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Protein Interactions in mRNA Methylation Complexes

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Protein Interactions in mRNA Methylation Complexes

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by

Yazan Ali Alqara

May 2013
Acknowledgments

I can wholeheartedly say that committing to this research project is one of the best decisions I have made to date. The determination it takes to commit to research is beyond comparison. Even the slightest possibility that I could contribute to the scientific realm of discoveries has kept my curiosity stimulated throughout the interval of my project. Research, at times, can be frustrating and failures are inevitable but making expected or unexpected discoveries alleviates all frustrations. I have learned a vast amount of knowledge from my research, and it has positively contributed to my character in so many ways. There are many people that have directly and indirectly contributed to my research.

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Abstract

Experiments were performed to test sequence and structural specific interactions of proteins with a conserved RNA modification enzyme, which is known as Ime4 in yeast and Mettl3 in mammals. Ime4 methylates N6-adenosine bases on mRNA molecules. The goal of this project is to gain direct insights into how novel proteins interact with Ime4 to form the methyltransferase (MTase) complex and to identify proteins that are essential for Ime4 activity. It has been recognized that there are two proteins that interact within the Ime4 complex, which are known as Mum2 (a cytoplasmic protein essential for meiotic DNA replication within yeast) and Slz1 (a transcription factor). We hypothesize that the N-terminal domain of Ime4 is the location of binding of the aforementioned proteins in this complex. Similarly, we tested whether the human ortholog of Ime4 (Mettl3) forms an analogous complex that includes an ortholog of Mum2, known as WTAP, and its binding partner WT1. The major approaches include in vivo genetic assays in yeast to test protein-protein interactions and the use of recombinant DNA technology to construct fusion genes/deletions. The results demonstrate that Mum2 interacts with a specific, non-conserved region in the Ime4 N-terminal domain. Furthermore, we discovered a new binding partner, Ygl036w, which also interacts with Ime4. Currently, several experiments are being carried out with the Mettl3 complex and its hypothesized protein binding partners to assess the interactions of this complex.

Key Terms: m^6A, IME4, METTL3, RNA Methylation, MUM2, WT1, WTAP,
Introduction

Importance of protein-protein interactions

Protein-protein interactions are essential for the regulation of many processes within cells. An understanding of how proteins function as complexes is crucial to understanding how organisms live. Proteins regulate all biological systems, and although some proteins perform independent functions, most interact with other proteins to perform necessary biological activities. Understanding the function of single proteins is essential to understanding the function of a protein complex. But because most proteins interact with other proteins to carry out their functions, it is important to study the interacting protein in its entirety to fully appreciate a protein’s exact function within a cell.

Protein-protein interactions are necessary for cell proliferation, nutrient uptake, morphology, motility, gene expression, intercellular communication, and control of cell homeostasis, among others. Many aspects of protein-protein interactions are necessary to understand how a protein carries out its functions. The amino acid sequence and its structure may be used to ascertain motifs that identify the likely function of a protein. Conserved sequences are especially important in revealing orthologous proteins from different organisms. Also, conserved sequences found in different organisms may help identify residues that are crucial to the regulation of protein function. Expression profiles can explain the specificity of cell type and how expression is modulated within cells. Also, the function of proteins may be examined by how they are post-translationally modified or how they modify other proteins. Post-translational modifications may regulate the activity of proteins, where they localize, and how they are activated (Protein-protein Interactions: a Molecular Cloning Manual 2005). Moreover, the localization of proteins may suggest the function of a protein or provide an understanding of the
proteins that a specific protein may interact with to help it carry out its function. Most importantly, the function of proteins can be elucidated by understanding their interactions with other proteins.

Protein-protein interactions appear in two forms, transient and stable. Stable interactions are characteristic of proteins that are involved in multi-subunit complexes, containing different or identical subunits. Proteins involved in stable interactions may help stabilize a complex of proteins. There may be many proteins interacting in a complex that centers around one protein. In a sense, different protein-protein interactions may help assemble a multi-subunit complex to strengthen the functions of specific proteins. Or proteins may interact with one another to regulate the catalytic activity of specific proteins.

In contrast, transient protein interactions are believed to govern most cellular processes (Protein-protein Interactions: a Molecular Cloning Manual 2005). A transient interaction is an interaction that is temporary. Transient interactions are also known as unstable interactions, because unlike stable interactions, transient interactions occur rapidly and usually include an association of two proteins. Transient interactions regulate cellular processes including transport, folding, cell cycling, and protein modifications. Protein-protein interactions are essential to cell viability. In general, cells perform many functions that are necessary for the survival of the entire organism as a whole. Without protein-protein interactions, cells will not be able to perform the functions necessary for maintenance and regulation of an organism. Ultimately, a cell depends on both stable and transient interactions for the upkeep of an entire organism.
**N<sup>6</sup>-methyl-adenosine (m<sup>6</sup>A)**

As explained, protein-protein interactions are important for the regulation of many cellular processes. One such process found in all organisms is mRNA methylation, an important posttranscriptional modification of adenosine residues known as N<sup>6</sup>-methyl-adenosine (m<sup>6</sup>A). As the most common modification of RNA molecules in eukaryotes, m<sup>6</sup>A may serve as a significant, novel epigenetic marker in all organisms (Niu et al. 2013). The m<sup>6</sup>A modification is catalyzed by specific proteins known as Ime4 in *Saccharomyces cerevisiae*, Mettl3 in *Homo sapiens*, Dm Ime4 in *Drosophila melanogaster*, and MTA in *Arabidopsis thaliana*. These four proteins have been extensively researched in terms of how this modification affects transcript, protein, developmental, and metabolic functions (Jia, Fu, and He 2013). The m<sup>6</sup>A modification seems to be evolutionarily conserved, and may function to regulate gametogenesis in eukaryotes (Clancy et al. 2002; Hongay and Orr-Weaver 2011). The Ime4 protein in yeast remains to be the most extensively studied and multiple Ime4 interacting proteins have been discovered.

*Saccharomyces cerevisiae* interacts with its environment and receives a variety of nutritional and genetic signals to activate cellular developmental pathways including sporulation and mating. Through many very involved and intricate experiments, it was found that Ime4 activated a variety of sporulation specific genes that ultimately lead to the formation of haploid spores through a specific modification of mRNA molecules (Shah and Clancy 1992). Once Ime4 becomes active, it initiates a mechanism by which the catalytic motif IV catalyzes methylation of N<sup>6</sup>-adenosine residues (m<sup>6</sup>A) in polyadenylated mRNA within yeast cells that are sporulating (Clancy et al. 2002). Modified targets may include mRNAs of IME1, IME2, NDT80, IME4 itself, and other sporulation specific transcripts at particular adenosine residues (Bodi et al.
The \( m^6A \) modification may cause alterations of these mRNAs, in which lead to changes in stability, splicing, efficient translation, or compartmentalization (Clancy et al. 2002).

In accord, Ime4 may be viewed as a meditator for the activation of these genes through this mechanism of methylating adenosine residues. Though the function of \( m^6A \) is still unidentified, it may cause the aforementioned functions. Strong evidence, through methylation inhibitor experiments, indicates that the \( m^6A \) modification may play a role in transcript splicing, stability, translation efficiency, or compartmentalization in mammalian cells. Unfortunately, the pleiotropic effects of these experiments cannot verify that \( m^6A \) causes these transcripts to alter their biogenesis (Clancy et al. 2002). Through the discovery of proteins involved in the protein complex of Ime4, it may become clearer as to what function \( m^6A \) coordinates. Two proteins have already been discovered to interact with Ime4, known as Mum2 and Slz1. This complex will be explained later in the Introduction. The human ortholog of Ime4, MT-A70 (also referred to as \textit{METTL3}), may also have similar roles to Ime4, and also functions in a protein complex.

The \( m^6A \) modification in humans is catalyzed by the MT-A70 subunit of a large protein complex. The catalytic Mettl3 subunit is the only one that has been identified to date. This catalysis by MT-A70 is believed to be involved in the regulation of many processes in humans including embryonic development, gonad development, mRNA metabolism, and protein expression (Niu et al. 2013). There is some evidence that the \( m^6A \) modification may play a regulatory role in the nuclear export machinery (Niu et al. 2013). This has been observed in experiments that involve HeLa cells treated with the methylation inhibitor S-Tubercidinylhomocysteine (STH), which showed that the retention time of mRNA in the nucleus increased by 40\% on average (Camper et al. 1984). Remarkably, high \( m^6A \) activity was detected in cells that underwent transformations as compared to non-transformed cells, indicating a
relationship between cancer and $m^6$A methylation (Tuck et al. 1996). Though $m^6$A may be involved in these processes, its exact function has yet to be elucidated. MT-A70 functions in a virtually unknown protein complex that is presumed to involve many specific protein-protein interactions. Discovering these protein-protein interactions in this unknown protein complex will be important in elucidating the functions of the $m^6$A modification.

![N6-Methyl-adenosine](image)

**Figure 1:** N$^6$-Methyl-adenosine ($m^6$A)

*Saccharomyces cerevisiae as a model organism*

*Saccharomyces cerevisiae* is a model organism to study the cellular processes of eukaryotes. *Saccharomyces cerevisiae* was the first eukaryote to have its genome sequenced (Botstein, Chervitz, and Cherry 1997). This was an amazing feat because by having all of its genome decoded, scientists have used yeast as a model to study human diseases such as cancer, infections, and hereditary diseases. Determining the function of yeast proteins is critical to understanding how human proteins function as well. The genome of yeast can be compared to
the human genome to assess the number of yeast genes that have noteworthy mammalian homologs (Botstein, Chervitz, and Cherry 1997). Yeast cells also share a common fundamental life cycle and cellular construction with more complex multicellular organisms, including humans and plants (Mell and Burgess 2002). Yeast may contain 31% of protein encoding genes that have robust mammalian homologous proteins (Botstein, Chervitz, and Cherry 1997). This homology between yeast genes and human genes that encode functional proteins is the most important reason *Saccharomyces cerevisiae* is used as the model organism for experimental study. Yet, there are many more incentives to using yeast as a eukaryotic model organism for experimental study.

*Saccharomyces cerevisiae* is in many ways analogous to the bacterial organisms scientist use to conduct experiments. Bacteria such as *E.coli*, are easy to manipulate and many *E.coli* strains are affordable for purchase. Yeast is similar to *E.coli* in this aspect, because yeast is also simple to manipulate and strains are also affordable (Botstein, Chervitz, and Cherry 1997). Also, segregation analysis and screening mutants are simpler to accomplish in yeast than in any other multicellular organism (Mell and Burgess 2002). Performing experiments on yeast, such as plasmid transformations, deletions, protein extractions and assays, mutagenesis of gene targets, and knock out of targeted genes is important for investigators because these methods help to identify functions of many different types of genes that may be similar to the human homolog of these genes. Also, by performing the aforementioned experiments on yeast, scientists may be able to solve complicated mechanisms of cellular pathways that are crucial for the understanding of human disease processes. By using yeast as an experimental model, scientists are able to induce mutations in yeast genes (e.g. metabolic genes) to demonstrate the importance of these
genes in control of cellular pathways and regulations of cellular mechanisms (Mell and Burgess 2002).

Another incentive for using Saccharomyces cerevisiae as an experimental model is that the time required to perform experiments on yeast is relatively short in comparison to multicellular organisms. A fundamental difference between using more complex eukaryotic models in comparison to yeast is the amount of time required to grow these organisms. The generation time is infinitesimal compared to the amount of time it would require to grow an organism such as a mouse for experimentation. Saccharomyces cerevisiae has a generation time of about 90 minutes, and this is important when scientists need to observe rare events such as genetic mutations (Mell and Burgess 2002). Because the generation time is so short, many millions of cells can be cultured rapidly, and this increases the probability that a genetic mutation can be identified, with its effects testable. For example, a mutagenesis experiment performed on yeast may require three days, which is short in comparison to doing mutagenesis experiments on mice which may require many months before a comprehensible result is obtained.

**Mating of Saccharomyces cerevisiae and its role in its life cycle**

Saccharomyces cerevisiae undergoes meiosis in nutrient-limiting environments. Specifically, meiosis is induced by media that lacks nitrogen but contains an adequate respirable carbon source, typically acetate. This is essential to the continued survival of yeast because they can undergo major physical transformations that allow them to pass on and assort their genetic information. Interestingly, yeasts are able to exist in both haploid and diploid forms. Glucose and nitrogen deprivation causes biochemical changes to occur in diploid yeast cells, and these alterations cause diploid yeast cells to change to their haploid forms through the process of meiosis and spore formation (Wagstaff, Klapholz, and Esposito 1982). In their haploid forms,
yeast cells express alleles that differentiate the mating types of yeast cells. Haploid cells can either be MATa or MATα, which are the alleles that phenotypically address the differences of the two different sexes in haploid yeast cells. The MATa and MATα alleles of the mating type locus regulate the expression of genes encoding pheromones known as ‘α pheromone’ and ‘α pheromone’ and other genes involved in cell fusion. Pheromones induce intracellular changes that cause the MATa and MATα cells to be attracted to one another, leading to the formation of cell projections and consequently to the formation of morphological structures called schmoos (Bardwell 2004). Once the haploid cells fuse to one another, the nuclei also fuse causing the 16 chromosome haploid to become a 32 chromosome diploid known as the MATa/MATα cell (Bardwell 2004). The resulting diploid does not mate, but can be propagated indefinitely mitotically.

This mating process, in all, turns two haploid cells into a functional diploid cell which may then undergo the process of meiosis to form four distinct haploid cells. Two of the four haploid cells are MATa and the remaining two are MATα. This process is crucial to the survival of yeast in harsh environments. Also, meiosis creates genetic variations through the process of crossing over and exchange of genes from two distinct cells. This allows yeast to acquire gene combinations that are necessary for survival in harsh habitats. The meiotic process that yeast undergoes is very interesting. This same process occurs in higher eukaryotic organisms such as mammals, but not in the same way. Meiosis only occurs in gamete cells in animals, but identifying the genes that mediate meiosis in yeast may lead to a better understanding of how meiosis operates in animals. Research has unequivocally identified the discovery of genes that induce meiosis in Saccharomyces cerevisiae such as IME4, IME1, and IME2.
IME4 and its role in sporulation in *Saccharomyces cerevisiae*

The expression of key activators is essential for ascosporogenesis and meiosis in yeast. Meiosis and ascosporogenesis are complex processes involving the creation of genetic variation, production of many new proteins, and turnover of foregoing cellular constituents (Clancy et al. 1983). “Early” genes such as *IME1* and *IME2* begin to be transcribed at about 1-2 hours in most laboratory strains following the nutritional deprivation of *Saccharomyces cerevisiae* and may decrease significantly thirteen hours following nutritional deprivation (Clancy et al. 1983). Some sporulation-specific mRNAs are synthesized at about 7 hours, such as *NDT80*, which encodes a transcription factor for genes involved in spore formation and exit from the pachytene stage of meiosis (Jia, Fu, and He 2013). Three genes known as *IME4*, *IME1*, and *IME2* are essential early activators of meiosis in yeast. The activation of these genes is precisely regulated, and their activation is essential for meiosis and spore formation. Though the mechanism of activation is not fully understood, it is essential that these three genes, in lieu of the activation of other sporulation-specific genes, be active for a certain time during meiosis.

Ime4 plays a very important role in the induction of meiosis in yeast that are nutritionally deprived of nitrogen and that are placed on respirable carbon sources (Clancy et al. 2002). *IME4* is located on the left arm of chromosome VII in between *ADE5* and *LYS5* (Shah and Clancy 1992). Its importance resides in the fact that it is finely regulated. *IME4* is transcribed in low quantities during the diploid lifecycle of MATα/MATα vegetative yeast cells (Shah and Clancy 1992). This is important because this transcript needs to be present in small quantities in order to activate sporulation-specific transcripts when yeast cells are starved of their required nutrients that maintain their viability. Ime4 is also known to methylate its own mRNA along with those of other genes known as *IME1* and *IME2*, among others (Bodi et al. 2010).
Ime1 is a transcriptional activator of meiosis, and leads to the transcription of genes that are required for sporulation (Kassir, Granot, and Simchen 1988). Ime1 induces a cascade of gene expression that regulates meiosis and sporulation under starved conditions (Bodi et al. 2010). Because of this cascade of gene activation, it is noted that more than five hundred new mRNA transcripts exist during yeast meiosis (Chu et al. 1998). This is important because yeast cells morph into entirely new cells and require different proteins to regulate this transformation. IME1 mRNA is not present in high amounts during the vegetative mitotic life cycle of yeast, and thus, its regulation is dependent on other proteins (Shah and Clancy 1992). Its expression increases rapidly during conditions where yeast cells are starved, and it leads to the activation of the two genes encoding IME2 and NDT80 among many others that are needed for meiosis, recombination, and chromosomal segregation.

Ime2 and Ndt80 play important roles in the regulation of meiosis. In order for sporulation to occur correctly, Ime1 and Ime4 need to be inactivated at certain times as cells transition into the meiotic process. In order for these proteins to be inactivated, they require the function of Ime2 and Ndt80, respectively. Ime2 is a serine/threonine protein kinase that mediates the stability of the Ime1 protein during meiosis, it is required for the degradation of Ime1, and also for the activation of Ndt80 (Guttmann-Raviv, Martin, and Kassir 2002). Ime2 itself is very unstable and is toxic in vegetative diploid yeast cells (Guttmann-Raviv, Martin, and Kassir 2002). By degrading Ime1, Ime2 down regulates the activity of Ime1. Ime2 phosphorylates the C-terminal domain of Ime1, leading to its degradation by a 26S proteasome (Guttmann-Raviv, Martin, and Kassir 2002). The interaction between Ime1 and Ime2 may be viewed as a negative feedback loop. The activation of Ime1 causes activation of the Ime2 protein, and when the Ime2 protein is active at a certain point, it causes the degradation of Ime1. Ime2 is likely to
phosphorylate other protein targets as well (Guttmann-Raviv, Martin, and Kassir 2002). Ndt80, on the other hand, is a meiosis-specific transcription factor that is essential for the progression of yeast cells through the pachytene stage of meiosis and is responsible for activating the “middle” group of sporulation specific genes (Xu et al. 1995). Ime4 may function to activate Ndt80, which in turn down regulates IME4 during the middle phase of meiosis (Agarwala et al. 2012). This may be achieved indirectly through the activation of Ime2, and/or directly by modification of adenosine residues of the Ndt80 transcript (Agarwala et al. 2012). This modification is an essential part of the Ime4 protein function. It functions to methylate the sporulation specific transcripts that are part of the meiosis cycle in yeast.

**The IME4 homolog, METTL3**

The human ortholog of Ime4 is METTL3, also known as MT-A70. There is a 72% sequence similarity between Ime4 and METTL3 proteins (Bokar et al. 1997). The METTL3 protein is found in human cells. This is significant because the homologs perform the same function, which is to methylate mRNA transcripts. The entire protein complex consists of two separable components known as MT-A and MT-B (Bokar et al. 1997). The total weight of these components is 1075 kDa (200 kDa for MT-A and 875kDa for MT-B) as estimated by gel filtration chromatography (Bokar et al. 1997). MT-A and MT-B contain multiple subunits, and the subunit with catalytic activity is encoded by METTL3 (Bokar et al. 1997). METTL3 is the gene that encodes the 70 kDa subunit of the human mRNA m⁰A MTase known as MT-A70, which is located in the protein component MT-A (Bokar et al. 1997).
The function of the catalytic subunit is to methylate adenosine residues on mRNA transcripts in humans, which is the exact function of Ime4 in yeast cells. This posttranscriptional modification is also known as m^6A in humans. The only difference is Ime4 methylates adenosine residues on mRNA transcripts in yeast exhibiting sporulation. The function of m^6A is still unknown, wherever it is found (Bokar et al. 1997). Past experiments using HeLa cells have shown that decreased levels of m^6A coincide with a decreased level of newly transcribed mRNA in the cytosol (Bokar et al. 1997). This finding suggests that this modification may play a role in regulating the transport of mRNA from the nucleus to the cytoplasm. Also, m^6A may play a role in further posttranscriptional modifications. The evidence for this notion lies in inhibition experiments on HeLa cells with decreased m^6A found on newly transcribed mRNA transcripts. It is shown that a decrease in the m^6A modification results in aggregation of unspliced pre-mRNA in the nucleus (Bokar et al. 1997). The modification by MT-A70 may also play a role in mRNA metabolism.

**Figure 2:** Sequence Alignment of IME4

The function of the catalytic subunit is to methylate adenosine residues on mRNA transcripts in humans, which is the exact function of Ime4 in yeast cells. This posttranscriptional modification is also known as m^6A in humans. The only difference is Ime4 methylates adenosine residues on mRNA transcripts in yeast exhibiting sporulation. The function of m^6A is still unknown, wherever it is found (Bokar et al. 1997). Past experiments using HeLa cells have shown that decreased levels of m^6A coincide with a decreased level of newly transcribed mRNA in the cytosol (Bokar et al. 1997). This finding suggests that this modification may play a role in regulating the transport of mRNA from the nucleus to the cytoplasm. Also, m^6A may play a role in further posttranscriptional modifications. The evidence for this notion lies in inhibition experiments on HeLa cells with decreased m^6A found on newly transcribed mRNA transcripts. It is shown that a decrease in the m^6A modification results in aggregation of unspliced pre-mRNA in the nucleus (Bokar et al. 1997). The modification by MT-A70 may also play a role in mRNA metabolism.
The system by which MT-A70 expression is regulated has yet to be elucidated. It may be regulated tissue specifically or developmentally, but research has yet to confirm this possibility (Bokar et al. 1997). Ime4 is regulated in a timely fashion and is only active in yeast cells during sporulation. Ime4 is also regulated by the decrease of antisense transcripts that is correlated with an increase of sense transcripts (Agarwala et al. 2012). It has been confirmed that when a cell undergoes a transformation of some sort, whether it is a viral infection or cellular transformation, the content of adenosine residues that are methylated increases substantially (Bokar et al. 1997). Transformations of adenovirus into rat embryonic cells resulted in a 7.5-fold increase in MT-A70 activity (Bokar et al. 1997). There may be a connection to the regulation of m^6^A modifications in humans, and in some way, this modification may change the fate of specific mRNA transcripts. More research should be done to understand this phenomenon.

**Mum2 and Slz1 in complex with Ime4**

Ime4 is known to interact with other proteins in order to carry out its function, which is to methylate N^6^-adenosine residues on mRNA transcripts. This is a fairly new discovery, and these protein-protein interactions complement one another. This protein complex, defined by two hybrid analysis, contains Ime4, Mum2, and a protein known as Slz1 (known as theMIS complex). Kar4 has also been reported to be included in a complex involving Ime4 and Mum2 (S. Morgan and J. Eugebrecht, Personal Communication). There is not much known about Mum2 and Slz1, but Mum2 was shown to be a crucial protein for the methyltransferase activity of Ime4 (Agarwala et al. 2012). Slz1 functions as an accessory protein to further enhance the catalytic activity of Ime4 (Agarwala et al. 2012).

Mum2 is a protein that is, at least partially, located in the cytoplasm, and it is necessary for sporulation in *Saccharomyces cerevisiae* (Davis et al. 2001a). Mum2 can be found in the
nucleus as well, where methylation of pre-mRNAs occurs in eukaryotic cells. Also, Mum2 (Muddled Meiosis 2) interacts genetically with Orc2p, which is a protein that is part of the origin recognition complex (Davis et al. 2001b). Mum2 is a protein with a coiled-coil region located at its C-terminus that may interact with Ime4 to form a scaffold where other proteins may bind and further strengthen the activity of the MIS complex (Davis et al. 2001b). Coiled-coil protein structures are formed by several alpha helical regions in complex with one another (Davis et al. 2001b). Mum2 may also activate Ime4 to perform its catalytic activity, or Mum2 could function to target Ime4 to mRNA molecules (Agarwala et al. 2012). The importance of Mum2 was revealed in deletion experiments. When MUM2 was deleted, yeast cells attained sporulation defects similar to when IME4 was deleted in previous experiments (Agarwala et al. 2012). This verifies the significance of Mum2 in sporulation. Even more fascinating is when Ime4 is not in complex with Mum2, Ime4 cannot abundantly modify mRNA transcripts (Agarwala et al. 2012). The prominence of Mum2 in the MIS complex was verified through these elegant deletion and mutagenesis experiments.

Slz1, on the other hand, is a sporulation-specific transcription factor that contains a leucine zipper domain (Agarwala et al. 2012). There is not much known on the direct function of this protein, but it is a known component of the MIS complex. With its association in the MIS complex, it appears to strengthen the activity of Ime4 (Agarwala et al. 2012). Through SLZ1 deletion experiments, it has been proven that Slz1 is not a critical component of the MIS complex (Agarwala et al. 2012). Ime4 is able to methylate adenosine residues in the absence of Slz1 when overexpressed in mitotic cells. Slz1 may be switched out with other transcription factors in the MIS complex, but experiments would have to be performed to confirm this possibility (Personal communication with Dr. Clancy).
**Wt1 and Wtap**

Wt1 (Wilms’ tumor 1) and Wtap (Wilms’ tumor associated protein) are important proteins found in humans. These proteins regulate a variety of functions in the human body. These proteins interact with one another to carry out their functions. Defects in these proteins cause major health issues in young children and adults, such as Frasier syndrome, Denys-Drash syndrome, Wilms’ tumor (embryonal malignancy of the kidney), nephrotic syndrome type 4, Meacham syndrome, and mesothelioma malignancies (Weizmann Institute of Science 2012).

Wtap and Wt1 proteins are known binding partners. The Wtap protein mostly interacts with Wt1 when a three amino acid sequence, KTS, is absent from the Wt1 protein sequence (Little, Hastie, and Davies 2000). Wtap is a protein that is ubiquitously located in the nucleus of human cells (Horiuchi et al. 2006). Interestingly, Wtap is important for the stabilization of mRNA transcripts that encode the protein cyclin A2, which is important for G2/M transition (Horiuchi et al. 2006). The knockdown of WTAP shows a substantial decrease in cyclin A2 mRNA transcripts, and also causes death of 6.5 weeks old rat embryos (Horiuchi et al. 2006). Also, the knockdown of WTAP leads to an increased activation of cell adhesion, metabolism, and inflammation (Horiuchi et al. 2006). The importance of this protein may therefore lie in its regulation of the cell cycle, and possibly the role it plays in its interaction to Wt1.

Wtap has been proposed to be the human ortholog of the yeast protein Mum2, and this homology indicates that Wtap and Wt1 may be interacting in a larger unknown protein complex (Agarwala et al. 2012). A homolog of Wtap was also identified in Drosophila melanogaster, known as Fl(2)d. Fl(2)d is regarded as a splice factor that regulates the alternative splicing of the sex lethal (SX1) gene (Penn et al. 2008). The splicing of the SX1 gene by Fl(2)d is known to regulate female specific programs (Penn et al. 2008). Fl(2)d is also known to interact in a protein
complex as a splicing factor, and this indicated that the human Wtap protein may also interact as a splice factor in association with a protein complex. Wtap has been observed to associate in functional human spliceosomes, but how it contributes to the splicing process is a mystery (Penn et al. 2008). More importantly, strengthening the notion that Wtap may interact in a protein complex with Mettl3, is the verification that the homolog of Mettl3, known as MT-A in *Arabidopsis thaliana*, interacts directly with a homolog of Wtap, known as At FIP37. Evidence for this is observed in coimmunoprecipitation assays that show MT-A and At FIP37 directly interacting with one another (Zhong et al. 2008). Also, knockout experiments of either MT-A and At FIP37 resulted in inhibition of embryonic development at the globular stage in *Arabidopsis thaliana* (Zhong et al. 2008). These experiments reinforce the notion that Wtap may interact as a splicing protein, and that Wtap is, in fact, a protein that binds to Mettl3 to regulate its function and affect cell fate. The experiments to prove the Wtap protein is directly interacting with Mettl3 is still being carried out by our lab, but what is known is Wtap interacts directly with Wt1.
Figure 3: Sequence alignment of MUM2

Wt1 is a protein that is involved in the development of the kidney and the reproductive organs (Little, Hastie, and Davies 2000). This protein is amazing because it may assume many functions. Wt1 protein is known to exist in 16 different isoforms (Little, Hastie, and Davies 2000). Yet, how can one protein assume many different roles from a single, primary transcript? The gene that encodes WT1 may include different types of exons that can alter different segments of the translated protein. This is a posttranscriptional modification of the encoded WT1 transcript. More specifically, WT1 has two alternating splice sites that encode different versions of the same protein. One splice site is encoded by exon 5, and if this site is included in the post modified transcript, it encodes a specific 17 amino acid sequence that may or may not be included in the final translated protein (Little, Hastie, and Davies 2000). Though not much is known about the function of the 17 amino acid sequence, it may be a transcriptional repression domain (Natoli et al. 2002).
The second alternatively spliced exon includes a lysine, threonine, and serine (KTS) consecutive sequence which is believed to inhibit the use of Wt1 as a DNA binding protein (Little, Hastie, and Davies 2000). This is because Wt1 has a zinc finger binding domain, which is used to bind DNA, that the KTS sequence hinders (Little, Hastie, and Davies 2000). Interestingly enough, many of the functions that Wt1 performs must incorporate the interaction of other proteins. Wt1 may also act as a transcription factor, but only if other proteins interact with Wt1 to allow it to carry out its function (Little, Hastie, and Davies 2000). Wt1 may also act as a posttranscriptional regulator, may play a role in splicing, and it may have a role in cell cycle regulation (Natoli et al. 2002). Through the use of yeast-two hybrid experiments, scientists have discovered that Wtap and Wt1 are definite binding partners (Little, Hastie, and Davies 2000). These proteins may also interact in an unknown protein complex. The importance of this interaction is still unknown.

**Protein complexes**

An explanation of protein complexes segues into the experimental approaches we have taken to understand how the MIS complex functions, and to discover new unknown proteins that may be associated with the MIS complex.

A protein complex is a single functional system consisting of multiple proteins that may assume different functions but all work to strengthen the activity of a central protein in the complex. The proteins that function as a single unit may interact transiently or stably. As mentioned, not much is known of the MIS protein complex. It is known that the proteins that function in this complex strengthen the function of Ime4, which methylates adenosine residues on pre-mRNA transcripts. Also, we believe that the homolog of Ime4, known as Mettl3, may function in a protein complex similar to the MIS complex. The homology between Ime4 and
Mettl3 strengthens this hypothesis. Also, we hypothesize that the homolog of Mum2 (Wtap) may be a binding partner in the proposed Mettl3 binding complex.
Materials and Methods

Materials

Primers were designed in lab and sent to Integrated DNA Technologies for their synthesis. Strataprep plasmid miniprep kits (The catalog number for the kit is 400763), Strataprep PCR purification kits (The catalog number for the kit is 400771), XL1-Blue competent and subcloning competent cells used for Escherichia coli transformations, GAL4 Two-Hybrid Phagemid Vector kits, and high fidelity Herculase II fusion Pfu polymerase used for PCR amplifications were purchased from Agilent/Stratagene Technologies. Most of the restriction enzymes, Taq polymerase, and their associated buffers were purchased from Promega. Some restriction enzymes, the T4 DNA Ligase, and their associated buffers were purchased from New England Biolabs.

Media used for Transformations and Cell Cultures

For E. coli transformations and streakings, we used Luria broth (LB) media. This media was made using 1.5% agar (0.015 g/ml), 1% bacto-tryptone (0.01g/ml), 0.5% yeast extract (0.005 g/ml), 0.5% NaCl (0.005 g/ml), and adjusted to a pH of 7.5. Accordingly, antibiotic resistance was included in the media depending on the requirement of the specific plasmid that was used. The antibiotics used were either ampicillin (100 µg/ml) or chloramphenicol (30 µg/ml). LB liquid media used for cultures include the aforementioned ingredients excluding the agar.

For yeast transformation and culture maintenance, a variety of media was used. Yeast extract peptone dextrose (YEPD) was used to streak and culture yeast colonies. This media included all the necessary supplements that yeast cells require for survival. Ingredients used to make this media include 1% yeast extract (0.01 g/ml), 2% peptone (0.02 g/ml), 2% dextrose
(0.02 g/ml), and 2% agar (0.02 g/ml) for plating. Liquid YEPD requires the above mentioned ingredients excluding the agar.

Nutrient limiting media was used for plasmid selection of transformed yeast. This media is known as synthetic complete (SC) media. The ingredients used to create this media include 10X Bacto-yeast nitrogen base (YNB) without amino acids (0.067 g/ml), 5X dropout mix (0.01 g/ml), 2% dextrose (0.02 g/ml), 2% bacto-agar (0.02 g/ml), and the supplemental amino acids which include: 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). The synthetic dropout mix without YNB was purchased from U.S BioLogical and does not contain adenine, histidine, leucine, tryptophan, and uracil.

**Primer Construction**

Primers were designed in lab and purchased from IDT according to the gene we were interested in amplifying for cloning or verification purposes. The amount of oligo we received from IDT was usually approximately 20 nmoles, and was delivered in the form of dry powder. Also, the oligos were desalted before they arrived. Forward and reverse primers were created by specific sequences found on the genes we used. The forward primer was a copy of the beginning of the gene to be amplified or the beginning of the section of the gene to be amplified (5’ end of the gene). The reverse primer needed to be copied from the end of the gene or specific region of the gene to be amplified. Then, the reverse primer needed to be complemented with its necessary bases, and was written in reverse. Primers used for cloning incorporated restriction sites that coincided with the restriction sites located on the vector to which the gene was ligated. Also, extra bases were added or removed to put the gene in frame with the GAL activation or the GAL binding domain encoded by the vector, so the incorrect protein was not translated when a yeast
two hybrid experiment was performed. We used the restriction sites on the ligated plasmid which verified a ligated gene was cloned to its correct vector. Restriction enzymes knicked the genes at the junction sites and dropped the gene from its vector. An agarose gel was used to view the restriction digested DNA.

Primers used for cloning are shown in the table below:

<table>
<thead>
<tr>
<th>Gene or Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BUR2</strong></td>
<td>TTTTTTCATCGAGATGTCTGCTCATCCTCA</td>
<td>TTTTGTGACCTATATTTTATTAGTTTTTTGCTGAGCTTCA</td>
</tr>
<tr>
<td><strong>IME4 1-600</strong></td>
<td>TTTTTGAATTCATGATTAACGATAAAATCA</td>
<td>TTTTGTGACCTGAGCAAAATATGTT</td>
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<tr>
<td><strong>IME4 1-316</strong></td>
<td>TTTTTGAATTCATGATTAACGATAAAACTA</td>
<td>TTTTGTGACATGTCGAGGAGC AATTGCC</td>
</tr>
<tr>
<td><strong>IME4 231-600</strong></td>
<td>TTTTTGAATTCACCACCAAGGATTATTGAGTGCATTCAA</td>
<td>TTTTGTGACTTAATTGCTAATTCTTTAGG</td>
</tr>
<tr>
<td><strong>IME4 131-225</strong></td>
<td>TTTTTGAATTCATTACATGATTAACGAAAGACCAA</td>
<td>TTTTGTGACGGGATATTTTGGACCATTG</td>
</tr>
<tr>
<td><strong>METTL3 1-302</strong></td>
<td>TTTTCCCGGGGGAATGTGGGACACGTGAGCTCTATC</td>
<td>TTTTGTGACCTATGATAATTCTGTCGAGGTAGAT</td>
</tr>
<tr>
<td><strong>METTL3 273-580</strong></td>
<td>TTTTTGAATTCATGATGAATGATTCGAGGAGGAG</td>
<td>TTTTGTGACCTAATTCTTTAGGAGTTAGAT</td>
</tr>
<tr>
<td><strong>MUM2</strong></td>
<td>TTTTTGACTAGCATTATATGAAATTACATGAGCT</td>
<td>TTTTGTGACCTCAATTAGCAACGTCC</td>
</tr>
<tr>
<td><strong>pAD 745-765</strong></td>
<td>AAGGATGTGTTAATACCAACTAC</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>pBD 816-836</strong></td>
<td>GTGCGACATCATTACATCGGAAG</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>WT1</strong></td>
<td>TTTTTGAATTCAAGGAGGTTACAGCAGCGGTC</td>
<td>TTTTGTGACCTAAAGCGCAAGCGCAAGGCAAGC</td>
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<tr>
<td><strong>WTAP</strong></td>
<td>TTTTTGAATTCACCAACGAGAACCTCCTTCACCTTTC</td>
<td>AAAAGTCGACTTAACAAATCTGACCCTGCTACTTCCT</td>
</tr>
<tr>
<td><strong>YGL036W</strong></td>
<td>ATTTGGATGCGAGAAGGATGCTGAG</td>
<td>TTTTGTGACCTTAATTGCTCCTTCT</td>
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</table>

Plasmids purchased for cloning:
<table>
<thead>
<tr>
<th>Plasmid Identity</th>
<th>Accession #/ Manufacturer</th>
<th>Plasmid Created by:</th>
<th>Transformation Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAD-GAL4-2.1 vector</td>
<td>AF033313.1/ Stratagene</td>
<td>Stratagene</td>
<td>LEU2, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>pBD-GAL4 Cam vector</td>
<td>U46126.1/ Stratagene</td>
<td>Stratagene</td>
<td>TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
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</tbody>
</table>

Plasmids created for transformations:

<table>
<thead>
<tr>
<th>Plasmid Identity</th>
<th>Accession #/ Manufacturer</th>
<th>Plasmid Created by:</th>
<th>Transformation Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUR2-GAL4AD</td>
<td>DAA09542.1/ Open Biosystems</td>
<td>Jenisha Ghimire</td>
<td>LEU2, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>IME4 1-600-GAL4BD</td>
<td>P41833.1/ Clancy Lab</td>
<td>Sanjeev Dahal and Pinithi Perrera</td>
<td>TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>IME4 1-316-GAL4BD</td>
<td>P41833.1/ Clancy Lab</td>
<td>Sanjeev Dahal and Pinithi Perrera</td>
<td>TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>IME4 231-600-GAL4BD</td>
<td>P41833.1/ Clancy Lab</td>
<td>Mary Clancy</td>
<td>TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>IME4 131-225-GAL4BD</td>
<td>P41833.1/ Clancy Lab</td>
<td>Sanjeev Dahal</td>
<td>TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>METTL3 1-302-GAL4BD</td>
<td>BC001650.2/ Clancy Lab</td>
<td>Yazan Alqara</td>
<td>TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>METTL3 273-580-GAL4BD</td>
<td>BC001650.2/ Clancy Lab</td>
<td>Yazan Alqara</td>
<td>TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>MUM2-GAL4AD</td>
<td>CAA85000.1/ Clancy Lab</td>
<td>Mary Clancy</td>
<td>LEU2, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT1-GAL4AD</td>
<td>BC032861.2/ Clancy Lab</td>
<td>Yazan Alqara</td>
<td>LEU2, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>WTAP-GAL4AD/ WTAP-GAL4BD</td>
<td>BC069192.1/ Clancy Lab</td>
<td>Yazan Alqara/ Yazan Alqara</td>
<td>LEU2, Amp&lt;sup&gt;r&lt;/sup&gt;/ TRP1, Cam&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>YGL036W-GAL4AD</td>
<td>P53185.1/ Clancy Lab</td>
<td>Yazan Alqara and John Williamson</td>
<td>LEU2, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Strain used for Yeast Two Hybrid assays:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reporter Gene</th>
<th>Transformation markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>YRG-2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MATα ura352 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112</td>
<td>lacZ, HIS3</td>
<td>leu2, trp1, his3</td>
</tr>
</tbody>
</table>
Restriction Digests

Restriction digests were used for verification that a gene had been cloned correctly, and was used to create the ends of vectors and inserts for cloning purposes. A typical restriction digest consisted of a mixture of restriction enzymes, their associated buffers, and the DNA that was used for cloning or verification purposes, in a 20 microliter total volume. A reaction included 2 microliters of 10X enzyme buffer provided by the supplier for optimal activity of the restriction enzyme. If two restriction enzymes were used in one 20 microliter mixture then one buffer that optimized the activity of both enzymes was used. The amount of sterile water used was variable, depending on the amounts of DNA and enzyme used. Typically, 0.5-1 microliter of enzyme was used (about 10 units of enzyme per microliter). The amount of DNA used was about <1000ng of DNA, and the amount of water used was between 11 and 14 microliters. Reactions were incubated at 37 degrees Celsius for approximately one hour for optimal enzyme activity.

Polymerase Chain Reaction

The PCR amplification technique was used to amplify specific genes from their vectors. First, the vector in which the gene is enclosed was linearized using the appropriate restriction enzyme. Second, a mixture was made that included the required polymerase (2 units of Pfu or Taq polymerase), its 5X associated buffer (10 microliters), forward and reverse primers for the gene of interest (50-100 pmol), deoxyribonucleotide triphosphates (200 µM of dNTPs), the DNA of interest (<1000 ng), and sterilized water to a total volume of 50 microliters. Finally, this mixture was put into the PCR machine at variable temperatures. For the first step (initial
denaturation) the mixture was heated to 95 degrees Celsius for 5 minutes. The second step is the first denaturation which was set for 95 degrees Celsius for a time between 15-40 seconds. The third step is the annealing step, which was set at 45-60 degrees Celsius for a time between 30-60 seconds. The fourth step is the extension step, which was set at 60-72 degrees Celsius for a time that corresponded with the length of the DNA (2 minutes/kb for Pfu and 1 minute/kb for Taq). The final step is the final extension step which was set at 72 degrees Celsius for 5 minutes. Steps 2-4 were repeated for 30 cycles.

**PCR Purification**

Minicolumns were used to purify DNA following a PCR or restriction digest to be used for cloning purposes. This kit includes a microspin cup with a filter that retains DNA equal to or greater than 100 bp. Also the kit includes wash buffer and DNA binding solution. DNA obtained from PCR amplification or restriction digests were mixed with equal volumes of DNA binding solution. The DNA binding solution and DNA mixture was transferred into a filtered microspin cup that was centrifuged (14,000 rpm) for 30 seconds. This separated the DNA binding solution from the DNA of interest. 750 microliters of wash buffer mixed with ethanol was added to the microspin cup and centrifuged in a microfuge for a maximum of 30 seconds to separate the wash buffer from the DNA. Then 1X TE was added to the column and incubated for 5 minutes. The microspin cup was then centrifuged as above to elute the purified DNA.

**Plasmid Purification from E. coli**

Transformed plasmids from *E. coli* competent cells were purified using Strataprep plasmid miniprep kits. We used the protocol that came with the miniprep kit. It includes solution 1, solution 2, solution 3, wash buffer, microspin cups, and 2 ml receptacle tubes. These solutions were used to break the cells, in order to extract the plasmid DNA and then to bind the DNA.
After cell lysis and centrifugation for a maximum of 5 minutes, the supernatant was transferred to a plasmid purification column (microspin cup seated in a 2 ml receptacle tube). Once the supernatant was added to the column, it was centrifuged for a maximum of 30 seconds. This separated the solutions from the plasmid DNA. Wash buffer was then added and separated from the DNA in the filter column; this assured the DNA was purified. Finally, 1X TE was added to the filter column, and left to incubate for 5 minutes at room temperature. The purified plasmid DNA was eluted with 1X TE.

**Cloning procedure**

Cloning is an experiment used to create a fusion protein by using an insert and a vector of choice. The cloning procedure requires many steps and is very involved. Many of the steps required involve the aforementioned methods, and since these methods were already explained in sections above, I have summarized the cloning procedure as much as possible. PCR amplification was required to obtain large quantities of insert that was used for the ligation. Once large quantities of insert were obtained, the DNA was purified as much as possible using the Strataprep PCR purification kit. Following the PCR purification, separate restriction digests were run on the amplified insert and on the vector in which the insert was ligated. The restriction digest was to ensure that the ends of the insert and vector were compatible and able to ligate to one another. After the restriction digest, another purification procedure (using the Strataprep PCR purification kit) was required to ensure that the vector and insert were purified. If any restriction enzymes remained in the insert or vector mixtures the ligation procedure was inhibited due to continuous cutting by the enzyme. To make sure no residual enzymes remained after the purification, the vector and insert were placed on the heating block at 65 degrees Celsius for 20 minutes. This denatured any residual enzymes and deactivated its activity.
After the vector and insert were placed on the heating block, a gel was run to verify the DNA had not vanished, and also guaranteed that the bands coincided with the correct number of base pairs that the vector and insert contained. Also, a gel was used to estimate the amounts of vector and insert used for the ligation procedure. Usually, while the gel ran, we used the nanodrop instrument to quantify the amount of DNA present in both the vector and insert mixture. The nanodrop instrument measured the absorbance of the DNA mixture at 260 nm and the determined amount of DNA in nanograms per microliter. With this data, we calculated the exact amount of DNA used in the ligation mixture. Also, if the correct bands were viewed on the gel, the ligation was not abandoned. If bands were not observed on the gel, it was a good indicator that the correct DNA was not present, and the cloning procedure was redone.

After calculating the amount of DNA, based on the data obtained from the nanodrop and gel analysis, the ligation was ready to be performed. The mixture contained a T4 DNA ligase, its 10X associated buffer, vector, insert, and sterilized water. The total volume used for our ligations was 40 microliters. The amount of 10X T4 DNA ligase buffer used was 4 microliters, and the amount of other components used in a ligation mixture was usually variable. The amount of T4 DNA ligase used was usually between 0.5 -1 microliter (400 units/μl). The amount of water used depended on the calculated amount of vector and insert used. After the mixture was created, it was incubated at room temperature for one hour. After this incubation period, the mixture was doubly incubated in a Styrofoam box filled with water at room temperature and put in the 4 degree Celsius fridge overnight. This is so the ligase had the opportunity to be active at many different temperatures because we were not sure of the temperature it reached its optimal activity. Once this incubation period was over, the ligation mixture was used for *E. coli* transformations on the correct antibiotic media (depended on the antibiotic resistant marker
found on the associated vector) and we retrieved the correct ligated plasmid. We found the correctly ligated plasmid by culturing many colonies from the E. coli transformation, miniprepped the cultured colonies to purify the recombinant DNA, used a restriction digest to drop the insert from the vector, and ran the restriction digested DNA on a gel that verified that the insert had dropped from the vector.

**E. coli Transformation**

E. coli transformations were done to produce sufficient plasmid DNA. These transformations were performed after cloning so cells procured the recombinant DNA and produced sufficient DNA used for future experiments. The colony was cultured and miniprepped, respectively. The transformation began by thawing XL1-Blue competent or sub-cloning competent cells. The sub-cloning grade competent cells (Stratagene) were used for quick transformations, but ultimately led to less efficient acquisition of the recombinant plasmid. The super-competent E. coli cells more efficiently acquired the transforming DNA, but required longer incubation times and used β-mercaptoethanol. β-mercaptoethanol was shown to increase transformation efficiency. I have explained the procedure for the sub-cloning competent cells for ease of understanding.

Once the cells were thawed, 50 microliters of cells were added to a 14 ml “Falcon” tube. After this, 0.1-50 ng of DNA was added to the 50 microliters of competent cells and incubated on ice for 20 minutes. Following the incubation period, the mixture was heat-pulsed for 45 seconds in a water bath that was 42 degrees Celsius. The duration of the heat-pulse was critical. The tube was then incubated on ice for 2 minutes. Then 0.9 ml of SOC (1ml of liquid LB media, 100 microliters of 2M MgSO₄, and 200 microliters of 20% glucose) medium was added to the competent cells mixed with plasmid DNA. The SOC, sub-cloning competent cells, and
recombinant DNA mixture was incubated at 37 degrees Celsius for 30 minutes with shaking at about 200 rpm. Afterwards, the mixture was plated on solid LB media with the correct antibiotic according to the antibiotic marker the plasmid contained. Usually, 100-200 microliters were used for each LB plate. Incubation overnight at 37 degrees Celsius was required for appearance of colonies.

**Yeast Transformations**

Yeast transformation experiments were used to transform plasmids into yeast that were obtained from *E. coli* transformations or bought directly from a manufacturer. The transformed plasmid contained an amino acid marker that yeast cells required for viability. Before the actual transformation began, yeast colonies were cultured in liquid YEPD (usually 5 mls) and incubated at 30 degrees Celsius overnight. The yeast culture was robust so the yeast transformation was efficient. Once the yeast culture was done incubating, it was diluted in 45 mls of liquid YEPD. This gave us a 1:10 dilution so the cells did not overgrow when transformed. The diluted yeast cells were incubated for 1-4 hours. Following the incubation, the yeast cells were centrifuged in a desktop centrifuge for 15 minutes, washed with 10 mls of sterile water, re-centrifuged, and centrifuged again in an ultra-centrifuge for 5 minutes at room temperature. The cells were then re-suspended in 1ml sterile water and transferred to a 1.5 ml microfuge tube.

The cells were centrifuged, and the supernatant was discarded. A 1X TE/LiOAc solution (1ml 10X TE pH 7.5, 1ml 1M lithium acetate and 8 mls sterile water) was made to resuspend the cells, and then the cells were centrifuged once again. The yeast cells were re-suspended in 0.25 mls of 1X TE/LiOAc solution. Then, 50 microliters were transferred to a different microfuge tube in which 5 microliters of carrier DNA (10 mg/ml) and transforming DNA were added. After this, a polyethylene glycol solution (4 mls 50% PEG, 0.5 ml 10X TE, 0.5 ml 10X LiOAc, and
equilibrated to a pH of 7.5) was made and 300 microliters was added to the transformed and carrier DNA yeast mixture. The mixture was incubated at 30 degrees Celsius for one hour, and after, 40 microliters of DMSO was added to increase transformation efficiency. The mixture was then heat shocked at 42 degrees Celsius for 15 minutes. Then the cells were washed and centrifuged in 1X TE. The cells were then resuspended in 1ml of 1X TE and 200 microliters were plated on solid selective media according to the plasmid used. The plates incubated for about 3 days at 30 degrees Celsius, or until colonies were fully grown. This high efficiency yeast transformation was performed according to the Linda Hoskins/Hahn Lab protocol (http://labs.fhcrc.org/hahn/Methods/genetic_meth/dmso_yeast_transform.htm).

Yeast-Two Hybrid to Test for Protein Interactions

The Yeast-Two Hybrid analysis is used to test for protein interactions (Molecular Cell Biology 2000). Two specific plasmids were used for this experiment. The pAD-GAL4-2.1 vector contains an ampicillin resistance marker for E. coli transformations, the LEU2 gene for selection in yeast transformations, and DNA encoding the activation domain of GAL4 for protein interaction. The pBD-GAL4 Cam vector contains a chloramphenicol resistance marker, TRP1 gene for selection, and DNA encoding the binding domain for protein interactions. Both plasmids contain a multiple cloning site where several restriction sites are found for cloning purposes. Before these plasmids were used for any transformation, the gene of interest (insert) was first ligated into these vectors, according to the cloning procedures previously explained. Then these recombinant plasmids were transformed into E. coli for overexpression of the plasmids, as explained in the E.coli transformation section. After the E. coli transformation, the plasmids were miniprepped and used for yeast transformations (as explained in the sections above).
The recombinant plasmids were transformed into the YRG-2 yeast strain, which contains HIS3 and the Lac Z reporter gene driven by GAL4 binding sites to assess verified in vivo protein interactions. Also, the YRG-2 strain carries a mutation which ensures that the GAL4 gene is not expressed. Similarly, the YRG-2 strain carries leucine and tryptophan auxotrophic markers to verify that the GAL AD and the GAL BD recombinant vectors are transformed into the YRG-2 strain. The YRG-2 strain also has a histidine auxotrophic marker to select for in vivo protein interactions. The HIS3 gene is only activated if the fusion proteins interact with one another. The Lac Z assay may also be used to test for protein interactions quantitatively.

The GAL AD and the GAL BD vectors were transformed simultaneously using the yeast transformation procedure explained in the previous section. To verify these plasmids were correctly transformed, the transformed cells were plated on SC solid media plates that did not contain leucine and tryptophan. Growth indicated that the cells contained the correct GAL AD and GAL BD vectors. These vectors should also contain the correct inserts which were translated in yeast cells to their protein products. The yeast cells that grew on the -leu,-trp plates were then patched onto SC plates that did not contain leucine, tryptophan, or histidine. If the cells grew on these plates, there was a protein interaction occurring. This experiment was done multiple times to acquire my results and to verify the results as well.
Results

Cloning the *IME4* gene and its fragments to test for specific protein-protein interactions

The *IME4* full length (amino acids 1-600) gene, the *IME4* N-terminal (amino acids 1-316) fragment, the *IME4* C-terminal (amino acids 231-600), and the *IME4* leucine (amino acids 131-225) rich fragment were cloned into the GAL 4 binding domain of plasmid pBD-GAL4 Cam using the EcoRI and SalI restriction sites located on the GAL 4 binding domain vector to test for protein interactions. The *IME4* gene and its aforementioned fragments were amplified using primers (see Materials and Methods section for specific sequence of primers) that contained EcoRI and SalI restriction sites that were used to ligate these inserts to the restricted vector, which contained the compatible sites as well. The purified PCR products were ligated to the pBD-GAL4 Cam vector to create in-frame protein fusions to the Gal 4 binding domain.

![Diagram of ime4 “bait” fusion proteins for testing interactions](image)

**Figure 4:** A representation of the fragmented inserts of *IME4* within the GAL4 binding domain.
Test for Auto activation of *IME4* and its associated fragments

The N-terminal Ime4 fragment was seen to auto activate when transformed into YRG-2 yeast strains with the empty pAD-GAL4-2.1 vector for yeast two hybrid assays. The empty pBD-GAL4 Cam vector, full length, leucine rich, and catalytic regions of Ime4 did not auto activate when transformed with the empty pAD-GAL4-2.1 vector. By this notion, we did not use the Ime4 N-terminal in the yeast two hybrid assays because it would have given us false positives for any pAD-GAL4-2.1 vector with its associated insert. Also, we strongly believe that the *HIS3* reporter gene is leaky in certain transformations and has resulted in the slight increase of growth in the YRG-2 strain when transformed with Ime4 full length and an empty activation domain vector. We sequenced these bait plasmids and verified that all of them were correctly cloned and in frame. The reason the *IME4* insert is used in the “bait” plasmid (binding domain) is because we are interested in validating novel protein interactions.
Motivation to study Mum2 protein interactions

Mum2 and Ime4 were verified to interact with one another in a protein complex by the Fink Lab (Agarwala et al. 2012). This protein complex is referred to as the MIS complex (Mum2, Ime4, and Slz). Mum2 is an essential protein in the MIS complex, and its interaction with Ime4 is crucial for Ime4 to carry out its catalysis. Deletion experiments of the MUM2 gene resulted in sporulation defects in Saccharomyces cerevisiae because of the consequent down regulation of Ime4 (Agarwala et al. 2012). Mum2 is essential for the catalytic activity of Ime4. We were interested in understanding the exact location of Mum2 binding with the Ime4 protein, and we used the fragments of IME4 to ascertain the sequence to which Mum2 binds to Ime4.

Cloning the MUM2 gene

The MUM2 gene was cloned into the GAL4 activation domain (AD) vector as the “prey” protein and we assessed its specific interaction with the IME4 gene and its fragments, which were considered the bait “proteins” in yeast two hybrid experiments. The restriction sites used in the PCR amplification of the MUM2 gene were EcoRI and a BamI/BglII junction. The BamI/BglII junction was used because ligating the MUM2 gene into the GAL 4 activation domain proved to be difficult with the use of other restriction sites.
Figure 6: A restriction digest was performed on MUM2 in its GAL4 activation domain vector which showed that MUM2 was correctly ligated. Also, restriction digests were performed on IME4 and its associated fragments that were ligated to the GAL4 binding domain. The GAL binding domain vector is 6.5 kb, the GAL4 activation domain vector is 7.0 kb, the MUM2 insert is 1.1 kb, the IME4 full length insert is 1.8 kb, the IME4 N-terminal is 0.95 kb, the IME4 catalytic region is 1.1 kb, and the IME4 Leucine rich segment is 0.28 kb. Also, we sequenced the fusion plasmid and verified the clone was in frame.

Mum2 and Ime4 Protein-Protein interactions

By using the yeast two hybrid assays, the cloned MUM2-GAL4 4 activation domain and the IME4 full length-GAL4 binding domain, with its fragments as well, were simultaneously transformed into YRG-2 yeast cells and plated on SC media that were lacking leucine and tryptophan to select for the two plasmids. The yeast cells present on the leucine and tryptophan deficient SC media grew for about three days before being patched onto leucine, tryptophan, and histidine deficient media to observe activation of the Gal4 dependent HIS3 reporter in the strain. We patched the yeast cells on this media and verified that Mum2 and Ime4 full interacted. We further investigated the interaction between Ime4 and Mum2.

Analogous experiments were completed with the Ime4 fragmented proteins and the Mum2 protein. We demonstrated that the Ime4 catalytic region (amino acids 231-600) did not
interact with the full length Mum 2 protein. The Ime4 N-terminal (amino acids 1-316) displayed an interaction with Mum2, but this result remains inconclusive because we validated that the N-terminal domain of Ime4 is auto activated in yeast two hybrid assays. We zoomed in on a leucine rich region located in the N-terminal of the Ime4 protein, which did not auto-activate in previous yeast two hybrid experiments. We termed this region the Ime4 leucine rich region, extending from amino acids 131-225 of the Ime4 protein. Interestingly, Mum2 interacted directly with the Ime4 Leucine rich region in yeast two hybrid assays. This led us to believe that the interaction between Mum2 and the Ime4 Leucine rich region may be even more specific. Presently, we are conducting experiments that will narrow down the interaction between Mum2 and the Ime4 leucine rich region.

**Motivation to study Ygl036w protein interactions**

Previous affinity capture and yeast two hybrid experiments have suggested that Ygl036w and Mum2 exhibit protein-protein interactions (TylersLab.com 2013). *YGL036W* is not an essential gene, and its function is unknown (TylersLab.com 2013). These interactions have implications that Ygl036w may interact with Ime4.

**Cloning the YGL036W gene**

The *YGL036W* gene was cloned into the GAL4 activation domain vector using BamHI and SalI restriction sites that were incorporated into the primers. The *YGL036W*-GAL4 activation domain fusion protein was transformed into YRG-2 yeast cells simultaneously with the *IME4* full length and fragments of IME4 that were cloned into the GAL4 binding domain vector as above. This was done to investigate the interaction between Ygl036w and the Ime4 protein, including its associated fragments.
Figure 7: A restriction digest was performed on YGL036W in its GAL4 activation domain vector which showed that YGL036W was correctly ligated. Also, restriction digests were performed on IME4 and its associated fragments that were ligated to the GAL4 binding domain. The GAL binding domain vector is 6.5 kb, the GAL4 activation domain vector is 7.0 kb, the YGL036W insert is about 2.7 kb, the IME4 full length insert is 1.8 kb, the IME4 N-terminal is 0.95 kb, the IME4 catalytic region is 1.1 kb, and the IME4 Leucine rich segment is 0.28 kb. Also, we sequenced the fusion plasmid and verified the clone was in frame.

Ygl036w and Ime4 Protein-Protein interactions

Though virtually nothing is known about the Ygl036w protein, we decided to test whether or not Ime4 interacts with this protein. We were able to confirm that the full length Ime4 protein does interact with the Ygl036w protein.

In discovering that the Ime4 full length protein and the Ygl036w protein interacted, we decided to do similar experiments that were done with the Mum2 protein, which narrowed down the interaction between Ime4 and Mum2 to specific leucine residues. The Ime4 catalytic region displayed no interaction with Ygl036w, and this result was consistent with those seen for all “prey” proteins. We then decided to test whether the leucine rich region of Ime4 interacted with Ygl036w, which Mum2 was verified to interact with. This experiment was inconclusive because
it interacted very weakly with the Ime4 leucine rich region. We believed that the *HIS3* reporter gene in the YRG-2 strain could be leaky when yeast two hybrid assay experiments are carried out. Further experimentation will be done to assess the results between the leucine rich region of Ime4 and Ygl036w.

**Figure 8**: a. Ygl036w in the pAD-GAL4-2.1 vector transformed with Ime4 Full in the pBD-GAL4 Cam vector. b. Ygl036w in the pAD-GAL4-2.1 vector transformed with empty pBD-GAL4 Cam vector.

**Motivation to study Bur2 protein interactions**

Bur2 is a cyclin for the Sgvp1 (Bur1) protein kinase, and they comprise a CDK-cyclin complex that is involved in transcriptional regulation (Yao, Neiman, and Prelich 2000). The regulation of transcription through this complex is controlled by phosphorylation of the carboxy-terminal domain of the largest subunit of RNA polymerase II (Yao, Neiman, and Prelich 2000). Fascinatingly, Bur2 used as the bait protein in yeast two hybrid analysis and Ime4 used as the prey protein was found to interact in two independent studies (Malmström et al. 2007). The function of this interaction is still unclear, and it was in our best interest to find out the specific interaction between these two proteins. This may further elucidate the function of Bur2 in the MIS complex.
Cloning the *BUR2* gene

The *BUR2* gene was cloned using restriction sequences that were added to the specific *BUR2* primer sequences. The restriction sequences are SalI and XhoI. We amplified this gene using the specific forward and reverse primers and cloned the insert into the GAL4 activation domain vector, which had compatible restriction sites.

![Image of restriction digest](image)

**Figure 9**: A restriction digest was performed on *BUR2* in its GAL4 activation domain vector which showed that *BUR2* was correctly ligated. Also, restriction digests were performed on *IME4* and its associated fragments that were ligated to the GAL4 binding domain. The GAL binding domain vector is 6.5 kb, the GAL4 activation domain vector is 7.0 kb, the *BUR2* insert is about 1.2 kb, the *IME4* full length insert is 1.8 kb, the *IME4* N-terminal is 0.95 kb, the *IME4* catalytic region is 1.1 kb, and the *IME4* Leucine rich segment is 0.28 kb. Also, we sequenced the fusion plasmid and verified the clone was in frame.

**Bur2 and Ime4 protein-protein interactions**

Yeast two hybrid screens were prepared to test the interaction between the Ime4 full length protein and the Bur2 protein. We used Bur2 as the prey protein and Ime4 as the bait.
protein in our yeast two hybrid assays, which opposed the two independent previous
experiments. Our results were similar; we found that the Bur2 protein and the Ime4 full length
protein interacted. We replicated the Ygl036w experiments to acquire a clearer representation of
the specific interaction between Bur2 and Ime4.

Bur2 and the catalytic region of Ime4 were screened for interactions. We discovered that
Bur2 and the Ime4 catalytic region did not interact when coupled in yeast two hybrid screens.
The catalytic region of Ime4 seems to be absent of protein binding. This is why we are convinced
that all the aforementioned proteins interact in the N-terminal portion of Ime4. To further
convince us that Bur2 does not interact in a similar manner to Mum2, we tested the interaction
between Bur2 and the leucine rich region of Ime4. These fusion proteins did not interact with
one another in our yeast two hybrid screens.

Figure 10: a. Bur 2 in the pAD-GAL4-2.1 vector transformed with Ime4 Full in the pBD-GAL4
Cam vector. b. Bur 2 in the pAD-GAL4-2.1 vector transformed with empty pBD-GAL4 Cam
vector.

Cloning the METTL3 gene and its fragments to test for specific protein-protein interactions

We tried numerous times to clone the full length METTL3 gene, but it proved to be
difficult. We tried many different restriction sites in association with the restriction sites found in
the GAL4 binding domain, but nothing seemed to work. We considered that the fusion protein of
the full length Mettl3 protein could be toxic in E.coli cells when transformed following a ligation (Personal Communication with Dr. Clancy). But even this conclusion could be debated because the ligations could have been erroneous in some manner (Personal Communication with Dr. Clancy). We decided the best scenario would be to clone half segments of the protein to test for interactions using the yeast two hybrid screens.

*METTL3* amino acids 273-580 was cloned into the pBD-GAL4 Cam vector using EcoRI and SalI restriction sites that were compatible with restriction sites situated in the multiple cloning site of the GAL4 binding domain vector. The primers (specific primer sequences can be viewed in the Methods section) contained the restriction sequences that were needed to create the flanking restriction sites for the *METTL3* 273-580 insert. The *METTL3* fragment was cloned into the pBD-GAL4 Cam vector known as the bait plasmid because we were interested in observing its interaction with different proteins. The genes we used to test protein interactions with Mettl3 were cloned into the pAD-GAL4-2.1 vector, which is considered the “prey” plasmid.

**Motivation to study Wtap protein interactions**

We postulated that the Wtap protein would interact with the Mettl3 protein because of their orthology to the yeast proteins Mum2 and Ime4, respectively. Furthermore, Mettl3 has been suggested to carry out its catalysis in a protein complex (Bokar et al. 1997). The elucidations of the proteins that interact in the Mettl3 complex are still being investigated.

**Cloning the WTAP Gene**

The *WTAP* gene was cloned into both the GAL4 activation and binding domain vectors. The restriction sites flanking the *WTAP* inserts were EcoRI and SalI. Only one pair of forward and reverse primers (specific sequences found in Materials and Methods section) with these
restriction sequences were required to successfully clone the WTAP inserts into their respective vectors. Once cloned, these plasmids were used for yeast two hybrid screens.

**Figure 11:** A restriction digest was performed on WTAP in its GAL4 activation domain vector which showed that WTAP was correctly ligated. Also, restriction digests were performed on IME4 and its associated fragments that were ligated to the GAL4 binding domain. The GAL binding domain vector is 6.5 kb, the GAL4 activation domain vector is 7.0 kb, the WTAP insert is about 1.2 kb, and the IME4 N-terminal is 0.95 kb. Also, we sequenced the fusion plasmid and verified the clone was in frame.

**Wtap protein interacting with itself**

We did experiments that showed Wtap interacting with itself. The WTAP gene was cloned into the binding and activation domain plasmids, and then we transformed the plasmids simultaneously into the YRG-2 yeast strains. We then did a yeast two hybrid assay and discovered that Wtap does interact with itself. Also we did controls to verify that Wtap did not auto-activate. The results are shown in Figure 12.
Figure 12: a. Wtap in the pBD-GAL4 Cam vector transformed with Wtap in the pAD-GAL4-2.1 vector to test for their interaction. b. Wtap in the pAD-GAL4-2.1 vector transformed with empty pAD-GAL4-2.1 vector as a control to ensure that it did not auto-activate. c. Wtap in the pAD-GAL4-2.1 vector transformed with empty pBD-GAL4 as a control to ensure that it did not auto-activate.

**Wtap and Mettl3 273-580 protein interaction**

Experiments were performed to test the interaction between Mettl3 amino acids 273-580 and Wtap in the pAD-GAL4-2.1 vector. We suspect that these proteins are interacting because of the growth on the plates shown in Figure 13. These protein interactions will have to be verified using coimmunoprecipitation assays.

Figure 13: a. Mettl3 amino acids 273-580 in the GAL binding domain (nicknamed “Bokar” by our lab) was shown to interact with Wtap in the GAL activation domain. b. Wtap in the
activation domain was transformed with empty GAL binding domain to ensure that it did not auto-activate. c. Mettl3 amino acids 273-580 in the GAL binding domain was transformed with GAL activation domain to make sure that Mettl3 amino acids 273-580 did not auto-activate.

**Motivation to study Wt1 protein interactions**

Wtap has been verified to be a binding partner of Wt1 in yeast two hybrid assays (Little, Hastie, and Davies 2000). We tested the interaction between Wtap and Wt1 as a positive control. Furthermore, we were interested in discerning the interaction between Wt1 and Mettl3.

**Cloning the WT1 gene**

The WT1 gene was ligated using EcoRI and SalI restriction sites, which flanked the WT1 insert. A PCR amplification using specific primers (primer sequence found in Materials section) contained the restriction sites that flanked the insert. The insert had the same restriction sites that its associated vector had so there were no compatibility issues. The vector was the GAL4 activation domain, which had the same restriction sites necessary for an efficacious ligation.

**Wt1 and Wtap protein-protein interaction**

We performed a yeast two hybrid assay to test the interaction between Wt1 in the pAD-GAL4-2.1 vector and Wtap in the pBD-GAL4 Cam vector. Interestingly, these proteins did not interact in the assays we performed. We also did a Wtap binding domain and empty Gal-4 activation domain plasmid control, and we did not see an interaction. Furthermore, we did a control of Wt1 in the Gal-4 activation domain vector and transformed it with empty Gal-4 binding domain control, and we they did not interact. The absence of an interaction is most likely due to the Wt1 isoform we used to test this interaction.
**Wt1 and Mettl3 273-580 protein-protein interaction**

Utilizing the yeast two hybrid assays, we assessed the interaction between Wt1 in the Gal-4 activation domain vector and the fragmented Mettl3 273-580 protein which was cloned into the Gal-4 binding domain vector. We did not observe an interaction between these proteins in our assays. We also verified that these proteins did not auto-activate by transforming them with empty vectors. Wt1 in the Gal-4 activation domain vector was transformed with empty Gal-4 binding domain plasmid, and no interaction was observed in this experiment. Mettl3 273-580 in its binding domain vector was transformed with empty Gal-4 activation domain plasmid, and this protein did not auto-activate in our assays.
Discussion

Protein complexes govern many processes that occur within cells. Discerning the proteins that regulate protein complexes is crucial to understanding the function of these proteins, and the function of the protein complex in its entirety. The N^6^-methyladenosine (m^6^A) modification is known to be regulated by a protein complex. The role of the m^6^A modification is still unclear, but this modification is incredibly significant. It is unlikely that there is one unifying role behind this modification, but it may play many roles in cellular processes such as effecting transcriptional fate and regulation, mRNA metabolism, translational efficiency and control, protein transport, and regulating cellular transformations. m^6^A is the most prevalent internal messenger RNA modification detected in eukaryotes and in RNA of nuclear replicating organisms (Fine-tuning of RNA Functions by Modification and Editing 2005). The m^6^A modification is catalyzed by methyltransferases found in all organisms (e.g. Ime4 in yeast and Mettl3 in humans). These methyltransferases are recognized to be in complex with other proteins that may regulate, enhance, or repress the catalysis of these methyltransferases. Understanding the exact function of m^6^A through experiments which directly invoke an alteration in this modification is perplexing, which is shown by many pleiotropic methylation inhibitor experiments. It appears more fathomable to elucidate the function or functions of m^6^A by illuminating the protein-protein interactions that are associated in the protein complex that catalyzes this modification.

Ime4 in yeast catalyzes the methylation of pre-mRNA transcripts. Ime4 was hypothesized to reside in a protein complex that facilitated its catalysis, which is to transfer reactive methyl groups bound to sulfur in S-adenosyl methionine (SAM). It has since been verified that Ime4 is in complex with two proteins known as Mum2 and Slz1. Mum2 is known to be an essential
protein in the Mum2-Ime4-Slz1 (MIS) complex. Our results show that Mum2 interacted in the amino acid region 131 through 225 within the N-terminal region of Ime4, which we considered the leucine rich region. In this thesis, we have hypothesized that Mum2 may scaffold other proteins to this complex. The leucine region of Ime4 contains alpha helices that may bind the Mum2 coiled-coil protein.

Mum2 was verified to interact with Ygl036w in two independent experiments, which were affinity capture and yeast two hybrid assays. With this information, we tested whether Ygl036w would interact directly with the full length protein (amino acids 1-600) and its fragments. We observed that Ygl036w interacted with the full length Ime4 protein. We were also convinced that Ygl036w specifically interacted within the N-terminal region of Ime4, but experiments to confirm this are still ongoing because we established that the N-terminal fragment of Ime4 self-activated in yeast two hybrid screens. Also, we observed that Ygl036w does not interact in the catalytic region of Ime4 (amino acids 231-600) or the leucine rich region of Ime4. Similar results were shown with the Bur2 protein. The Bur2 protein interacted with the full length Ime4 protein, but does not interact with the leucine rich or catalytic region of Ime4. Experiments are still partial in concluding whether Bur2 interacts in the N-terminal of Ime4 because of its difficult screening due to the N-terminal self-activating in yeast two hybrid experiments. We do not know the importance of these interactions with Ime4. We will be conducting gene deletion experiments to assess whether these interactions play a role in yeast cell viability or the sporulation process. We hypothesize that Bur2 may, in some manner, target Ime4 to RNA transcripts because of its interaction with RNA polymerase. Nothing can be said about Ygl036w and its interaction with Ime4 because there is virtually nothing known about this protein.
The human homolog of Ime4 is termed Mettl3, which is hypothesized to reside in an enormous protein complex. However, to our knowledge, the proteins that interact in this complex remain elusive. Mettl3 catalyzes the same function that Ime4 catalyzes, which is to methylate adenosine residues of mRNA transcripts internally. The mechanism by which this occurs is still unclear, and the proteins that regulate this modification catalyzed by Mettl3 are still unknown. Mettl3 amino acids 273-580 in the GAL binding domain was suspected to interact with Wtap in the GAL activation domain in our yeast two hybrid assays. We will need to perform future pull down assays to confirm this interaction. Wtap may act in a similar manner to Mum2, which is hypothesized to scaffold proteins to the Ime4 complex. Or Wtap may act as a splicing factor and affect the fate of methylated transcripts. Experiments are ongoing to elucidate these hypotheses. Also, it is necessary to make the connection that Wtap associates with the Wilms’ tumor protein 1 (Wt1), which has an essential role in the normal development of the urogenital system. Mettl3 may play a regulatory role in the function of Wt1 if it is found to interact with either Wt1 or Wtap.

Importantly, the m^6A modification has remained evolutionarily conserved in all organisms over time. This is significant because natural selection conserved the genes that function to catalyze this modification. This conservation proves necessary for organisms to sustain their continued survival overtime. The most studied proteins that catalyze the m^6A modification include *Saccharomyces Cerevisiae, Homo sapiens, Arabidopsis thaliana*, and *Drosophila melanogaster*. These proteins are termed Ime4, Mettl3, Mta, and Dm Ime4 respectively. Deficiency experiments have revealed the importance of these proteins. Induced experimental deficiency of Mettl3 is detrimental and leads to apoptosis in *Homo sapiens* (*Fine-tuning of RNA Functions by Modification and Editing* 2005). Developmental arrest was observed
in *Arabidopsis thaliana* when Mta was inhibited of its activity (Zhong et al. 2008). Defects in gametogenesis were verified when Ime4 and Dm Ime4 were silenced in *Saccharomyces Cerevisiae* and *Drosophila melanogaster*, respectively (Clancy et al. 2002; Hongay and Orr-Weaver 2011). Discovering the exact function or functions of m$^6$A and the proteins that interact in these complexes that catalyze this modification may prove to be medically relevant.

In *Homo sapiens*, experiments have revealed that the knockdown of Mettl3 resulted in apoptosis of Hep2G cells (Dominissini et al. 2012). Cells undergoing transformations, whether it may be normal cellular transformations or harmful ones, the methylation of adenosine residues in mRNA transcripts increases. The reason this occurs remains unclear, but it may have to do with an increased amount of translated proteins that cells need for specific transformations. Silencing the m$^6$A methyltransferase is also known to considerably affect gene expression and alternative splicing patterns (Dominissini et al. 2012). Experiments performed by silencing *METTL3* resulted in the inactivation of specific isoforms of *MDMX*, which is a key gene that regulates the activation of the *p53* gene (a tumor suppressor gene). *MDMX* is a gene that is needed for the inactivation of the *p53* gene (Dominissini et al. 2012). The p53 protein is critical in regulating the cell cycle and preventing cancer in humans. When this gene is mutated, cells become immortal and begin to divide sporadically. This leads to metastatic cancers and death as a result of these cancerous cells affecting regular organ function. On this notion, altering the manner in which transcripts are methylated may one day serve to be therapeutically beneficial in treating cancers and other diseases that may be caused by proteins that are not translated by the lack of m$^6$A modified transcripts. Down-regulating transcripts that are overexpressed in diseases such as trisomy 21 by inhibiting the m$^6$A modification may prove to be a noteworthy treatment. Remarkably, a protein called FTO (Fat mass and obesity associated protein) catalyzes oxidative
demethylation of $m^6A$ residues in mRNA transcripts in humans (Jia et al. 2011). FTO has been revealed to affect energy homeostasis, human obesity, and is associated with Alzheimer’s disease (Jia et al. 2011). Exactly how the demethylation of these mRNA transcripts is associated with these factors remains to be clarified. By lowering the amount of $m^6A$ residues on transcripts, translation of proteins, regulation of mRNA metabolism, and transportation of transcripts to and from the nucleus may be affected in some manner. These reasons may justify targeting $m^6A$ or increasing the catalysis of the proteins that modify transcripts may serve as being medically beneficial.
Bibliography


APPROVAL SHEET

This is to certify that Yazan Ali Alqara has successfully completed his Senior Honors Thesis, entitled:

Protein Interactions in mRNA Methylation Complexes

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