaliana. The relative concentration of m6A varies with species and tissues but has been estimated to occur in approximately half of the total transcripts (Bodi et al. 2010). The group of enzymes that have the ability to methylate their substrate are collectively called methyltransferases. The first pre-mRNA adenosine methyltransferase was isolated from HeLa cell extracts (Bokar et al. 1997). The methyltransferase was named MT-A70. MT-A70 in human, MTA in plants and IME4 in Saccharomyces cerevisiae are the experimentally proven mRNA m6A- methyltransferases.

The N6-methyladenosine (m6A) Modification

S-adenosylmethionine (SAM, AdoMet) serves as a methyl donor for methyltransferase enzymes in general, including the enzymes catalyzing the ubiquitous N6-methyladenosine (m6A) formation in eukaryotic mRNA (Wertheimer et al. 1980; Ping et al. 2014). m6A
modification is the most prevalent internal modification in mRNA and accounts for 80% of all RNA base methylation. m6A modification is shown to be highest in the coding region of the mRNA particularly in the 3’ end of coding sequences (CDS) and first quarter of the 3’UTR with an average of one m6A residue per 2000 ribonucleotides (Meyer et al. 2012; Niu et al. 2013). Although, this modification occurs at a highly conserved consensus site RRACH (R = G or A; A=m6A; H = A, C or U), only a fraction of these sites are methylated, an estimated 3-5 m6A sites per one eukaryotic mRNA (Wei and Moss 1977; Schibler et al. 1997). This is 10 times less than the expected prevalence of RRACH, so there is the possibility of other mechanisms determining the sites to be methylated (Meyer and Jaffrey 2014). Even among the confirmed methylation sites, not all carry the m6A modification; when one site is abolished in an individual mRNA species, another nearby site can take over its role (Bokar et al. 2005). Bioinformatic analysis of high throughput data has shown m6A modification to be random but enriched in long exons and 3’ UTR of mRNAs with frequency peaking near stop codons (Meyer et al. 2012; Dominissini et al. 2012). In some transcripts, inhibition of m6A modification has shown to decrease their translation efficiency (Tuck et al. 1999). This together with the enrichment of m6A near stop codons suggests that m6A plays a role in mRNA translation. This idea is also supported by its enrichment in the 3’UTR. Furthermore, Wang and group in their two studies published in 2014 have demonstrated that m6A not only plays a role in mRNA stability but also targets mRNA for degradation (Wang et al. 2014a; Wang et al. 2014b).

In all organisms tested, deletion of methyltransferase protein is detrimental to various aspects of the cells. In Homo sapiens, deletion of methyltransferase METTL3 (MTA-70 when first identified) led to apoptosis. Similarly, deletion of the METTL3 ortholog in Arabidopsis thaliana, MTA, resulted in developmental arrest during embryogenesis and knock out of the
ortholog IME4 in *Saccharomyces cerevisiae* and *Drosophila melanogaster* caused defects in gametogenesis (Bodi et al. 2012; Clancy and Shah 1992; Hongay and Orr-Weaver 2011). In mammalian stem cell lines, knockdown led to premature entry into differentiation pathways and loss of proliferation (Wang et al. 2014; Batista et al. 2014). Although the mechanism of action as well as actual roles of methylation of mRNA are still largely unknown, recent research has provided some insights in to the complexity and variety of mechanisms that involves m6A. It has been recently demonstrated that methylation of primary microRNAs (pri-miRNA) by METTL3 is necessary for their further processing of microRNAs (Alarcón et al. 2015).

Similarly, another study has shown the importance of m6A regulation by fat mass and obesity associated (FTO) protein in the splicing and expression of genes involved in adipogenesis. The demethyltransferase, FTO, expression and m6A levels are shown to be inversely proportional during adipogenesis. This research has shown that m6A sites overlap with splice sites and cis-regulatory elements. It has also been demonstrated that the m6A level increases at the edges of exons as a response to FTO depletion. FTO is also shown to regulate m6A levels around splice sites resulting in the isoform expression of the adipogenic regulatory factor RUNX1T1 (Zhao et al. 2014). Similarly, demethylation activity of another RNA demethyltransferase AlkB Homolog 5 (AlkB5) shows significant effects on the mRNA export and RNA metabolism. The knockout of the protein resulted in apoptosis affecting spermatocytes causing infertility in male mice (Zheng et al. 2013). Hence, m6A plays many diverse roles in eukaryotic cells and is important during miRNA processing, mRNA processing, splicing and splice site usage in higher eukaryotes.

Methylation and demethylation can also alter interactions between proteins and nucleic acids as well as secondary structure of RNAs (Wlodarski et al. 2011).
Figure 1: N6-methyladenosine (m6A) formation in eukaryotic mRNA

Figure 2: A schematic representation of life cycle of Saccharomyces cerevisiae under different nutrient conditions. (Modified from Strudwick et al., 2010)
**Role of Ime4 in cell fate decisions**

*Saccharomyces cerevisiae* is a unicellular organism which can exist in three forms; the haploids (MATα and MATα) and the diploid (MATα/a) (Fig 2). Both haploids and diploids grow mitotically by budding, but they differ in whether they can undergo cell fusion (mating) or sporulation. Haploids are the mating cells which undergo nuclear fusion to produce diploid cells which subsequently give rise to daughter diploid cells by budding. The MATα/a diploid cells are incapable of mating with either MATα or MATa haploid cells but at the time of nutritional starvation can undergo sporulation (meiosis and spore formation) to produce four haploid meiotic progeny existing inside a spore sac, the ascus (Herskowitz 1988). Sporulation in *Saccharomyces cerevisiae* is equivalent to gametogenesis in higher organisms. It is a multistep complex differentiation process that is induced by nitrogen and carbon starvation. This stressful condition stimulates a series of complex biochemical, genetic, and morphological changes that ultimately leads to the formation of haploid stress resistant tetrads of spores (Piekarska et al. 2010). Depending on the nutritional environment, yeast cells can also undergo pseudohyphal development. When the yeast cells are starved for nitrogen in the presence of fermentable carbon (e.g. glucose), they form filamentous chains called pseudohyphae (Gimeno et al. 1992). Ime4 is involved in sporulation, pseudohyphal growth and mating. It is essential for sporulation, and is induced by pheromone in haploid cells. It is also inhibitor of pseudohyphal growth.

The diploid cells produce a1/α2 that repress haploid specific genes. Among these genes is *RME1* which codes for the protein that inhibits expression of Inducer of Meiosis 1, *IME1* and *RME2*, which produces an antisense transcript that reduces the expression of *IME4* in haploid cells. Together, these two repressors guarantee that haploid cells do not enter the meiotic program (Clancy and Shah 1992; van Werven et al. 2012). Both of these proteins exert their
effects by means of non-coding RNAs. Expression of *IME4* and reduction of *RME1* lead to accumulation of Ime1. Ime1 is a transcription activator for many genes essential for sporulation and meiosis of yeast, including Inducer of meiosis 2, *IME2*. Ime2 is a serine/threonine protein kinase which is necessary for the stabilization and regulation of Ime1. Ime1 accumulation which is essential for initiation of sporulation, in turn, requires Ime4 expression for a reason that is not yet known (Shah et al. 1992). Ime4 is expressed highly in diploid cells in starvation conditions and is shown to methylate *IME1, IME2* and even its own mRNA (Bodi et al. 2010).

Recent studies have demonstrated that the maintenance of normal level of m6A during the induction of meiosis requires not only Ime4 (and Kar4) but also two other poorly understood proteins, Muddled Meiosis 1 (Mum2) and Sporulation-Specific Leucine Zipper 1 (Slz1). The interactions between these proteins were identified by yeast two hybrid assays and confirmed by co-immunoprecipitation assay (Agarwal et al. 2012).

**Conserved Eukaryotic Adenosine Methylase Complex**

Ime4 functions as part of a conserved adenosine methylase complex consisting of at least two proteins, Mum2 and Kar4. Mum2 has been reported primarily to be cytoplasmic protein but recent reports have demonstrated its presence in the nucleus in yeast cells. However, in other eukaryotic cells, it is seen in the nucleus where it plays important role in methylation of pre mRNA (Schwartz et al. 2012). The structure of Mum2 consists of two coiled-coil region on its C-terminus. It has been suggested that the coiled coil region could interact with catalytic domain of Ime4 and act as a scaffold for other proteins in the complex. Alternatively, Mum2 could also directly activate the catalytic activity of Ime4 or regulate its localization in the cell. It is still not known what Mum2 does in yeast cells, but deletion of the protein resulted in sporulation defect
similar to that of Ime4 mutant cells. Furthermore, Ime4 couldn’t catalyze m6A formation in mRNA without the presence of Mum2 in the cells, confirming the need of Mum2 for methylation and sporulation.

Kar4 is a catalytically inactive ortholog of Ime4 and is required for sporulation. In the absence of KAR4, the diploid yeast cells show defects in meiotic progression and stay arrested as mono-nucleate cells with mono-polar spindles, a phenotype similar to IME4 mutant cells (Kurihara et al. 1996). KAR4 transcripts accumulates in the G1 stage and is essential for the efficient exit from this stage to meiosis. Microarray analysis has shown that IME4 is dependent on KAR4 for expression in response to mating pheromone (Lahav et al. 2006).

Another protein, Slz1, is also suggested to be in the adenosine methylase complex. The protein is not well characterized but appears to have a role in meiotic progression (Agarwal et al. 2012). Slz1 orthologs have not been observed in other organisms. Along with these proteins, Kar4, is also reported to have regulatory function in multiple meiotic pathways in yeast and could be in complex with Mum2 and Slz1. Kar4 is anomalous in that it has mutations in conserved catalytic motifs but is nonetheless essential for meiosis and mating (Bujnicki et al. 2002). KAR4 is the closest yeast ortholog to METT14 despite the lack of catalytic residues.

The complex is highly conserved throughout eukaryotic evolution through humans, Drosophila and Arabidopsis (Fig 3). The Arabidopsis homolog of MUM2, AtFIP37, has been shown to interact with IME4 homolog in that organism. Other homologs of MUM2 include FI (2) D in Drosophila and WTAP-1 in human. These homologs are highly conserved in their C terminal regions which is proposed to be the binding site for Ime4 protein (Agarwal et al. 2012). The subunit in human methyltransferase that recognizes and binds to SAM to catalyze the m6A formation is called MTA-70 or METTL3. The analysis of MT-A70 led to the identification of its
orthologs which revealed that the MT-A70 family comprises of four subfamilies within varying degrees of interrelatedness. One of the subfamilies is a small group of bacterial DNA: m6A methyltransferases. The remaining three are paralogous eukaryotic lineages (Bujnicki et al. 2002).

The MTase region is common to all MT-A70 family members including some divergent paralogs, such as KAR4, a member of a different branch of the MT-A70 family in yeast (Lahav et al. 2006). KAR4-like genes are present in higher eukaryotes including human’s METTL14. METTL14 is demonstrated to be in a complex with METTL3 and WTAP1 (Ping et al. 2014). The invariant cysteines and cysteine-rich region N-terminal to the MTase catalytic region is common to all original MT-A70 family members, including IME4 (Clancy et al. 2002). The extreme N terminus and C terminus region in MTase are not conserved; the former includes the Mum2-binding domain (our unpublished data). MTases dependent on AdoMet have a conserved structural core that helps in binding with AdoMet and orients it for methyl transfer. Other MTases have conserved insertions in their core regions that mediate interaction of methylation targets. Similarly, bacterial DNA: m6A MTase also show a conserved circular permutation of the core catalytic domain (Bujnicki et al. 2002).
Downstream effectors of m6A modification: Roles for YTH-domain proteins

The binding of proteins to RNA is a fundamental part of biology and is required for gene expression and cell function. RNA-binding proteins (RBPs) are crucial during post-transcriptional regulation of RNAs which contribute to gene expression during growth and development. Splicing, polyadenylation, mRNA stabilization, mRNA localization and translation all involve RNA binding proteins that carry out and regulate various aspects of these processes (Dreyfuss et al. 2002; Mazumdar et al. 2003; Gerber et al. 2004; Lee and Schedl 2006). The different families of RBPs are defined by the motifs present in them. Commonly found RNA binding motifs or domains include the RNA recognition motif (RRM), the double-stranded RNA binding domain, the Piwi Argonaught and Zwille domain, and the heterogeneous nuclear ribonucleoprotein K homology domain, among others. Recent research has identified a new
RNA binding domain, called YTH (YT521 homology) because it was first identified in splicing factor YT521. This domain, found only in eukaryotes, is 100 to 150 amino acids long and is characterized by 14 invariant and 17 highly conserved residues. There are 174 identified YTH domain RBPs including the founding member YT521-B (Zhang et al. 2010; Wang and He 2014). The homologue of human \textit{YTHDF2}, Ydr374c (Pho92), is the only protein in \textit{Saccharomyces cerevisiae} that has an YTH (\textit{YT521-B} homology) domain.

\textbf{m6A readers, YTH domain RBPs}

The significance of m6A regulation is also confirmed by the identification of methyltransferase and demethylases. In humans, the methyltransferase complex METTL14-METTL3-WTAP has been identified. Similarly, existence of demethylases FTO and ALKBH5 show that m6A is a reversible process. Hence, if methyltransferase writes and demethylases deletes, a mechanism that can recognize and bind to the m6A should also exist. While potential methyl-specific binding proteins or “readers” have been suggested for a long time based on immunoprecipitation data, it has only recently been demonstrated that some YTH domain proteins are, in fact, the m6A readers (Li et al. 2014; Xu et al. 2014). An experiment performed with control versions of RNA bait followed by mass spectrometry was used to identify the m6A binding proteins, \textit{YTHDF1}, \textit{YTHDF2} and \textit{YTHDF3}, in human. All of these proteins contained the highly conserved YTH domain. Furthermore, among the three, \textit{YTHDF2} and \textit{YTHDF3} exclusively bound to the methylated bait while \textit{YTHDF1} showed significantly higher affinity to it, confirming that they are m6A readers (Dominissini et al. 2012). The crystal structure of the YTH domain of \textit{YTHDF2} protein in association with m6A shows an aromatic cage that specifically interacts with the methylated adenosine (Li et al. 2014) which is thought to be conserved through the YTH domain family. One particular protein, \textit{PHO92}, of \textit{PHO} regulon, is
shown to have the YTH domain and can possibly be an m6A reader in yeast. Pho92, also known as methylated RNA binding protein 1(MRB1), is not regulated by any other PHO regulon genes. However, it is essential for the destabilization of mRNA of transcription activator, PHO4, making it a negative regulator of PHO pathway. This suggests that Ime4 might also affect the mechanism in some way (Kang et al. 2014).

PHO92 and PHO Regulon

There are around 20 genes in PHO regulon in Saccharomyces cerevisiae that are essential for phosphate homeostasis in yeast but also contribute to other processes such as response to potassium starvation and the alkaline stress response (Serra-Cardona et al. 2014; Barreto et al. 2012). Under circumstances of low concentration of phosphate, the PHO regulon induces the expression of genes that encode a high-affinity transport system (PHO84 and PHO89) and genes that encode secreted acid phosphatases (PHO5, PHO11 and PHO12). These
genes are regulated by PHO-specific regulatory proteins Pho2 and Pho4, the transcriptional activators, and Pho80–Pho85, the cyclin–CDK complex. A simple representation of the pathway is given in the Figure 5. These genes are repressed in the presence of inorganic phosphate and are expressed at high levels only during phosphate starvation.

Figure 5: PHO regulon pathway in yeast, *S. cerevisiae*. When phosphate is low in the yeast cells, Pho81 inhibits the Pho80-Pho85 cyclin-CDK complex that phosphorylates transcription activator Pho4. Pho81 binds constitutively to Pho80/Pho85, but is turned into an inhibitor only during phosphate starvation. The non-phosphorylated form of Pho4 localizes in the nucleus and along with its cofactor Pho2 activates the transcription of target genes of PHO regulon including the transporters (*PHO84* and *PHO89*) and phosphatase (*PHO5* and *PHO11*). In contrast, when phosphate is present, Pho81 doesn’t inhibit Pho80-Pho85 kinase which in turn phosphorylates Pho4. The phosphorylated Pho4 loses affinity for Pho2 and other proteins that are necessary for its export to nucleus. Hence, Pho4
localizes to the cytoplasm and the cell expresses the target genes of the \textit{PHO} regulon at low levels (Kang et al. 2014; Korber and Barbaric 2014). Recent research has shown Pho92, the YTH domain protein, to have an important regulatory function in \textit{PHO} regulon, adding another layer of regulation to this pathway. It was shown that Pho92 binds to the 3’ UTR region of \textit{PHO4} mRNA and can destabilize it (Kang et al., 2014). In the cells with \textit{PHO92} deletion, the target genes of the \textit{PHO} regulon were up-regulated also suggesting that Pho92 destabilization of \textit{PHO4} mRNA may be phosphate dependent.
Motivation for This Work

N6 methyl adenosine (m6A) is the most common post transcriptional modification in eukaryotic mRNA. The process, which involves transfer of methyl group from S-adenosyl methionine (SAM) molecule to adenosine on RNA oligonucleotide with consensus sequence RRACH, is performed by a protein called methyl transferase. Inducer of Meiosis 4 (Ime4) is the methyl transferase in yeast, *S. cerevisiae*, which is induced during mating and is necessary for sporulation and meiosis. Ime4 is conserved throughout eukaryotes as Methyl transferase (MTA) in *Arabidopsis*, Ime4 in *Drosophila* and Methyl transferase like 3 (Mettl3) in humans.

Recent work has discovered m6A “readers”, YTHDF1, YTHDF2 and YTHDF3 identified in humans, which share the highly conserved YTH domain that preferentially recognizes and binds to m6A. One YTH domain family protein was recently discovered in *S. cerevisiae*. Pho92, also known as Methyl RNA Binding Protein 1 (MRB1) is suggested to play an important role in the yeast Pi starvation pathway called *PHO* regulon. Δ*pho92* cells show elevated level of mRNA that encodes for transcription activator, *PHO4* and also other *PHO* regulon genes. Pho92 protein is believed to be essential for the destabilization of *PHO4* target mRNAs. Ime4, being a methyltransferase, may affect the *PHO* regulon via Pho92 which binds m6A in mRNAs. Hence we hypothesized that Ime4 affects the *PHO* regulon through *PHO92* via a single pathway.

In order to test our hypothesis and analyze the effect of Ime4 in the *PHO* regulon target genes, we constructed cells mutant in *IME4, PHO92* and both *IME4* and *PHO92*. We also constructed wild type cells overexpressing *IME4* and *MUM2* to study the phenotype of yeast cells in the presence of excess *IME4*. We performed RT-qPCR assays on the RNA extracted from mutant cells (Δ*ime4, Δpho92* and Δ*ime4Δpho92*) as well as cells overexpressing *IME4 and MUM2* (*M2, I4* and *M2 & I4*). We quantified the RNA abundance of *PHO* regulon target genes.
in mutant and overexpressing cells relative to the wild type. We predicted that if our hypothesis is correct, the relative increase in PHO regulon in transcripts will be the same in Δime4, Δpho92 and the double mutant Δime4Δpho92 with no additivity. Similarly, the single pathway theory will also show decrease in the PHO regulon genes in haploid cells overexpressing with IME4. Based on our results, we confirm our hypothesis and conclude that IME4 interacts with PHO92 in a single pathway to affect the PHO regulon target genes in Δime4, Δpho92 and Δime4Δpho92 cells. However, our overexpressing cells demonstrated that IME4 may affect the PHO regulon target genes through multiple mechanisms in different conditions.
Materials and Methods

Growth and Transformation of *Escherichia coli*

Competent *Escherichia coli*, XL1 Blue strain subcloning grade cells from Agilent Technologies, were used for plasmid propagation. Transformation of the cells was performed following the protocol provided with the competent cells. *E. coli* was grown at 37°C in Luria Broth (LB; 1% Bacto Tryptone, 0.5% Yeast Extract and 0.5% NaCl, pH 7.5) supplemented with ampicillin (100 μg/ml) or kanamycin (30 μg/ml) depending on the selectable marker of the plasmid transformed. The plates used for streaking and storage were made using same recipe with the addition of 1.5% agar. Plasmids were isolated from small scale (1-5 ml) cultures using Agilent Stratagene Plasmid Miniprep kits after the cells were grown overnight in the 37°C incubator. Isolated plasmids were evaluated by restriction enzyme digestion and 0.8% agarose gel electrophoresis using 1X TBE buffer. Restriction enzymes were purchased from Promega and New England Biolabs and used according to directions from the supplier.

Growth and Transformation of *Saccharomyces cerevisiae*

A variety of media were used for growth, transformation and culture maintenance of different strains of *Saccharomyces cerevisiae*. Yeast Extract Peptone Dextrose (YEPD; Yeast Extract (0.01 g/ml), Peptone (0.02 g/ml), Dextrose (0.02 g/ml)) was the primary media used to grow and store yeast strains under nonselective conditions. Synthetic Complete Casamino Acids (SC Casamino Acids, Yeast Nitrogen Base (YNB) without amino acids (6.7 mg/ml), dextrose (20 mg/ml), casamino acids (10 mg/ml), adenine (4 mg/ml)) was also used for the growth of yeast. Nutrient limiting Synthetic Complete (SC Dropout; 10X YNB without Amino Acids (67 mg/ml), 5X Dropout Mix (10 mg/ml), dextrose (20 mg/ml)) were used to select for plasmids with URA3 and/or LEU2 selectable markers. 2% agar was added for semi solid media on plates. The yeast
extract, dextrose and casamino acids were purchased from Fisher Scientific. Peptone was purchased from Bacto Laboratories. The agar and the synthetic dropout mix was purchased from U.S Biological. Yeast nitrogen base without amino acids was bought from Difco Laboratories. The 10X YNB and 5X Dropout mix were prepared by filter sterilization. The drop-out mix does not contain adenine, histidine, leucine, tryptophan, or uracil. Supplemental amino acids (5 mls leucine (10 mg/ml), 5 mls tryptophan (10 mg/ml), 5 mls histidine (10 mg/ml), 10 mls uracil (2 mg/ml), 5 mls adenine (4 mg/ml), 5 mls lysine (10 mg/ml), and 5 mls methionine (10 mg/ml)) were added to the media as needed. The modified form of LiAC/ss carrier DNA/PEG method was used for highly efficient yeast transformation. The heat shock step was shortened to 15 minutes from 40 minutes (Ito et al. 1983; Gietz et al. 2007).

**Primer Construction**

Primers for Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) were constructed and ordered from Integrated DNA Technologies (IDT). The amount of oligo we received from IDT was usually approximately 25 nmoles, and was delivered in the form of dry powder that was dissolved in 1XTE (pH 7.5) before use. Extreme precaution was taken during the design of primers. The software Primer3plus was used for the selection of primer pairs. Mfold software was used to confirm the primers. Mfold was also used to avoid regions of the amplicons with high secondary structure, unwanted hairpins and primer complementarity with self or each other. The primers were also Blasted through *Saccharomyces* database to ensure that they targeted the desired sequence amplification and that no other genes will be detected by the primer pair.
Primers used for RT-qPCR are given in the table below.

Table 1: Primers used in this study with the amplicon size and the identity of genes they amplify.

<table>
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<tr>
<th>Gene or Gene Fragment Identity</th>
<th>Forward Primer Sequence 5’to3’</th>
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<td>5’ – CCG AAA CCG AAA CCG AAA CC –3’</td>
<td>5’ – GCG CAA TTA CAG GAG ACT CA –3’</td>
<td>142</td>
</tr>
<tr>
<td><strong>PHO5</strong></td>
<td>5’ – TGA ACG CCA AGA GAC ATG CT –3’</td>
<td>5’ – CGG CAA AAC TGG TTT GGT TT –3’</td>
<td>75</td>
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<td><strong>PHO11</strong></td>
<td>5’ – CTT GAC CGC CGA ACA TGT TC –3’</td>
<td>5’ – AAC ACG AGC ACC TTG TGG AA –3’</td>
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<tr>
<td><strong>PHO84</strong></td>
<td>5’ – AGG TCG GTG CCA TTA TTG CA –3’</td>
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<tr>
<td><strong>PHO85</strong></td>
<td>5’ – AGC AAC GAC CAC CAA GAG AC –3’</td>
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<td><strong>PHO89</strong></td>
<td>5’ – ATC GCC GTT GGT GGT ATT GT –3’</td>
<td>5’ – CCG GCA ATC AAA CCA GCA AT –3’</td>
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</tr>
<tr>
<td><strong>ACT1</strong></td>
<td>5’-ATG GTC GGT ATG GGT CAA AA -3’</td>
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<td><strong>ACT2</strong></td>
<td>5’-TCG CCT TGG ACT TCG AAC AA -3’</td>
<td>5’-CAA AGC TTC GGC TCT GA -3’</td>
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RNA extraction and RT-qPCR

For RNA extraction, cells were grown overnight in appropriate media, either SC or dropout media according to the experiment. The overnight cultures were then diluted in fresh media and allowed to grow for 3-4 hours to ensure the cells were in the exponential phase of growth prior to breakage and RNA extraction. The cell growth was quantified using hematocytometer and light microscopy. At the time of harvest, the cells varied in number from $5 \times 10^7$/ml to $6 \times 10^7$/ml or $5 \times 10^8$ to $6 \times 10^8$ cells per extraction. Total RNA was extracted using Qiagen Midiprep Kit reagents according to the manufacturer's mechanical disruption protocol for isolation of total RNA from yeast. Breakage was assessed microscopically. RNase-Free DNase Set from Qiagen was also used for efficient on column digestion of DNA during RNA purification to ensure that genomic DNA was minimized in the final RNA preparations. Quality and quantity of the mRNA extracted was assessed using the NanoDrop from Thermoscientific and Experion RNA StdSens and HighSens Analysis Kits. Typical yields of total RNA from 10 ml cultures were approximately 400-800 ng/ul. 1 µg of total RNA was used for cDNA synthesis using iScript cDNA synthesis kit from Bio-Rad (#170-8890). For RT-

| PHO5 (Z. Liu) | 5’-GTC ACT CGA GCA GCG GCT AGA ACC GAA -3’ | 5’-GTC AGG ATC CAT GTT TAA ATC TGT TGT-3’ |
| PHO85 Splice | 5’-CCA TGA ATT CTG AGC AAT ACC AAT GTC TT -3’ | 5’-ACT TCT GCA GGG TGT ACC TTC CTC TGA ATC CA -3’ | 213 |

Table 1 cont.
qPCR amplification of the cDNA, Real Time PCR detection system was used with iQ SYBR Green Super Mix from Bio-Rad (#170-8880). After an initial denaturation of cDNA at 95°C for 3 minutes, an amplification using required primers was done with 40 cycles of denaturation at 95°C for 10 seconds followed by primer annealing at 55°C for 30s. Real time data was accumulated for further analysis. iCycler iQ software provided with the instrument was used for data analysis. The $2^{\Delta\Delta ct}$ method was used to analyze relative changes in gene expression in different conditions. The software Prism was used to obtain statistical difference in mRNA abundance of Pho regulon genes between different genotype of cells (http://www.graphpad.com/scientific-software/prism/). The P value between two groups have to be less than 0.05 for the difference between the groups to be significant. The significant difference is denoted by an asterisk (*) in the graphs.

**Yeast Strains**

Different strains of yeast with unique deletions were made in our lab, bought from commercial source or obtained from various labs. The strains with their background, description and the source are described in the Table below.
Table 2: Yeast strains used in this study with their genetic markers.

<table>
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<tr>
<th>Strain</th>
<th>Description</th>
<th>References/Source</th>
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</tr>
<tr>
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<td>mum2::KanMX</td>
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</tr>
<tr>
<td>BY4741</td>
<td>ime4::KanMX</td>
<td>P. Dehon</td>
</tr>
<tr>
<td>BY4741</td>
<td>ime4::HIS3</td>
<td>Z. Liu</td>
</tr>
<tr>
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</tr>
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<td>BY4741</td>
<td>ime4::KanMX pho92::HIS3</td>
<td>Z. Liu</td>
</tr>
<tr>
<td>BY4741</td>
<td>pho92::KanMX ime4::HIS3</td>
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<td>ime4::KanMX pho4::HIS3</td>
<td>Z. Liu</td>
</tr>
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<td>Z. Liu</td>
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Table 3: Plasmids used in this study to construct different yeast strains.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
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</tr>
</thead>
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<tr>
<td>pRS315</td>
<td>Amp; CEN LEU2</td>
<td>Sikorski and Hieter, 1989</td>
</tr>
<tr>
<td>Yep351-MUM2</td>
<td>Amp: 2u LEU2</td>
<td>J. Engebrecht</td>
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<tr>
<td>pRS416 GPD</td>
<td>CEN6/ARSH4 URA3 Amp</td>
<td>Z. Liu</td>
</tr>
<tr>
<td>pRS416 ADH1-GFP</td>
<td>CEN6/ARSH4 URA3 Amp</td>
<td>Z. Liu</td>
</tr>
<tr>
<td>pRS416 ADH1-GFP-PHO5</td>
<td>CEN6/ARSH4 URA3 Amp</td>
<td>This Study</td>
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</tbody>
</table>
Construction of \textit{pADH1-GFP-PHO5}

Polymerase chain reaction was performed to amplify the \textit{PHO5} ORF and 3’UTR from a genomic tiling library using primers \textit{PHO5} \cite{Z. Liu} mentioned in table. Herculase II Fusion DNA Polymerase enzyme and 5× Herculase II Reaction Buffer from Agilent Technologies with nucleotide mix from Promega were used to obtain high-fidelity PCR reaction. StrataPrep PCR Purification Kit from Agilent Technologies was used to purify the PCR product from PCR primers, enzymes and non-specific amplification products \cite{#400771}. The protocol provided with the kit was followed for the purification. After the PCR product was purified, it was digested if necessary using restriction enzymes BamHI and XhoI \cite{Promega}. The digested products were analyzed by agarose gel electrophoresis using Tris Acetate-EDTA buffer (TAE). The required bands were separated from the gel using QIAquick Gel Extraction Kit from QIAGEN and protocol provided with it \cite{#28704}. Vector pRS416 \textit{ADH-GFP} was digested with the same enzymes and gel purified. For the ligation of two plasmids (vector and insert), T4 DNA ligase and Ligase 10X Buffer from BioLabs was used for ligation at room temperature for 3 hours and transformed into \textit{E.coli}: XL1-Blue as above. Minipreps were screened by digesting with the same enzymes. Three isolates having the desired inserts were sequenced to verify that no mutations were introduced. The amount of plasmids used depended on the plasmid concentrations.

\textbf{Splicing Assay}

An assay was performed to compare splicing efficiency of \textit{PHO5} mRNA in cells mutant in as well as overexpressing \textit{IME4}. The templates we used for the splicing assays were obtained from enzymatic conversion of 1 ug RNA to cDNA. 1 ul of the cDNA was used for 25 ul of PCR reaction. The primers used for the amplification was designed to flank a portion of the 5’
region of \textit{PHO85} including the single intron. Taq polymerase was used for PCR which was stopped after 25 cycles to avoid the plateau stage. The amplified samples were then analyzed on 1.5% and 2.0% agarose gels through electrophoresis. 1X TBE was used to pour and run the gel.

\textbf{Total Cell Extraction for Western Blot (TCA Method)}

The “quick-kill” method was used for Western blot experiments to quantify the expression of \textit{ADH1-GFP-PHO5} in wild type (BY 4741), \textit{Δime4, Δpho92} and \textit{Δime4Δpho92} cells. The strains were grown overnight in 5 ml SC Casamino Acids with uracil (2 mg/ml) at 30°C. All cultures were determined to have similar ODs. 1 ml of cells was added to 160ul of β-mercaptoethanol (bME)-NaOH ((740 ul water, 74 ul bME, 185 ul 10 N NaOH) and incubated on ice for 10 minutes before adding 82 ul of 100% trichloroacetic acid (TCA). The mixture was again left on ice for another 30 minutes after which it was centrifuged for 5 min at 21000g. The supernatant was aspirated before adding 50 ul of SDS loading dye to re-suspend the cells. The loading dye was prepared by adding 950 ul of 2x Laemmli Sample Buffer from Bio-Rad with 50 ul of bME. If the color didn’t change to blue, 1 ul of 1M unbuffered Tris was added stepwise until the color changed. The samples were boiled at 95° C for 10 mins to prepare them for SDS electrophoresis (Dr. Zhengchang Liu).

\textbf{SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot}

Total protein extracts were separated using SDS-PAGE. 15 ul of cell extract was added to the well of a 10% Tris-HCl Ready Precast Gel from BioRad. The extracts were separated at approximately 150 Volts in 1X SDS gel running buffer (25 mM Trizma Base, 200 mM glycine, 0.1% SDS). After the loading dye interface reached the bottom of the gel, the gel was transferred to a nitrocellulose membrane. A cassette was assembled with two filter papers on both side and gel over the membrane between them. The gel, membrane and filter papers was soaked for about
15 minutes in the transfer buffer (25 mM Trizma Base, 14.4% glycine, 0.05% SDS dissolved in 80% deionized water and 20% methanol) to equilibrate. The transfer was done using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell from BioRad. Transfer was done at 15 Volts and 150mA for 60 minutes. The membrane was then blocked using blocking buffer (5 g nonfat dry milk, 0.1 mL Tween, 100 mL 1xPBS) for one hour. The membrane was washed in 1X PBS-Tween (11.5 g/L Na₂HPO₄ (dibasic), 172.96 g/L Na₂HPO₄ (monobasic), 5.84 g/L NaCl, 0.1% Tween; pH 7.5) for 10 minutes. The primary antibody against GFP (Santa Cruz Biotechnology) was diluted 1:200 in blocking buffer and incubated overnight at 4°C while mixing. The membrane was washed in PBS-Tween twice for 10 minutes. The secondary antibody HRP coupled goat anti-mouse IgG was diluted 1:5000 in PBS-Tween and incubated with the membrane for one hour while mixing. The membrane was washed three times in PBS-Tween for 10 minutes each before being detected. Proteins on the membrane were then detected using Clarity Western ECL blotting Substrate from BioRad. An equal volume of each solution was mixed and poured onto the protein side of the membrane and incubated for 1 minute. Excess detection reagent was drained before the membrane was wrapped in Saran Wrap and exposed to chemiluminescence. Phosphoglycerate kinase (PGK) was used as a loading control and was detected using a rabbit polyclonal antibody kindly provided by Dr. Zhengchang Liu. In this case, the secondary antibody was HRP- coupled goat anti-rabbit IgG. After an image was obtained, ImageJ software was used to quantify the bands.
Results

Development of RT-qPCR assay for PHO regulon genes.

We chose to use real-time quantitative PCR (RT-qPCR) to determine whether IME4 regulates PHO regulon RNA abundance. With this method, RNA is extracted from cells of various genotypes. Then, following enzymatic creation of cDNA from the starting mRNAs, the specific targets are amplified by RT-qPCR. The sequences of the Open Reading Frames (ORFs) of PHO transcripts of interest were retrieved from Saccharomyces Genome Database (http://www.yeastgenome.org). Then, they were entered into the primer design software Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). The software provided the length of primer, primer melting temperature, annealing temperature, product size, Mg++ concentration, stability and GC clamps. This information was used to identify the best primer pair for the amplification of each transcript. The software also showed any primer dimers that could form in the given option of primers.

Once the primer pairs were selected, the amplicon regions were analyzed using Mfold software (http://mfold.rna.albany.edu). This software predicts any secondary structures of the amplified transcript region by the given primers. Excessive secondary structure in the amplicon leads to inefficient amplification of the target. The target transcripts themselves may also be very similar to others encoded by the genome. In our study, different phosphatases, transcription activators and transporters do share very similar sequences. Hence, the primers were then analyzed by BLAST search of Saccharomyces genome database to be certain that they would not amplify unwanted transcripts. The Basic Local Alignment Search Tool (BLAST) presented the number and the positions of matched bases between the primers and different transcripts as well.
as primer orientations and distance between forward and reverse primers. Primer sequences and the length of the amplicons are shown in Table 1.

Lastly, the amplification efficiency of the designed primer pairs was verified. RT-qPCR data was analyzed using ΔΔCt method which assumes the RT-qPCR assay has 100% amplification efficiency. An efficient primer pair produces a two fold increase in the target DNA with each cycle of the PCR, and as the primers deviate from the ideal, fold differences increases exponentially. To determine the amplification efficiency of the primers, serially diluted templates of known concentrations were amplified using the primers. The Ct values versus the initial amount of the template was calculated and a logarithmic calibration curve was plotted. The curve closest to the slope of -3.33 is expected to give the highest amplification efficiency. Such logarithmic calibration curves to test the efficiency of four of our designed primers is shown in Fig 6. The results showed that all four primer pairs performed well in the amplification reaction, with efficiencies close to 100% in all cases.
Figure 6: Calibration curve to verify the amplification efficiency of designed primers.

**RNA Extraction and Quality**

RNA was extracted from yeast cells using Qiagen MidiEasy Purification System followed by on-column DNase I digestion. The integrity and quality of the extracted RNA was assessed before performing any experiments on RNA abundance. Low-quality RNA will compromise data obtained from downstream experiments. Hence, different methods were used in this study to verify the RNA quantity and quality prior to RT-qPCR. The NanoDrop instrument (Thermo Scientific, USA) was used to obtain the 260/280 absorbance value for each sample to assess the purity of the RNA. A ratio of ~2.0 is generally accepted as “pure” for RNA. Values
that are significantly lower than 2 indicates the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. NanoDrop also measured 260/230 absorbance values for the samples. These values were used as a secondary measure of nucleic acid purity. The results from a typical experiments are shown in Figure 7 and Table 4.

Since NanoDrop only gave information on the quantity of the RNA, another method, Experion (Bio-Rad Laboratories, USA), was also used to confirm the quality of RNA samples. Experion is a microfluic-based electrophoresis technique which has the ability to assess quantity and quality of the RNA in a single step. Experion uses on-a-chip technology (Calipher Life Sciences Inc.) to perform electrophoresis in channels of a microchip. It allows assessment of the quality of RNA by visual inspection of the resulting electropherogram. It also compares the area of peaks in the electropherogram corresponding to rRNAs and provides a ratio value, 18S/28S, which if close to 2 is indicative of intact RNA. Experion also calculates the RNA Quality Index (RQI) by comparing the electropherogram of RNA samples run on the chip with series of standardized degraded RNA samples, and gives a value between 10 (highly intact) and 1 (highly degraded) for each sample. Figure 7 represents the electropherogram of RNA samples from one of our experiments and the following table demonstrates some of the information obtained from NanoDrop and Experion that attests the quality of same set of sample RNAs. The yields shown are typical for 10 ml cultures grown in SC medium to a cell density of 3-5X10^7 cells/ml.
Figure 7: Electropherogram for RNA samples obtained using Experion electrophoresis method.

Table 4: Information obtained from the electropherogram for RNA samples using Experion electrophoresis method.

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<tr>
<th>Well ID</th>
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Analysis of RT-qPCR data.

Effects of mutation of IME4 and PHO92 in haploid S. cerevisiae cells.

Expression of PHO regulon genes in wild type and Δime4 cells.

We tested the hypothesis that IME4 interacts genetically with PHO92 by asking whether ime4 mutations lead to elevated levels of PHO regulon target mRNAs. We examined this question using the RT-qPCR assays described above. RNA was extracted from wild type cells and haploid cells mutant for IME4 (Δime4). The extracted RNA was used to study the expression of the PHO4 target genes in the presence and absence of IME4. Two different primer pairs specific for actin (ACTIN1 and ACTIN2) were used for normalization. Actin is a “housekeeping gene” that is standard for qPCR assays in S. cerevisiae. The gene is expected to be similarly expressed in all cells and hence are apt for normalization.

Analysis of relative gene expression data from RT-qPCR showed an increase in PHO5 expression in Δime4 cells when compared with the wild type strain, BY4741 (Fig 8). The x axis represents the genotype of the cells and y axis represents the normalized expression data. The
results showed that PHO5 RNA levels were approximately 2 fold higher in the Δime4 cells vs. the wild type control. We also performed a t test using the software Prism on the mean and standard deviation of expression of the PHO5 gene in Δime4 cells compared to the expression of the same gene in the control, wild type, and statistically confirmed that absence of IME4 increases the abundance of the PHO5 mRNA (http://www.graphpad.com/scientific-software/prism/). Hence, Ime4 protein affects the expression of phosphatase PHO5 of the PHO regulon pathway in *S. cerevisiae*.

![Relative Abundance of PHO5 RNA](image)

**Figure 8:** Expression of the PHO regulon gene, PHO5, in Δime4 cells compared to wild type (BY4741) cells of *S. cerevisiae*.

Relative RNA abundance of other PHO regulon genes *PHO11, PHO84* and *PHO 89* was also analysed through RT-qPCR of wild type and Δime4 cells, using primers that target these genes. *PHO11*, like *PHO5*, codes for a phosphatase. *PHO84* and *PHO89* both encode phosphate transporters and are expressed at high levels during Pi starvation in *S. cerevisiae*.

As shown in the Figure 9, RNA transcript levels of PHO regulon genes, phosphatase *PHO11* (Fig. 9a) and phosphate transporters *PHO84* and *PHO89* (Fig 9b and 9c), increased in
the Δime4 mutant strain. The t-test performed using the software Prism also showed significant amplification differences in the levels of the expression of the PHO regulon genes in the Δime4 cells. Hence, Ime4 affects the expression of PHO regulon genes in haploid cells of S. cerevisiae. The magnitude of expression change varied from gene to gene, in these preliminary experiments, with PHO5 and PHO84 increasing 1.5 to 2 fold and PHO89 increasing more strongly by approximately 4 fold. PHO11 levels were more modestly higher, approximately 50%, in the experiments shown.

Figure 9: Expression of PHO regulon genes, PHO11, PHO84 and PHO89, in Δime4 cells compared to wild type (BY4741) cells of S. cerevisiae.
Expression of PHO regulon genes in Δpho92 and Δime4Δpho92 cells.

PHO92, also known as MRB1, plays an important role in the PHO regulon. Δpho92 cells show elevated level of mRNA that encodes the transcription activator, Pho4 and also other PHO regulon genes. Hence, Pho92 protein is believed to be essential for the destabilization of PHO4 and/or its target mRNAs (Kang et al. 2014). Pho92 contains an YTH domain characteristics of m6A binding proteins and is the only S. cerevisiae protein of this class. We hypothesized that, Ime4, being a methyltransferase, may affect the PHO regulon via Pho92. We tested the hypothesis that Ime4 affects the PHO regulon through PHO92 via a single pathway. We predicted that if this idea is correct then the relative increase in PHO regulon transcripts will be the same in Δime4, Δpho92 and the double mutant Δime4Δpho92 with no additivity. Models for possible mechanism of action of Ime4 in PHO regulon is illustrated below.

If IME4 and PHO92 work together in the same pathway, the phenotypes of the knockout mutants will be the same as each other because either mutation will be sufficient to block the pathway. Alternatively, if the two act on separate pathways, the combine phenotype of the double mutant will be more extreme than either mutation alone.

Model 1.

IME4 → PHO92 → Wild Type Level of RNA Targets
IME4 ↔ PHO92 → Increase in Target RNAs
IME4 → PHO92 ↔ Increase in Target RNAs
IME4 ↔ PHO92 ↔ Increase in Target RNAs

Figure 10: Models for the possible mechanism of action of Ime4 protein in PHO regulon.
Model 2.

![Diagram of Models for the possible mechanism of action of Ime4 protein in PHO regulon.](image)

Figure 10: Models for the possible mechanism of action of Ime4 protein in PHO regulon.

RNA was extracted from the haploid cells mutant in Pho92 (Δpho92) and double mutant in Ime4 and Pho92 (Δime4Δpho92) along with single Δime4 mutant. RT-qPCR was performed on the RNA samples from these cells and compared to wild type to determine the relative gene expression of PHO regulon genes PHO5, PHO11, PHO84 and PHO89 (Fig 11). Analysis of RT-qPCR data showed increases in the expression of PHO5, PHO84 and PHO89 genes in Δpho92 and Δime4Δpho92 cells as were previously observed for Δime4 alone. The signal obtained from PHO11 genes were very low which made it difficult to convincingly say that PHO11 expression changed in these cells.

As shown in Figure 11, expression of PHO5 (Fig 11a), PHO84 (Fig 11c) and PHO89 (Fig 11d) increased in the Δime4, Δpho92 and Δime4Δpho92 cells compared to the wild type strain approximately 4, 3 and 2 fold, respectively. The evidence obtained from the gene expression data of PHO11 (Fig 11b) showed slight increase in its expression in Δime4 cells. The t
tests performed on the Prism software statistically corroborated our findings, demonstrating significant increases in the expression of PHO5, PHO84 and PHO89 and no change in expression of PHO11 in Δime4, Δpho92 and Δime4Δpho92 cells compared to wild type. Hence, Ime4 and Pho92 both affect the expression of PHO regulon genes PHO5, PHO84 and PHO89.

The two way ANOVA test and posttest analysis performed on expression of target genes in Δime4, Δpho92 and Δime4Δpho92 cells relative to wild type and each other confirmed the results obtained from RT-qPCR software and the t-tests. For PHO5, there was a significant difference in the expression of the gene in Δime4 and Δpho92, in Δime4 and Δime4Δpho92 as well as in Δpho92 and Δime4Δpho92. The ANOVA test showed that the expression of PHO11 did not differ significantly among the genotypes. PHO84 expression was significantly higher in Δime4Δpho92 when compared to Δpho92 but the difference in PHO84 expression between Δime4Δpho92 and Δime4 was insignificant. ANOVA test showed significant increase in the expression of PHO89 in Δime4Δpho92 cells compared to Δpho92 but no significant change of expression between Δime4 and Δime4Δpho92 cells.

The results show that the increase in the expression of PHO regulon genes is similar in Δime4, Δpho92 and Δime4Δpho92 cells considering the variability in the measurement. Hence, Ime4 and Pho92 interact genetically and the data favors model 1 where Ime4 and Pho92 act in a single pathway. There is no additivity in expression of PHO genes in Δime4 and Δime4Δpho92. Although PHO5 shows decrease in expression in Δpho92 cells when compared to Δime4Δpho92 cells, the relative gene expression of other PHO genes, PHO84 and PHO89, between these genotypes is non-additive consistent with the idea that IME4 and PHO92 work together in the same process. On the other hand, the lower levels of the transcripts in Δpho92 strain vs Δime4 hints that IME4 could have some effects that are independent of PHO92. PHO11 expression in
this experiment is consistent with the previous experiment. Similar to $\Delta ime4$ cells, $PHO11$ shows very modest increase in $\Delta pho92$ and $\Delta ime4\Delta pho92$ cells when compared wild type, BY4741, and no significant difference among the four strains overall.

Figure 11: Expression of $PHO$ regulon genes $PHO5$, $PHO11$, $PHO84$ and $PHO89$ in haploid $\Delta ime4$, $\Delta pho92$ and $\Delta ime4\Delta pho92$ cells compared to wild type (BY4741) cells of $S. cerevisiae$.

**Expression of $PHO4$ in wild type, $\Delta ime4$, $\Delta pho92$ and $\Delta ime4\Delta pho92$ cells**

Pho4 is a transcription activator that is responsible for expression of $PHO$ regulon genes in $S. cerevisiae$ during phosphate starvation. When the phosphate is low, a non-phosphorylated form of Pho4 localizes to the nucleus and along with its cofactor Pho2 activates the transcription of target genes of the $PHO$ regulon including $PHO5$, $PHO11$, $PHO84$ and $PHO89$. In contrast,
when phosphate is present, Pho4 is phosphorylated by the \textit{PHO85} cyclin dependent kinase. It then moves to the cytoplasm with the result that the expression of its target genes is reduced. It has been shown that the \textit{PHO92} regulates \textit{PHO4} RNA levels and that \textit{PHO4} mRNA half-life is increased in the \textit{Δpho92} mutant (Kang et al. 2014). Given the role of this transcription factor in expression of downstream genes, it is possible that Ime4 exerts its effects on these genes through \textit{PHO4}; elevated levels of this transcription factor could lead to higher \textit{PHO4} activity, thereby activating target genes.

RT-qPCR experiments were performed with RNA samples extracted from wild type, \textit{Δime4}, \textit{Δpho92} and \textit{Δime4Δpho92} to study the relative expression of the \textit{PHO4} gene. As shown in Figure 12, the data demonstrated an increase in relative gene expression of \textit{PHO4} in \textit{Δime4} and \textit{Δpho92} cells compared to wild type. The t-test performed using Prism software also showed p value very close to 0.05 consistently when \textit{PHO4} RNA levels in \textit{Δime4} and \textit{Δpho92} cells were tested against \textit{PHO4} gene expression in wild type. The RT-qPCR data and Prism software showed significant increase of \textit{PHO4} RNA in \textit{Δime4Δpho92} cells compared to wild type cells. Two way ANOVA test performed also showed significant changes in \textit{PHO4} expression between wild type and \textit{Δime4Δpho92} cells. There was no additive increase in the expression of \textit{PHO4} gene in the double mutant confirming our assertion that Ime4 and Pho92 work in a single pathway. We conclude that increased Pho4 protein could be responsible for some or all of the increase in \textit{PHO} target genes in mutant strains.
Expression of PHO85 in wild type, Δime4, Δpho92 and Δime4Δpho92 cells

We decided to study the expression of a crucial regulatory protein that is further up on the pathway. Pho85 is a cyclin dependent kinase (CDK) that interacts with cyclin protein Pho80 to regulate localization of PHO4 transcription factor, Pho4. Pho80-Pho85 complex phosphorylates Pho4 when phosphate is sufficient in the cell, leading to the removal of the Pho4 protein from the nucleus. However, during Pi starvation, Pho80-Pho85 is inhibited by Pho81. This results to a decrease in Pho4 phosphorylation causing Pho4 to localize to the nucleus consequently increasing the target gene transcripts. Thus, change in the expression of PHO85 in haploid Δime4, Δpho92 and Δime4Δpho92 could explain the increase in PHO regulon target genes under these conditions. We hypothesized that IME4 and MUM2 affects PHO85 expression in haploid S. cerevisiae cells.

RT-qPCR experiments were performed with RNA samples extracted from wild type, Δime4, Δpho92 and Δime4Δpho92 to study the relative expression of PHO85 gene. As shown in Figure 13, the data demonstrated an increase in relative RNA abundance of PHO85 in Δime4, Δpho92 and Δime4Δpho92 cells compared to wild type. The t-tests performed using Prism software showed significant increase in all three mutant strains compared to wild type cells.
Two way ANOVA test performed also showed significant change in *PHO85* expression among all genotypes of cells. Moreover, there was no additive increase in the expression of *PHO85* gene in double mutant confirming our assertion that Ime4 and Pho92 work in a single pathway.

The elevation of *PHO85* mRNA was somewhat surprising given that Pho85 kinase acts to repress *PHO* gene transcription rather than activate it. We can explain their apparent discrepancy in several ways. Most importantly, *PHO85* activity is regulated at the level of the protein by its cyclin partners and inhibitors such that more Pho85 protein does not necessarily lead to more kinase activity toward Pho4. Finally, it is not necessarily true that higher mRNA levels lead to more protein.

![Relative Abundance of PHO85 mRNA](image)

**Figure 53:** Expression of *PHO* regulon gene *PHO85* in haploid Δime4, Δpho92 and Δime4Δpho92 cells compared to the wild type (BY4741) cells in *S. cerevisiae*.

In summary, with the exception of *PHO11*, the relative expression of *PHO* regulon genes increases significantly in Δime4, Δpho92 and Δime4Δpho92 cells when compared to wild type in haploid *S. cerevisiae* cells. *PHO11* gene expression increases very modestly but not significantly in the mutants relative to the wild type. Also, excluding *PHO5* expression which shows higher levels in Δime4Δpho92 than in Δpho92 cells, the increase in the expression of other *PHO* regulon genes, *PHO84* and *PHO89*, in Δime4Δpho92 isn’t additive relative to the single
mutants. *PHO4* shows modest but insignificant increase in Δ*ime4* cells and significant, non-additive expression in Δ*pho92* and Δ*ime4Δpho92* cells relative to the wild type. *PHO85*, higher on the *PHO* regulon, shows increase in expression in Δ*ime4*, Δ*pho92* and Δ*ime4Δpho92* cells. Moreover, the increase is not additive in double mutant cells. These results confirm that *Ime4* protein affects the gene expression of *PHO* regulon genes in *S. cerevisiae* by genetically interacting with Pho92 protein in single pathway.

**Overexpression of IME4 and MUM2 in haploid *S. cerevisiae* cells.**

**Expression of PHO5 in haploid cells with overexpression for IME4 and MUM2 genes.**

Since we saw increase in the *PHO* regulon target genes in the haploid cells lacking Ime4 in previous experiments, we hypothesized that overexpression of *IME4* in these cells would decrease the expression of the target genes. Muddled Meiosis Protein I (Mum2) is a scaffold protein that forms a complex with Ime4 in yeast *S. cerevisiae*. Ime4 cannot catalyze m6A formation in mRNA without the presence of Mum2 in the cells. *IME4* and *MUM2* were transformed in to wild type (BY4741) *S. cerevisiae* cells separately and together with appropriate vector-alone controls required for selection purposes. The vector used for *IME4* transformation was pRS 416 and the *IME4* gene was driven by the *ADH1* promoter for expression while *MUM2* was carried on a high copy *YEp351* vector. Total RNA was extracted from the transformed cells. RT-qPCR experiments were performed on the cDNA from the extracted RNA to analyze the expression pattern of *PHO* regulon genes in haploid *S. cerevisiae* cells overexpressing *IME4* and *MUM2*. As for the mutant haploid cells, two different primer pairs specified for actin (*ACTIN1* and *ACTIN2*) were used for normalization.
We examined the RNA levels of the same *PHO* regulon genes as before. Contrary to our prediction, the RT-qPCR data showed the relative gene expression of *PHO5* in haploid cells overexpressing *IME4* and *MUM2* to be amplified rather than repressed. As shown in Figure 14, the increase of the expression of *PHO5* RNA was non-significant in M2 (overexpression of *MUM2*) and significant in I4 (overexpression of *IME4*) cells compared to wild type cells containing vectors only. However, *PHO5* expression was much higher in M2 & I4 (overexpression of *MUM2* and *IME4*) cells.

The t-tests performed on expression of *PHO5* gene in M2, I4 and M2 & I4 cells compared to wild type cells with vectors only (none) showed no increase of *PHO5* gene expression vs M2 and significant increase in I4 cells and in M2 & I4 cells. The abundance of the RNA *PHO5* in I4 and M2 & I4 was significantly higher consistently through multiple experiments. The two way ANOVA test showed significant difference in the expression of *PHO5* gene in I4 and M2 & I4 when compared to wild type with vectors only. While the change in expression of *PHO5* between wild type with vectors vs M2 as well as M2 vs I4 was non significant, the increase in between M2 and M2 & I4 as well as I4 and M2 & I4 was very significant demonstrating that both *MUM2* and *IME4* over expression is required for higher expression of the *PHO5* gene. Hence, overexpression of *IME4* and *MUM2* increases the *PHO* regulon gene *PHO5*. We interpret this result to mean that *IME4* could affect *PHO5* through more than one process.
Figure 14: Expression of *PHO5* in haploid *S. cerevisiae* cells overexpressing *IME4* and *MUM2*.

**Expression of *PHO11*, *PHO84* and *PHO89* genes in haploid wild type cells with overexpression for *IME4* and *MUM2* genes.**

Relative gene expression of other *PHO* regulon target genes were studied in wild type cells in which *MUM2* and *IME4* genes were over expressed. Expression of the target genes was analyzed relative to the expression in control wild type cells with vectors controls. As shown in Figure 15, the RT-qPCR data showed no change in the expression of phosphatase, *PHO11*, (Fig 15a) and phosphate transporter, *PHO84*, (Fig 15b) in M2, I4 and M2 & I4 cells when compared to wild type with vectors only. However, another phosphate transporter, *PHO89*, showed increased relative gene expression in M2, I4 and M2 & I4 (Fig 15c).

The t-test performed on the expression of *PHO89* in M2, I4 and M2 & I4 showed significant differences, when compared to wild type with vectors only. Confirming the data from the RT-qPCR software analysis, t-tests showed the expression change of *PHO11* and *PHO84* genes in M2, I4 and M2 & I4 cells as non-significant relative to wild type with vectors only. The two-way ANOVA test showed the same results as the t-tests. The differences in RNA level of *PHO11* and *PHO84* is non-significant across all genotypes. However, the ANOVA test showed
the change in gene expression of $PHO89$ to be significant between not just wild type with vectors and among all genotypes for overexpression but also between M2 and I4 as well as between I4 and M2 & I4. Hence, overexpression of $IME4$ and $MUM2$ genes increases the expression of $PHO89$ in haploid wild type cells. The expression of other target genes of $PHO$ regulon, $PHO11$ and $PHO84$, was not affected by the overexpression of $IME4$ and $MUM2$, either in a positive or negative way.

Figure 15: Expression of $PHO11$, $PHO84$ and $PHO89$ in the haploid cells overexpressing $IME4$ and $MUM2$. 
Expression of *PHO4* in haploid wild type cells overexpressing *IME4* and *MUM2* genes.

As our previous experiments showed increase in the expression of *PHO* regulon genes in cells mutant in *IME4*, *PHO92* and both *IME4* and *PHO92*, we had expected that overexpression of *IME4* in the haploid cells would produce the opposite effect and decrease the expression of *PHO* regulon genes. Contrary to our belief, the expression of two of the *PHO* regulon genes (*PHO5* and *PHO89*) increased in the haploid cells overexpressing *IME4* and *MUM2* whereas two others (*PHO11* and *PHO84*) remained constant. We had grown the haploid cells in similar condition as previous experiments but in different media. For the selection of cells transformed with different plasmids, SC dropout media selective for Leucine and Uracil was used for the overexpression experiments. The anomaly may be attributed to the media difference. Having said that, the increase in *PHO* regulon genes in cells overexpressing *IME4* and *MUM2* grown in different media hints at separate ways *IME4* gene can affect *PHO* regulon pathway depending on growth conditions. We wanted to analyze the expression of *PHO* regulon genes upstream in the pathway to correctly identify which protein is affected by *IME4* directly in the overexpressing haploid cells. Hence, we studied the relative gene expression of transcription activator, *PHO4*, in M2, I4 and M2 & I4 relative to wild type with vectors only. As shown in Figure 16, the RT-qPCR software analysis showed the expression of *PHO4* decreasing among all genotypes relative to wild type with vectors only. However, t tests and ANOVA test performed on the relative gene expression data showed no difference in the expression of *PHO4* between any of the genotypes studied. Hence, statistically, *MUM2* and *IME4* overexpression doesn’t affect the expression of *PHO4* in haploid yeast cells.
Expression of PHO4 in the haploid cells overexpressing IME4 and MUM2.

**Expression of PHO85 in haploid wild type cells with overexpression of IME4 and MUM2 genes.**

Similar to haploid mutants, we decided to study Pho85, a cyclin dependent kinase (CDK), higher up in the PHO pathway that interacts with cyclin protein PHO80 to regulate PHO4 in PHO regulon. In those experiments, expression of PHO85 increased in haploid cells mutant in Ime4 and Pho92 while the PHO regulon genes downstream to it also increased in expression. Contrary to what we had expected, two of the PHO regulon genes (PHO5 and PHO89) also increased in the haploid cells overexpressing IME4 and MUM2 while others remained unchanged. We hypothesized that overexpression of IME4 and MUM2 affects PHO85 expression in haploid S. cerevisiae cells. As shown in Figure 17, our experiments showed that expression of PHO85 decreases significantly in haploid yeast cells overexpressing IME4 and MUM2. The t-tests performed on relative gene expression data obtained from RT-qPCR experiments with cDNA from RNA of wild type with vectors only, M2, I4 and M2 & I4 shows significant decrease in the
expression of \textit{PHO85} among the genotypes relative to wild type with vectors only. The ANOVA test performed on the same data showed significant differences in expression of \textit{PHO85} between wild type with vectors only and M2 & I4. Hence, \textit{PHO85} expression is affected by \textit{IME4} and \textit{MUM2} expression and may be responsible for increase in \textit{PHO} regulon target genes in \textit{S. cerevisiae}.

![Relative Abundance of PHO85 RNA in Overexpressing Cells](image)

Figure 17: Expression of \textit{PHO85} in haploid cells overexpressing \textit{IME4} and \textit{MUM2} in \textit{S. cerevisiae}.

**Expression of \textit{PHO5} gene in \textit{PHO92} mutant haploid cells overexpressing \textit{IME4} and \textit{MUM2}.**

Relative gene expression of \textit{PHO5} was studied in haploid cells lacking \textit{PHO92} transformed with vectors (pRS 315 and pRS416), \textit{IME4} and \textit{MUM2} individually and in combination. These cells were grown in identical media and transformed in same conditions as wild type from previous experiments. Ideally, wild type cells, BY4741, have some \textit{IME4}, \textit{MUM2} and \textit{PHO92} RNAs in normal conditions. Hence, there will be some interaction between \textit{IME4}, \textit{MUM2} and \textit{PHO92} in wild type transformed with each plasmid individually, which could result
in expression of some PHO regulon target genes in wild type cells carrying vectors only. Based on results from our previous experiments, overexpression of IME4 and MUM2 may inhibit the expression of PHO85 in haploid yeast cells, leading to the decreased phosphorylation of PHO4 and subsequent increase in PHO regulon target genes as shown in wild type cells.

If mRNAs that are methylated by Ime4 are subsequently degraded in a Pho92 dependent process, the deletion of PHO92 should elevate target RNAs irrespective of the plasmids that are present (overexpression or not). Thus, overexpression of IME4 and MUM2 also should have no effect on the expression of PHO regulon target genes in haploid yeast cells without Pho92 (Δpho92). Pho92 consist of YTH domain which binds preferentially to m6A modification produced by methyltransferase, Ime4, in mRNAs. It is also shown to regulate transcription activator Pho4 by destabilizing its mRNA. In this mechanism of regulation of PHO4, absence of Pho92 should stabilize and activate PHO4 gene which could then initiate the transcription of PHO regulon target genes.

In wild type cells, presence of Pho92 has some effect on PHO4 regulation. Although PHO85 is expected to decrease due to over expression of IME4 and MUM2 increasing the stabilization of PHO4, the constitutive presence of Pho92 may counteract this effect, destabilizing PHO4 mRNA to some extent, leading to modest increase in the PHO regulon genes. However, over expression of IME4 and MUM2 cells in Δpho92 cells may lead to increased activation of Pho4 through both mechanisms of regulation. Overexpression of IME4 and MUM2 leads to decrease in PHO85 transcript levels. That together with absence of Pho92 leads to stabilization of PHO4. If our hypothesis is correct, this should lead to increased expression of PHO regulon target genes in Δpho92 cells compared to wild type cells.
RNA was extracted from haploid \textit{Apho92} cells containing vectors only (none) and \textit{Apho92} cells containing both \textit{MUM2} and \textit{IME4} (\textit{Apho92 M2 & I4}). The RNA was then used for RT-qPCR assay to study relative gene expression of phosphatase, \textit{PHO5}, of \textit{PHO} regulon. We expected to see the phenotype of \textit{PHO92} conserved through the genotypes. If the \textit{PHO92} protein is removed from the cell, \textit{PHO4} is stabilized, \textit{PHO4} is able to initiate transcription of \textit{PHO} regulon genes. Hence, when compared wild type cells, \textit{Apho92} cells should always have increased expression of \textit{PHO5} in all genotypes. As shown in Figure 18, the expression of \textit{PHO5} increases in \textit{Apho92} with vectors only (none) and in \textit{Apho92} (M2&I4). The t-test and ANOVA test was performed on the relative RNA abundance of \textit{PHO5} in wild type and \textit{Apho92} cells containing vectors only vs both background of cells overexpressing \textit{IME4} and \textit{MUM2}. The results showed significant increase in \textit{Apho92} cells containing vectors only and \textit{Ap92} (M2&I4) when compared to wild type cells containing vectors only as well as wild type cells with M2 & I4.
Mechanisms for IME4 effects on PHO RNAs

Increase in relative transcript abundance results from changes in RNA synthesis (transcription) or RNA decay (degradation) or both. Because we have only measured relative RNA levels, we can’t attribute any of the changes that we see to either or both of their mechanisms. For PHO4 target genes PHO5, PHO84 and PHO89, increases could be due to increase in PHO4 activity, with no direct effect on the PHO transcripts themselves. Alternatively, the PHO transcripts could be direct targets, with PHO4 activity playing a minor role or none at all. Kang et al. attributes increase in PHO RNA abundance in Δpho92 mutants to PHO4 stability but their results do not eliminate the possibility that the remaining PHO genes may also be the targets.
On the other hand, the PHO85 RNA is likely to be a direct target of IME4 and possibly PHO92. Fewer than 300 of the approximately 6000 protein-coding genes contain introns. Methylation is most commonly associated with RNA destabilization through sites near to the stop codon and within 3’UTR. Methylation also regulates alternative splicing and occur in high level in exon borders and especially in splice sites leading to exon exclusion. We considered that PHO85 splicing could be altered by IME4 genotype. PHO85 contains several potential methylation sites within and near its single intron. One of these sites is immediately adjacent to the AG dinucleotide of the 3’ splice junction (Fig 19).

We performed an assay for detection of spliced and un-spliced PHO85 DNA in haploid cells of different genotypes. The size of the intron in PHO85 is about 102 nucleotides. PCR primers to amplify the portion of the PHO85 pre-mRNA that includes the intron were designed.

Figure19: cDNA of 5’ region of PHO85 with intron. The 5' splice site and 3' splice site are indicated by an arrows. The forward and reverse primer are highlighted as bold and the start codon ATG is in italics. The m6A methylation sites are in bold and the adenosine is shown with asterisks.

We performed an assay for detection of spliced and un-spliced PHO85 DNA in haploid cells of different genotypes. The size of the intron in PHO85 is about 102 nucleotides. PCR primers to amplify the portion of the PHO85 pre-mRNA that includes the intron were designed.
cDNAs corresponding to unspliced mRNAs that include the intronic region and are expected to produce a 252 bp PCR product, the same length as genomic DNA. Mature mRNAs lacking intronic sequences are expected to yield a product that is 150 bp in length. We examined haploids deleted for IME4, PHO92 or both for relative amounts of un-spliced vs spliced RNAs (Fig 20a). The gel images were quantified using ImageJ and the fraction of unspliced molecules calculated for each strain was obtained. We also examined PCR products from RNA amplified from wild type cells that were overexpressing IME4, MUM2 or both (Fig 20b). We concluded that the splicing efficiency of the PHO85 pre mRNA is not altered by the deletion of IME4 or PHO92 (Fig 21a). The ratio of unspliced PHO85 ranged from 0.12 to 0.16 in wild type mutant in IME4 and PHO92. However, the splicing efficiency of pre mRNA seems to decrease with over expression of IME4 and MUM2 (Fig 21b). The corrected ratio for unspliced PHO85 in wild type overexpressing IME4 and MUM2 and grown in SC Drop-out ranged from 0.02 to 0.07 with the highest fraction of unspliced pre-mRNA occurring in the cells overexpressing IME4 and MUM2. This suggests that methylation of PHO85 pre-mRNA could inhibit its splicing. This result will be pursued in future work. We also observed that the splicing efficiency also appeared to be somewhat altered by the growth conditions of the cells. The fraction of unspliced RNA was significantly lower in cells grown in drop-out vs the SC casamino acids media.
Figure 20: Amplification of PHO85 region (with intron) in wild type cells mutant in IME4 and PHO92 (A) and wild type cells overexpressing IME4 and MUM2 (B). In Figure 20 A the samples considered were from wild type (lane 1), Δime4 (lane 2), Δpho92 (lane 3) and Δime4Δpho92 (lane 4) cells. In Figure 20 B, samples considered were from overexpressing cells with vectors only (lane 1), with IME4 (lane 2), with MUM2 (lane 3) and with both IME4 and MUM2 (lane 4).
We also cloned and sequenced a small number of molecules (12 total) to determine whether the correctly spliced products were formed in wild type cells carrying the vectors only vs wild type cells overexpressing IME4 and MUM2. The sequences showed no differences in the mutation and appeared to be spliced correctly.

**PHO5: Promoter Swap and GFP fusion.**

If elevated RNA levels are the results of reduced degradation (higher stability) of the PHO5 mRNA, then constructs that encode PHO5 RNA sequences but no PHO4-dependent promoter should lead to elevated RNA Levels in the Δime4 and Δpho92 mutant strains. In this scenario, it is the mature mRNA sequence that dictates degradation rate or relative stability.
Conversely, changes in *PHO4* levels (either activity or amount) will depend on the promoter, not the mRNA sequence. Either mechanism or both could be acting on any particular RNA.

To test this idea, we constructed a plasmid in which *GFP* is fused in frame to the full length *PHO5* gene, beginning with the ATG and including approximately 500 base pairs downstream of the stop codon. Transcription is driven by the *ADH1* promoter, which is not known to be dependent on *IME4*. We transformed the plasmid into wild type, *Δime4, Δpho92* and *Δime4Δpho92* cells. To analyze the effect of deletion of *IME4* and *PHO92* on these cells, we performed a western blot assay on the cell extracts obtained from each of the transformed cell strains using anti-GFP antibody as primary antibody and HRP-conjugated anti-mouse IgG for the secondary antibody. We also used anti-PGK1 antibody to ensure that equal amount was loaded for each sample. We saw an increase in the *PHO5* expression in *Δpho92* and *Δime4Δpho92* cells (Fig 21). We didn’t see any significant change in *PHO5* expression in *Δime4* cells. However, the signal from PGK shows lower cell extract loading in the lane with *Δime4* which could have resulted in decreased signal from the sample. This results concludes that in the absence of *IME4* and *PHO92*, the expression of *PHO5* increases despite being driven by *ADH1* promoter instead of its own. Therefore, the increase of *PHO5* is due to direct methylation of the mRNA by *IME4*. The increase in transcription activity may play a role but is not solely the reason for *PHOS5*’s elevated expression.
Figure 22: Western Blot of ADH1-GFP-PHO5 extracted from wild type (lane 1), Δime4 (lane 2), Δpho92 (lane 3) and Δime4Δpho92 (lane 4) using a primary antibody against GFP and an HRP-conjugated anti-mouse IgG secondary antibody. PGK1 was used as a loading control.
Discussion

N-methyladenosine (m6A) modification was first discovered in 1970. It is a highly conserved and one of the most abundant internal modifications in mRNAs and long non-coding RNAs (lncRNAs) in higher eukaryotes. In 2012, two different groups applied m6A RNA immunoprecipitation followed by high throughput sequencing (MeRIP-seq) to map m6A RNA methylomes. These studies showed that m6A is distributed in more than 7,000 mRNA and 300 non-coding RNA (ncRNA) transcripts in human cells. The m6A sites are enriched around stop codons, in 3′ untranslated regions (3′UTRs) and within internal long exons. There are also sites near intron/exon borders (Meyer et al. 2012; Dominissini et al. 2012). A MeRIP-seq experiment performed on short mRNA fragments in the yeast mutant in the methyl transferase, Ime4, showed elevated methylation sites around 3’UTRs with the same consensus sequence as in human demonstrating that the distribution pattern of m6A is conserved from yeast to humans (Fu et al. 2014).

m6A can be recognized and bound by YTH domain family protein through different selective reading mechanisms. These m6A-specific RNA-binding proteins can directly bind to the m6A and lead to post-transcriptional regulation of gene expression. The human YTHDF2 regulates methylation-dependent RNA degradation. Other reader proteins may exist that affect RNA splicing, storage, trafficking and translation among other mechanisms. The presence of m6A could also affect the mRNA stability. It could increase or decrease the affinity of mRNA for other RNA binding proteins. The presence of m6A may also change the secondary structure of RNA and make binding sites for other RNA binding protein available (Liu et al. 2015; Fu et al. 2014).
In yeast, Pho92 of PHO regulon pathway is an YTH domain family protein and is expected to be the m6A reader. In this study, we analyzed the role of the methyltransferase, Ime4, and possible m6A reader, Pho92, in the PHO regulon pathway in *S. cerevisiae*. We performed RT-qPCR assays and analyzed the change in RNA abundance in PHO regulon genes in the presence and absence of IME4 and PHO92. We haven’t measured the levels of m6A directly. Hence, this is an indirect approach to quantify the effect of Ime4 in the PHO regulon genes. As a direct approach, we tried to quantify the protein level corresponding to PHO genes in IME4 mutant and overexpressing cells through phosphatase assay. Our results were inconclusive as none of the cell genotypes showed significant differences in phosphatase expression relative to the wild type. This may be due to presence of various phosphatases that are expressed constitutively in yeast cells. The m6A in the mRNA can also be directly quantified through methyl ImmunoPrecipitation (IP). The destabilization of mRNA can also be evaluated directly using RNA polymerase inhibitors.

Before RT-qPCR assay, we had to confirm the efficiency of the primers that we had made against each target gene. Primer efficiency and specificity are very important for the success of RT-qPCR. Extreme precaution was taken to make sure that the primers were efficient and specific for the desired targets. Poorly designed primers may lead to mispriming including self-hybridization of primers and also to unintended targets in the template. In both of these cases, the SYBR Green that binds to any dsDNA present in the reaction mix will detect these mispriming events as amplifications and will produce invalid data. In some cases, poorly constructed primers may also form primer dimers increasing fluorescence and resulting in inaccurate quantification of the amplicons. Other factors that might affect the specificity and efficiency of primers are amplicon length and primer quality. Hence, a successful RT-QPCR requires efficient
and a highly specific primer pair that doesn't produce primer dimers. For validity, RT-QPCR should produce short amplicons with results that are consistently reproducible (Fig 6, Fig 7, table 4).

Through RT-qPCR of haploid Δime4, Δpho92 and Δime4Δpho92, we analyzed the RNA abundance of the PHO genes in the absence of IME4 and PHO92. We saw increase in all PHO regulon genes (PHO5, PHO11, PHO84, PHO89) in Δime4 cells and concluded that Ime4 does affect the expression of these genes (Fig 8 and Fig 9). The RNA abundance was higher for PHO regulon genes PHO5, PHO84 and PHO89 in Δpho92 and Δime4Δpho92 as well (Fig 10 A, B, D) while the PHO11 didn’t show any significant difference (Fig 10 C). However, the elevation in RNA abundance of PHO genes was less in Δpho92 cells than in Δime4Δpho92 but not statistically significant in all cases. This could mean that IME4 leads to transcript degradation in a PHO92 dependent pathway but could also do so by a mechanism that is independent of PHO92 in lower levels. They are certainly not additive as the double mutant is the same as Δime4 itself. This increase can be attributed to more transcription of the PHO genes due to more active transcription of transcription factor PHO4 or less degradation of the PHO genes themselves or both of these. The experiments do not distinguish whether these RNAs are directly targeted by IME4 methylation or if they are elevated indirectly due to the methylation of PHO4 gene upstream in the pathway.

Dr. Zhengchang Liu and the group performed beta-galactosidase assays using different PHO regulon genes promoters in wild type, Δime4 and Δpho92 cells. The data showed increased activity of the PHO11 and PHO84 promoters while PHO5 didn’t change significantly. If the effect of Ime4 on the PHO RNAs were direct on RNA and not relying on the promoter, beta-galactosidase fusions using only the promoter with no other PHO sequences (no RNA) should
not show any activity. This is not what one would expect if the only relevant target of Ime4 is the RNA itself. These results demonstrate that the promoters of these genes are more active in the Δime4 and Δpho92 mutant strains consistent with the idea that there is increased PHO4 RNA abundance levels and more PHO4 activity). However, the activity elevation was modest for these genes, close to 50%, in beta-galactosidase assays which is much lower than 2 to 5 fold increase in RNA abundance seen through RT-qPCR assays. Hence, the increase in the PHO genes seen through beta-galactosidase assay and RT-qPCR assay may be due to both increase in transcription as well as stability of the RNAs due to the absence of IME4 and PHO92. Again, this can be tested by actually determining the methyl level on these RNAs.

We also have to consider that each of the PHO regulon genes have different promoters. Some of these promoters like in PHO5 are longer than the rest. The arrangements of the Pho4 binding sites on each of the promoters are also different. These promoters could be modified through other mechanisms. For example, PHO5 is protected by nucleosomes in repressed condition which gets disrupted on activation (Venter et al. 1994). Similarly, these promoters can be activated under other conditions besides phosphate starvation. The prime example of this scenario is Pho regulon gene PHO85 that codes for Cyclin Dependent Kinase that can couple with ten different cyclins and is involved in many separate transduction pathways (Carroll and O’Shea, 2002). Hence, the binding site of PHO4-PHO2 within the promoter varies in each PHO mRNA which might affect the transcription activity of the mRNAs causing some mRNAs to result in more proteins than others.

PHO85 RNA was detected among approximately 1300 methylated RNAs in a genome methyl IP experiment in sporulating yeast cells, SK1 (Schwartz et al. 2013). PHO85 was enriched approximately 16 fold in extracts from cells wild type for IME4 vs Δime4 mutant cells.
None of the other *PHO* RNA were detected in that study, although two of the *PHO85* cyclins *PCL5* and *PCL6* as well as the *PHO81*, inhibitor of *PHO85*, were observed. To better understand the mechanism of Ime4 methylation, we examined RNA sequences for *PHO85, PHO5, PHO4, PHO84* and *PHO89* for potential methylation sites within the coding region but especially near stop codon, in the 3’ UTR and near the intron/exon junction of *PHO85*, the only intron containing transcript that we studied. All of these RNAs contained potential methylation sites in regions of potential interest (Table 3).

There is some debate about the actual consensus site of Ime4 methylation. Most studies agree on RRACH while others insist RGAC or RRACU to be required for m6A modification. We found all the *PHO* genes have GAC consensus sites. The frequency of the consensus sites usually increased in 3’UTR close to stop codon which is characteristic to m6A modification. The sequence of all the genes studied with methylation consensus sites highlighted is presented as supplemental information.

Among the *PHO* genes, *PHO5* sequence showed the highest number of sites with 10 consensus sites for m6A within the coding region including 3 within 50 nt upstream of the stop codon. One of these 3 sites is a perfect match to the extended GGACU consensus. There are no consensus site in *PHO5* within the annotated 3’ UTR length upstream of the polyA sites. All these consensus sites are in the location characteristic of m6A modification. The sequence of other *PHO* genes, (*PHO4, PHO11, PHO84, PHO85* and *PHO89*) were also analyzed. *PHO4* showed 7 methylation consensus sites in the coding region and two in the 3’UTR, 138 nt before polyA sites. *PHO11* has 9 consensus sites in the coding region, none in 3’ UTR and none within 500 bp of the stop codon. *PHO84* has 5 sites in the coding region including one site 120 nt upstream of the stop codon which could both be methylated. Similarly, *PHO85* has 4 sites in the
coding region among which 2 are in the intron. There is one consensus site approximately 130 nucleotides downstream of stop codon but further than the annotated polyA sites and UTR length. None of these sites are close to the stop codon or 3’UTR but methylation can still take place in coding region. Lastly, there are 6 consensus sites in the PHO89 in coding region, but the nearest consensus site to the stop codon is around 250 nt upstream. There is no consensus site in 3’UTR. The UTR for PHO89 is quite long with numerous polyA sites but no m6A sites (Table 5).

**Table 5: Methylation Consensus site on PHO regulon genes.**

<table>
<thead>
<tr>
<th></th>
<th>Coding Region</th>
<th>Near Stop Codon</th>
<th>3’UTR Region Upstream of PolyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHO5</td>
<td>10</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>PHO4</td>
<td>7</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>PHO11</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PHO84</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>PHO85</td>
<td>4 (2 in intron)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>PHO89</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

All of the mRNA for these genes could possibly be direct targets of IME4. Some, like PHO11 are not likely to be direct targets for IME4. The nonexistent m6A sites in the 3’UTR and near stop codon suggests low methylation of PHO11. The non-significant change in RNA abundance of PHO11 in Δime4, Δpho92 and Δime4Δpho92 cells compared to the wild type in RT-qPCR assays also suggests that PHO11 isn’t affected by the absence of IME4 and PHO92 (Fig 11b). Based on only methylation sites, PHO89, PHO85 and PHO4, due to lower number of m6A sites at expected methylation region of mRNA, may or may not be the direct targets.

PHO5, with placement of 3 consensus sites within 50 nt of the stop codon is more likely to be a direct target of IME4 than others. One of the consensus near the stop codon is perfect consensus for the methylation. The increase in RNA abundance of PHO5 in Δime4, Δpho92 and
Δime4Δpho92 cells compared to wild type also supports elevated level of methylation in PHO5. In order to determine if PHO5 is direct target of IME4, we constructed a fusion plasmid where PHO5 was driven by another promoter, ADH1, attached to GFP. We transformed this plasmid in to BY wild type, Δime4, Δpho92 and Δime4Δpho92 cells, and performed a western blot on cell extracts obtained from these transformations. There is no known effect of IME4 on ADH1, so, any change in the expression of GFP is due to PHO5 coding sequences rather than its promoter. We used an anti-GFP antibody to analyze the blot. The results showed increase in PHO5 expression in Δpho92 and Δime4Δpho92 (Fig 22). Since the expression isn’t driven by PHO5 promoter, it is certain that the transformed PHO5 RNA sequence is actually a direct target of Ime4. Since we know the positions of methylation consensus sites for PHO5, we can determine the actual methylation sites. We can construct another plasmid with the same ADH1 promoter and GFP but ligate it to only the region surrounding the stop codon and 3’UTR of the PHO5. If 3’UTR region is the methylation site for IME4, there shouldn’t be any difference in the expression of GFP vs previous experiments. We can also determine if one or multiple of the 3 consensus sites close to the stop codon is methylation site in PHO5 through site directed mutagenesis. Analyzing the expression of PHO5 with different mutants could decide which consensus sites is most likely to be methylated. These experiments can be corroborated using methyl IP to look at changes in enrichment of PHO5 transcripts in the immune precipitates.

In our study, we also saw an increase in the PHO85 RNA abundance in Δime4, Δpho92 and Δime4Δpho92 cells relative to the wild type (Fig 13). The elevation of PHO85, the kinase, should be repressive in the mutants. It is a negative regulator of Pho4. In high phosphate, Pho80-Pho85 (cyclin/CDK) complex phosphorylates PHO4 resulting in its localization in the cytoplasm. However, when phosphate is low, Pho80-Pho85 is inhibited by Pho81. As a result,
Pho4 isn’t phosphorylated and moves to nucleus where it activates transcription of \textit{PHO} genes. This should result in the decrease of \textit{PHO} regulon target genes instead of the increase we saw.

Having said that, there is more than one ways in which Pho85 activity can be altered. We used SC media to grow our cells. The phosphate present in the media was calculated to be 7.4 mM. The saturating phosphate concentration in a growth media for \textit{S. cerevisiae} is shown to be around 10 mM (Springer et al. 2003). Although the amount isn’t saturating in our media, there is sufficient phosphate that Pho85 will not change even when RNA abundance is high. In our study, we are looking at the basal expression under repressive conditions. So, if there is sufficient phosphate present in the growth media, Pho4 may already be fully phosphorylated and no increase in \textit{PHO85} kinase activity will matter because system is already fully repressed. Also, higher RNAs does not necessarily mean more protein if the RNA is not translated efficiently. \textit{PHO85} has around 10 cyclin partners. The expression of \textit{PHO85} is regulated by these cyclin partners and inhibitors. There may be enough inhibitors to cope with any excess \textit{PHO85} that is produced.

On the other hand, \textit{PHO85} RNA abundance seem to decrease in BY cells overexpressing \textit{IME4} and \textit{PHO92} (Fig 17). Further investigation through a splicing assay on the \textit{PHO85} gene showed decreased splicing efficiency of \textit{PHO85} mRNA in cells overexpressing \textit{IME4} (Fig 21). Several conditional protein splicing systems have been reported so far in which splicing of mRNAs are affected by change in temperature, light, protease activity and the presence of molecules (Cheriyan and Perler 2009; Mootz 2009). The presence of increased level of m6A in cells overexpressing \textit{IME4} could negatively affect the splicing efficiency of \textit{PHO85} mRNA resulting in fewer mature \textit{PHO85} mRNA molecules.
As mentioned before, Pho85 interacts with different cyclins and performs multiple functions in S. cerevisiae. It is involved in several transduction pathways including cell-cycle progression and metabolism of nutrients. Deletion of Pho85 has resulted in series of pleiotropic responses in yeast. Δpho85 cells constitutively express *PHO* regulon genes. Also, cells without Pho85 demonstrate slow and poor growth with G1 delay, hyper accumulation of glycogen, abnormal morphology and sporulation defects among other phenotypes (Carroll and O’Shea, 2002). Although these responses require the phosphorylation of different substrates by Pho85 and the effects of the phosphorylation may vary according to the substrates, all appear to be involved in responses to changes in environmental conditions.

Ime4 is also expressed or repressed according to environmental changes. In nutrient rich condition, yeast cells undergo mitosis. However, when the nutrient availability limits mitotic growth, diploid yeast cells undergo sporulation or filamentation. Also, during the presence of pheromone, the yeast haploid MAT a and MAT α can mate to form diploids. Ime4 is induced during mating and is required for the initiation of sporulation. It is repressed during filamentation. More than thousand genes are expressed during sporulation. Interestingly, *PHO* regulon genes are repressed during the process. This is odd due to the fact that the sporulation media that induces sporulation in yeast consist of no phosphate. Therefore, it is safe to assume that sporulation in vivo also occurs in low phosphate condition. This should increase the expression of *PHO* genes during sporulation contrary to what has been demonstrated. This phenomenon can be explained if *PHO85* is a direct target of Ime4. Since *PHO85* tends to mediate responses during environmental stress, Pho85 activity and cyclin partners change during nutrient starvation and sporulation. That could explain the repression of *PHO* genes in these conditions. It would be interesting to see how *PHO* genes respond in Δime4, Δpho92 and
Δime4Δpho92 cells if they were grown in Sporulation Media (SPM) where IME4 is naturally induced and phosphate is minimal.

In summary, IME4 affects the PHO regulon target genes through several mechanisms. We suggest that PHO5 is a direct target for IME4. This could also be true for other PHO regulon genes and future experiments are required to confirm this. The increase in the PHO regulon target genes in cells mutants for IME4 and PHO92 may also be due to increase in transcription activity of these genes when PHO4 mRNA stabilizes in the absence of IME4. Although surprising, the increase in some PHO regulon genes observed in cells overexpressing IME4 could be attributed to decreased splicing efficiency of PHO85 in the presence of IME4. Also, all the PHO regulon genes have different promoters. These promoters vary in weather they contain binding sites for other activators and repressors. They can also be activated in response to different stimuli. The number of characteristic m6A methylation sites in PHO regulon mRNAs also differ. Hence, the transcription activity of PHO regulon genes can be affected by cellular conditions other than phosphate starvation. Our results are primarily based on the mRNA abundance of different PHO genes in IME4 mutants relative to the wild type. A more direct approach in quantifying the m6A level and translated protein amount is required to better understand the mechanism with which IME4 affects PHO regulon.
Conclusion

We concluded that Ime4 does have an effect on PHO regulon pathway mRNA. These effects may vary according to the conditions of growth and construct of the cell strain. The increase of PHO regulon RNA abundance in Δime4 cells demonstrates that Ime4 affects the PHO genes. The non-additive increase in PHO genes RNA abundance in Δpho92 and Δime4Δpho92 suggests that Ime4 may affect the PHO regulon target genes through Pho92 in a single pathway. The increase in the PHO regulon genes in wild type cells overexpressing IME4 and MUM2 hints that there might be multiple mechanisms with which Ime4 is regulating the PHO regulon. The increase of PHO85 RNA abundance in the mutant cells and decrease of it in overexpression cells supports the idea that Ime4 may affect the PHO regulon through Pho85 protein upstream in the pathway. Since Pho85 is involved in many pathways and can couple with ten different cyclins, it might be regulated differently in the mutant strains vs cells that are overexpressing IME4. We looked at the splicing efficiency of PHO85 in cell mutant in IME4 as well as cells overexpressing it. These preliminary results indicate that PHO85 efficiency is intact in mutant cells and lowered in overexpression cells. Hence, IME4 could affect splicing efficiency of PHO85 when overexpressed. Along with PHO85, Ime4 may also affect the PHO regulon genes through direct methylation of their RNAs. A western blot was performed using PHO5 coding sequences cloned in to ADH1-GFP expression vector confirms this theory PHO5 RNA is a direct target of the IME4-PHO92 pathway.
Bibliography


and its analogs. Structural features correlated with synthesis and methylation of mRNAs of cytoplasmic polyhedrosis virus.


Supplemental Material

PHO sequences, ATG to annotated polyA sites downstream. Possible sites boldface ital. Stop and pA underlined.

PHO5

1  ATGTTTAAAT CTGTGTTTTA TTCAATTTTA GCCGCTTCTT TGGCCAATGC
51  AGGTACCATT CCCTTAGGCA AACTAGCCGA TGTCGACAAG ATTTGTACCC
101 AAAAAGATAT CTTCCCATTT TTGGGTGGTG CCGGACCATATA CTACTCTTTC
151 CCTGGCGACT ATGGTATTTT TC intriguate TTCAATTTTA GCCGCTTCTT TGGCCAATGC
201 GCAACTGCAATGGCTGTTGGTGACGTGACCATAAAGATACCATCCTGGTGCCAA CACTTTGAGT GCTTGTAACT CATGTCCTGC
251 TGGGCTAAGAC TATCAAGAGT ACATGGTATA AGTTGAGCAA TTACACTCGT
301 CAATTCAGC GCTCATTTGCT ATACTCTTTC
351 CCGTGATGAC GATGATTTTG AAATGGGAAC CACTTTTGCC AACTGCGGACG
401 ATGGTTTGAA CCCATACTG ATGGTGAATGA ACGGATGACAC GACTTTCCGT
451 GACTTCTTTG CTTCAATACGG TTACACTGTC GAAAACAAAA CAGGTTTCCGC
501 CGTTTTTACC TCTAATTCTCA AGAGATGTC AAGATGCTGCT AGTACAGCTTCA
551 TTGATGGGTTT AGGTGACCGAA TTCAACATCA CTTGCAGAC TATCATGTA TGTCAAGTAA ACGTTGAAGC
601 ACTTTGAGT GATGGAAGAGA AGTAAGGAA AAGGATGACAC GACTTTCCGT
651 TTGGAGACT TAC GATGCCAATG ATGACATTGT AAATGAAATAC GACACAACCT
701 ACTTTGAGT GATGGAAGAGA AGTAAGGAA AAGGATGACAC GACTTTCCGT
751 TTGACCTCAA CTGACGCTAG TACTTTATTC TCTGTGGTGTG ACTGTCCTGC
801 GAACGTAAAT GGTTACAGTG ATGTCTGTGA TATTTTACGT AAGGATGAAAT
851 TAGTCCATTA CTCTCTACTAC CAAGACT TGC ACACCTATTA CCATGAGGGT
901  CCAGTTACG ACATTATCAA GTCTGT CGGT TCCAACTTGT TCAATGCCTC
951  AGTCAAATTA TTAAAGCAAA GTGAGATTCA AGAC CAAAG GTTTGGTTGA
1001 GTTTTACCCA CGATACCGAT ATCCTAAACT TTTTGACCAC CGCTGGTTATA
1051 ATTGACGACA AAAACA AACTGCGGCA TACGTTCCAT TCATGGGCAA
1101 CACTTCCAC AGATCCTGGT AGTTCCTCTCA AGGTGCTCGT GTCTACACC
1151 AAAAATCCA ATGTTCTAAC GACACCTACG TCAGATACGT CATTAACGAT
1201 GCTGTTGTTC CAATTGAAAC CTGTTCCTACT GGTCAGGGTT TCTCTTGTTGA
1251 AATCAATGAC TTTCAGGACT ATGCTGAAAA GAGAGTAGCC GGTACTGACT
1301 TCCTAAAGGT CTGTAACGTC AGCAGCGTCA GTAACCTAC TGATGGGACC
1351 TTCTACTGGG ACTGGAACAC TACTCATTAC AACGCCAGTC TATGAAAGAC
1401 ATAGTTTGTTGATAAAT GIF ATATTTGAAA ACTAAATACG AATACCACCA
1451 TTTTTATCT AAATTTTGGCC GAAAGATTAA

PHO4

1 ATGGGCCGTA CAACCTCTGA GGGATAACAC GGTATTGTTGG ACGATCTAGA
51 GCCCAAGAGC AGCATTCTTG ATAAAGTCGG AGACT TTATC ACCGTAAACA
101 CGAAACGGCA TGATGGGC CGGAGGACTTCA AGACGCAGTC TTCGAGCTC
151 AACAGTCAGG AGAACCACAA CAGCAGTGAC GATGGGAACG AGAATGAAA
201 TGAACAAGAC AGTCTCGCCGT TGGACGCAC CT AGACCGGCCT TTTGAGCTGG
251 TGGAAAGGTAT GGATATGAC TGGCCGACGT CTCACCAGCC GCACCACTCC
301 CCAAGCTACAA CTGCTACAAT CAAGCCGCGG CTATTATATT CGCCGCTAAT
351 ACACACCGCA AGTAGGGCGTT CCGTAACCCT TCGCCTTGAAC TGGCTGCTTA
401 CTGCTACTTC CACCACATCC GCTAACAAGG TCCATTAAAAA CAAAGTATAT
451 AGTAGTCCGT ATTTTGAACAA GCGCAGAGGT AAACCCGGGC CGGATCATC
501  CACTTCGCTG TTCGAATTGC CCGACAGCGT TATCCCAACT CCGAAACCGA
551  AACCGAACC AAAGCAATAT CCGAAGTGA TTCTGGCCTC GAAACAGCACA
601  AGACGCCTAT CACCGGTCAAC GGCACAAGACC AGCAGCAGCG CAGAAAGGCGT
651  GGTGAACGGTAC ATGGTAGTCTC CTGTAATTGG CCGCACTGGA TCGAGCCATT
701  CGCGGCTGCT GAGTAAGCGA CCGTCATCGG GCGCGCTCCTTG GAACGATGAC
751  AAGCGCGAAT CACACAAGCA TGCAAGACAA GCACGCGTGA TCACGATTAGC
801  GGTGGCGGTCA CACGACTGGG CACGTTCTTAAT CCCGCAGGAG TGGAAACAGC
851  AAAATGTGTC GGCACCGCGCG TCCAAAGCGA CCACGCGTGA GGCAGGGTCTG
901  CGGTACATCC GTCACCTACA GCAGAAACGG AGCAGCTGAGC CGTGCAACCC
951  TGGGAAGCAG TTTCCGGGCGA TATCGAAGCTG GGGCGCGCCT CCCCTGCGCG
1001 GTGCTTTTGGT AAGAGGGCGG TTTGGCTGGAA AGTGGGCCCAC ACCGGGGTTT
1051 CGAGATTA GGA CTTACTCAG TCTTAAGGCG AGTATTTGGT GCGGCTTTAT
1101 TGCACTATTT GTATAACGCA ACTCAACATTA AAGAGACGAC ACATAAAGAC
1151 TTACACCTAC ACA

**PHO85**

1  ATGTCTTCTT CTTCACAGTA TGTAGTTTTTC TAGTCAAGTA TCATTGGAAA
51  GTAAAAAGACT AGAAATGTATG AATACTAACA TATATACGAG AAAAAATCAA
101  CCTCGAGCTC TAT GACACA TTAAGCAGT TAGAAAAGCT TGGCAATGGA
151  AGGTATGCCA CAGTGTACAA GGGACTGAAC AAAACCACAG GGGTATATGT
201  TGCCCTGAAA GAGGTAAAGC TGGATTCAGA GGAAGGTACA CCCTCTACGG
251  CCATCCGTGA GATCTCCCTA ATGAAAGAAT TGAAACATGA GAACATTGTT
301 AGACCTTAAGT ACGTTATTCA CACAGAGAAG AAGTTGACTT TGGTTTTTGA
351 ATTCATGGAC ACGATTAA AAGAAATACAT GGATCCCAGG ACCGTGGGCA
401 ACACACCAAG AGGGCTAGAA CTAAAACTTG G T TAAATACTT CCAGTGGCAA
451 CTACTGCAAG GGCTGGCCTTT TTGCCCATGAA AACAAAGATT TCACCACGTGA
501 TTTAAAAACCT CAAAACCTAT TAATCAACAA GAGAGGCCAG TTGAAATTGG
551 GTGATTTCCGG TCTGGGCCCCGT C GCTTTCGGTA TTCCGGTCAA CACATTTTTA
601 AGCCGAAATCG TAAACGTTGTG GTACCGTGCT CCTGATGTGC TAATGGGGTC
651 TAGGACGTAC TCCACATCCA TTGATATATG GTCGTGTTGG TGCATTTCTTG
701 CGGAAATGAT AACGGGTAAG C CTTTGGTTTC CTCGCCACCAA CGACGAAGAA
751 CAACTGAAAT TGATCTTTGCA CATCATGGGA ACTCCTAATG AGTCCCTATG
801 GCCCAGTGT ACAAAGTTAC CC AATACAAC CCCAAATATC CAGCAACGAC
851 CACCAAGAGA C TACGTCAA GTATTGCAAC CACACACCAA AGAACCCTGA
901 GACGGGAATC AGCGCCAAGC AGGCTCTGCA TCACCTTTTG TTTGCAGAGT
951 TATGAGGCTG AGCGCCAAGC AGGCTCTGCA TCACCTTTTG TTTGCAGAGT
1001 ACTACCACCA CGCTTCAGAGAGA CCTACGTCAA G T ATTATGCAAC CACACACCAA AGAATCTGA
1051 GTAATTATAA TGATAATAAT CATCTATAAT ACACTACTAT TACTTTAGTC
1101 TCATTTACG ACAGCCTTTA CGACGGCTTT TACCGCCTTT AAGGCGGAC
1151 PHO84
1 ATGAGTTCCG TCAAATAGAA TACCTTTCAT GTTGCTGAAA GAAATCTTCA
51 TAAAAGAACCT C TACCCGAAAG GTGCTAACAT GGCCTTCCAC AACCATTGGA
101 ATGATTTTGCC CATATTGAAA GCCTTCTGG AAAGAAAGAAG ATTGGCTTTG
151 GAGTCCATCG ATGACGAAAGG TTTGCGTTTG GAACAGATTA AGACCATCTGC
201 CATTGCCTGGT GTTGCTTTCT TGACAGATTC TTAATGATIT TTTGCCATTA
251 ATTTGCGCAT GATATGATG TCCACGCTTT ACTGCCCAAG TAGTATGCCA
301 GGTCAAAGTC AAAACCTTGT GAAGGTTTTCC ACTCTTGTG GTACTGTGAT
351  TGGTCAATTT GGTTTTGGTA CTTTTAGCTGA TATTGTGGT CGTAAGAGAA
401  TTTATGGTAT GGAACCTATT ATCATGATTG TCTGTACCCT TCTGCAAACC
451  ACTGTTGCTC ATTCTCCTGC TATTAACCTT CTTGCTGTTT TAAACATTCTA
501  CCGTATTGTC ATGGGTTATG GATACGGTGG TGAATACCCCA CTATCTTCTA
551  TTATTTACTC TGAATTGGCC ACTACCAAAT GGAGAGGTGC CATCATGGGT
601  GCTGTTTTTG CTAACCAAGG TTTGGGCTCA ATCTCCGGTG GTATCATCGC
651  TCTTATCTTG GGGCTCTGTT ACAAGGGGCGA ACTAAGATAC GCAAACCTCTG
701  GTGCTGAATG TGATGCTAGA TGTTAAAAAG GTTGTGAAGA AATTGTGGAGA
751  ATCCTTATTG GGGTTGGGTCA CGTTCTAGGG TGCTGATGTT TGTATTTCAG
801  ATAACCTATT CCAGAGATCTC CTGATATATCA ATTGAGATGT AAGCCTAAGT
851  TGGAACCTTG GCCTGCGGCA CAAGAACCAAG ATGGCGAAAA GAAATTCAC
901  GACACCAGTG ATGAGACAT GCAGAAATACGG GTTTTGAAA GAGCTTCTAC
951  TGCCGTCGAA TCTCTTGACCT ATCATCCTCC AAAGCTTTCG TTTAAAGATT
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1151  ACAAGAAAAT GTATGATACG CTGTCCCGTGA ATCTGATTTT GATTTGTGCT
1201  GGTTCATTAC CTAGTTCGTC TGATCGCCTC TTCACTCGCC ATATAATCGG
1251  TAGAAACCCA ATTAATTTAG CCGTCTTTCAT CATTTGGACC GCTTTGGCTT
1301  ATGATCGGAT CTCTCCTCTG GTATGTTGCT TTTCTCCGAG GTGACCATGC
1351  CTTTACGTCA TTTGTTCAAT CTCTCCAAAA TTTGCTCCAA ACACAACCAC
1401  ATGATTGTGT TCTGGTTGAGT GTTTTCCAAA TCTGTACAGA TCTACTGCTC
1451  ATGGTATTTTC TGCTGATCTG GGTTAGGCTG GTGCCATTAT TGCAACAAACC
1501  GCTTTGGGTA CTCTAATCGA CCATAACTGT GCTAGAGACG GTAAGCCAAAC
1551  CAACTGTTGG TTACCTCAG TCTAGGAAAAT TTTCGCCTTA TTCTCTTGTT
1601  TGGGTATCCTTT CAAACCTTG TGATCCCAG AAACAAAGAG AAAAGCTTA
1651  GAAGAAATTA ACGAGCTATA CCACGTGAA ATCGATCCTG CTACGCTAAA
1701  CTTCAGAAAC AAGAATAATG ACATTGAATC TTCCAGCCCA TCTCAACTTC
1751  AACATGAAGC AAAAAACGCT CAAAGATGCA CTAAAACTTG TAAACTAGAA
1801  CAAATAATAC AAAAAACATT TTATAAACTT ATTATCA AACCCCTTACATA
1851  ATCTATAAAT ACTGTCAGGT TACATATTTA TTTGCATATT TCTTTTAATT
1901  TCATTAC

PHO89

1  ATGGCCTTTC ACATATTTTG CTAATATTCT GCCATTGCAA TGTTATTGTC
51  ATTTTTTGAT GCCCTTAACA TCGGGGCAAA CGACGTGGCG AACTCATTTCG
101  CGTCTCCGAT CTTTCCTAGA TCTTCAACG ACTGGCAAGC CATGGTTTTG
151  GCGGTCTCTG TGTAGGCTTT GGCTGTCGGC TTGCAAGGC CAAGAGTTTC
201  TGTCATCTC AAGAACAACA TTATAAGATC CTCCATTTTT ACCAACGACC
251  CCGCTTTT AATGCCCTACT ATGACCAGTG CTTTGAATGG CTCGFTTTGT
301  TGATTAACAG TTGCTACTGC AATCGGAATG CCAGTTTCTA CTACACATTC
351  CATAACTTGG GTGCAATTGG GCGCTGGGAG GGTCAACTTC ATTTATATTC
401  GTATCCTTTTC ATGTGGAGAT GGTCACTTCT AAATTAATTG GCACTGATGC
451  ATATCCGAA TTTGGCCAGG CGGACTCCGA GCTATAGTTG TTTCAAATTTC
501  TAGGTTTTCT GTCCCGGAAG TTAAGTCTTT GGAAGAGCAT ATTAAGAATG
551  CCTAATCGCT GGTGGGTGTTT TTAAGTCTTT TTGAAGATCG TTTGAACATCA
601  ATGTGGATTG TCTGGAAAGGG TTCTCCAACG CTACACTTGG AGAATTATTC
TGAAACTGAA ACTGCCGTAT CTAATGTTTCT CACAGGTGCA ATGCTTTCGA
TCGTTTACTT CATCTTTTTC TACCCATTCT ACAGAAGAAA AGTTTTGGAC
CAAGATTGGA CACTAAAGTT GATTGATTT TTCAGAGGTC CATCTTTTTTA
CTTTAAGTCC ACTGATGCTA TCCCACCAAT GCCAGAAGGC CATCAATTAA
CAATCGATTA TTACGAGGGC AGAAGAAATC TTGAACACAC TGTTTCTGTT
GAAGACGAAG AAAATAAGGC AGCGAGTAAT TCTAATGATT CCGTCAAAAA
CAAAGAACAC ATCCAGGAAAG TGGACCTGGT TAGAACTGAA ACTGAACCAG
AAAAACAGTT GTCAAACGAAA CAATATTGTT GGTCATTATT AAGCAGAGA
CCTAAAAAT GGCCTTTATT GTTCTGGTTA GTAATCTCTC ATGGTTGGAC
TCAAGACGTC ATTCATGCTC AAGTTAATGA TAGAGATATG TTATCTGGTG
ACCTGAAAGG AATGACGCAA AGATCTAAAT TTTACGACAA AGTACTGAA
TTATCTATT CGGTTCTCCA AGCCATTACT GCAGCCACTA TGCTCTTCGA
TCATGGAGCT AATGACGTTG CCAACGCTAC TGGTCCTTTG TCTGCTGTGT
ATGTGATCTG GAAGACTCAG TGGACCTGGT TAGAACTGAA ACTGAACCAG
GCTACCCAAT TAGGTATTCC TACCTCAACA ACCCAGATCG CCGTGAGGTG
ATTGTCGCT GTTGGTTATG GTAACAAGGA TCTCAAATCA GTTAATTGGA
GCTACCCAAT TAGGTATTCC TACCTCAACA ACCCAGATCG CCGTGAGGTG
TATTGTCGCT GTTGGTTTAT GTAACAAGGA TCTCAAATCA GTTAATTGGA
GAATGGTAGC CTGGTGGTAT TCTGTTGGGT TTATACTTT ACCAATTGCT
GGTTGATTTG CCGATCATG AAATGGATTT ATTTTGAATG CTCCTCGCTT
TGTTGTGGGA TACCAAATGA CATAACATC ATGTGTTTCG GTTATGAATA
CAGTTTTCACA ACATTAGGTA GAACCTTTAT TGCTCAGGTA CTTAAGGTT
1801  AAAACATTTG TAATTTAAAA CCTTTAAGAT GCATTAATTT TTTTCTAATT
1851  TATCAGTTGA TCTGCAAATT CCTGGCAATGG CTATGTGTAT TTTTTTTTTT
1901  TATGAATTTCC ATCAATTTAA ATAGAAAACACT AAACAAATA ATAAATAGAG
1951  TATTACAGAG TCGTAAAAAA AAAAAAAAGAA AAGAAAAAAG AAAATCTACA
2001  ATCAAAATAGC AGCAGTACCT

PHO11

1  ATGTTGAAAGT CAGCCGTTTTA TTCAATTTTA GCCGCTTTTT TGGTTAATGC
51  AGGTACCATA CCCCTCGGAA AGTTATCTGA CATTGACAAA ATCGGAAACTC
101  AAACGGAAAAT TTTCCCCATT TTGGGTGGTT CTGGGCCATA CTACCTTTTC
151  CCTGGTGATT ATGGTATTTTC TCGTGATTTT CCGGAAGTT GTGAAATGAA
201  GCAAGTGCAG AATGTTGGTA GACACGGTGA AAGATACCCC ACTGTCAGCA
251  AAGCCAAAAG TATCATGACA ACATGGGTCA AATTGAGTAA CTATACCGGT
301  CAATTCAGCG GAGCATTGTC TTTCTTGAAC GATGACTACG AATTTTTCAT
351  TCGTGACACC AAAAACCTAG AAATGAAAC CACACTTGCC AATTCGGTCA
401  ATGTTTTT GAA C CCCATATACC GGTGAGATGA ATGCTAAGAG ACACGCTCGT
451  GATTTCTTGG CGCAATATGG CTACATGGTC GAAAACCAAA CCAGTTTTGC
501  CGTTTTTACG TCTAACTCAGACAGATGTCA TGATACTGCC CAGTAATTTCA
551  TTGACGGTTT GGGTGATAAA TTCAACATAT CCTTGCAAAC CATCAGTGA
601  GCCGAGTCTG CTGGTGCCAA TACTCTGAGT GCCCACCATT CGTGTCCTGC
651  TTGGGGACGAT GATGTCACACG ATGACATTTT GAAAAATAT GATACCAAAT
701  ATTTGAGTGG TATTGCCAAG AGATTAAACA AGGAAAAAAA GGGTTTGAAT
751  CGTTTTTACG TCTAACTCAGACAGATGTCA TGATACTGCC CAGTAATTTCA
801  AAACGGCTTAGA GTGGTAACAGT GCATCTGTAA CATCTTCACC AAGATGAAT

80
851  TGGTCCGTTT CTCTACGGC CAAAGACTTGG AAACCTTATTA TCAAAACGGA
901  CCAGGCTATG ACGTCGTCAG ATCCGTCCGGT GCCAAGTTGT TCAACGCTTC
951  AGTGAAACTA CTAAAGGAAA GTGAGGTCCA GGA AGC AAAAAAG GTTTGGTTGA
1001  GTCACCCCA CGATACCGGAT ATTCTGAACCT ATTTGACCAC TATCGGCATA
1051  ATCGATGACA AAAATAACTT GACCGCGGAA CATGTCCCAT TCAATGGAAAA
1101  CACTTCCAC AGATCTCGGT AGCTTCCACA AGGTGCTCCT GTTTACACTG
1151  AAAAGTTCCA GTGTCCATG GACACCTATG TTAGATACGT CATCAACGAT
1201  GCTGTCGTTT CAATTGAAAAC CTGTCTCTACT GGTCAGGGGT TCTCCTGTGA
1251  AATAATGACT TCTACGACT ATGCTGAAAA GAGAGTAGCC GGTACTGACT
1301  TCCTAAAGGT CTGTAACGTC AGCAGCGTCA GTAACCTTAC TGAATTGACC
1351  TTTTCTGGG ACTGGAATAC CAAGCACTAC AACGACACTT TATTTAAAACA
1401  GTAAATAGAT AATATGATTATG TGAATTTTTA GAAACTAATT ATGAATACCG
1451  A
Vita

The author was born in Kathmandu, Nepal. She obtained her Bachelor’s degree in Biological Sciences University of New Orleans in 2012 with honors. She joined the University of New Orleans Biological Sciences graduate program to pursue a Masters in Biological Sciences, and became a member of Professor Mary J. Clancy research groups in 2013.