Regulation of Yeast Development by mRNA Methylation

by

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THESIS ABSTRACT:

The internal methylation of mRNA post-transcriptionally is an essential component of the mRNA editing machinery in virtually every eukaryotic system. Despite this ubiquity, little is known about the relevance, consequences or machinery involved in this process. The recent demonstration of this modification in the brewers' yeast, Saccharomyces cerevisiae, has allowed the study of this modification using the vast array of genetic and biochemical tools available in the organism.

In the second chapter of this thesis, we show that diploid cells of the yeast Saccharomyces cerevisiae experiencing nutrient limitation undergo a restriction of cellular potential and commitment in which the cells cease vegetative, mitotic growth and commit to meiosis. We show that the period prior to commitment can be divided further into two distinct phases: an early stage of initial starvation followed by a commitment to differentiation. Cells that are in the initial starvation phase revert to yeast-form mitotic growth if shifted to nutrient-rich conditions. Cells that are in the commitment to differentiation phase are incapable of returning to yeast-form growth if shifted to nutrient-rich medium, but instead synchronously engage in pseudo-hyphal budding—a nutrient foraging response. Co-ordination of meiosis and PH development in the commitment to differentiation phase is regulated by mRNA methylation.

We dissect this mRNA methylation upon nutrient starvation in the third chapter of this thesis. We identify Ime4, Mum2 and Slz1 as the components of a protein complex that catalyzes mRNA methylation in yeast. These components are necessary for m^6^A accumulation during nutrient starvation; mutation of any one of these components results in defects in meiotic and PH development. Furthermore, we find that ectopic expression of these components under nutrient-rich conditions is sufficient to catalyze this methylation of mRNA. Finally, we provide evidence that this modification is necessary for the activation of translation of genes under starvation conditions. These findings provide evidence for a method of fine-tuning translation under nutrient-stress conditions.

Together, our results support the notion that the yeast starvation response is an extended process that progressively restricts cell fate and reveal a broad role of post-transcriptional RNA methylation in regulating these decisions.

Thesis advisor: Gerald R. Fink
Title: Professor of Biology
To my grandmother, Kanak Lata Adhya,

whose mortar and pestle always proved more ample and welcoming than the temple bell,

and my grandfather, Shanti Bhusan Adhya,

whose patience was so great as to instill in us the pleasure of finding things out.

*In memoriam.*
ACKNOWLEDGEMENTS

Like some detective out of a Nabokov novel, Gerry has, in some form or another, been lurking in the background of my biological education since I formally started in college: whether it was taking classes with professors who were his former postdocs, reading his work in undergraduate seminars or working with auxin signaling in Arabidopsis roots for my college thesis, Gerry has been influencing my education since the beginning. As a graduate student in his lab, I have not only enjoyed the opportunity to witness the elegant science in the work of my colleagues that engaged me as student at the very beginning of my career, but have had the pleasure of being compelled towards those ends myself. To be sure: the creation of this institution that can train a student from the very beginning is far greater a testament to Gerry’s unique ability to nurture intellect and breed curiosity anything I can articulate here; it is with sincere gratitude and joy that I can look back on my time here.

Collaborators have played another essential role in the work presented here. Terry Orr-Weaver and her lab members not only welcomed me into their fly room during my early days studying fruit fly immunology, but also group meetings, lab outings and celebrations, fully including me in my role of interloper. Terry has also stayed on as part of my committee even after I switched projects and started work with yeast and was a valuable resource not only during the transition, but throughout all my subsequent work. I am also constantly amazed by Andreas Hochwagen and Hannah Blitzblau, collaborators with my yeast work: despite the immense pressures placed on a Whitehead Fellow, Andreas has always shown a curiosity for my work, made time to read over my writing and think about my work. Hannah is an incredible scientist, thinker and—at least to me—educator. She has made time for not only the silly questions about protocols and antibody concentrations, but also the important parts of any project: the lengthy discussions, the probing questions, and the critical eyeing of data. I am fortunate to have these collaborators not only as colleagues, but also role models.

I would be remiss not to mention my committee members: despite my often talking about subjects outside of his field, Phil Sharp has met my work not only with a constant enthusiastic curiosity, but—more importantly—genuine interest. Angelika Amon joined the committee at the very end, and I am indebted to her insight and engagement with my project. I am also thankful for Dennis Kim’s help at the very beginning of my graduate career. I was fortunate to have him as part of my initial committee and am still fortunate to be able to speak with him about graduate life and my future career. Uttam Rajbhandary and Fred Winston come as members to the final committee, but are, by no means, strangers: Tom’s advice on molecular techniques have been invaluable to my research, and his encouragement during our meetings have been a constant help. Fred Winston has been one of the authors I have followed throughout my studies, reading about his work on histones as an undergraduate, to his work with suppressors of transposition with Gerry in my graduate courses, to reading his work on transcriptional interference in my own research.

Graduate school has been challenging; classmates, roommates and outsiders have been invaluable. Cambridge, MA in the aughts has been an exciting time and I will remember it
fondly: for this, I owe much to Biograds and Biograd 2004, and especially cherish the friendships I made there. Outside of school, I will never be able to express my immense gratitude to Bill Cutter, who introduced me to so much wonderful music and musicians in Boston and has been a constant both in times of crisis and hilarity. There are also those friends who have constantly kidnapped me, distracted me and insisted on my attention: Sarah and Christine Chester, Sydney Gross and my old roommates Will Kuhlman, John Fiorenza, Mike Baffi and William Lopes have kept me balanced.

In addition, it is a shocking realization, but a good one, to see you are supported by people you wholly admire. I count myself lucky to have read the early cell cycle papers with Madhu Kumar during IAP my first year in our personal journal club, and look forward to talking about science with him every chance I get. I miss judging science fairs with Margaret Ebert and will find Lessons and Carols at Memorial Church from now on a little empty. I miss having lunch with Jane Kim, too, but—happily—still have ample opportunity look forward to her very important complement to my dysfunction. Losing Tal Gross first to Miami, then to New York still pains me a little—as does losing Anne Rounds to Manhattan. And despite chronically being out of touch with them, I consider Maggie Samuels-Kalow and Will Baude not only valuable and dear friends, but also important role models. You would too. This is not to forget or say less about Michael Zawada—also chronically out-of-touch—who has been playing patient Aeschinus to my wild-eyed Ctesipho since the eighth grade. Earlier, even. Nor will I forget Paul Kosmin, who—although arriving later to the game—remains my most important motivator, critic, aggravation and—most importantly—comfort at the end of the day. I could want nothing more in a partner, and do not ask.

Last, I thank my family: my parents and sister—their understanding, sacrifice, patience and wisdom have been the canvas that allowed all this to happen. I do not have words.
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Chapter I: Introduction
The internal methylation of messenger RNA at the N6 group of adenosine is a post-transcriptional modification conserved throughout eukaryotes. The implications of various post-transcriptional modifications, such as splicing, cap formation, and polyadenylation as well as secondary structure and sub-cellular localization on mRNA stability and function have been well-characterized in various organisms under various conditions. However, the functional significance of internal methylation of adenosine in mRNA remains unclear as yet. Specific questions as to the fate of the messages that bear this internal modification, or—more broadly—why a cell might initiate this modification to messenger RNAs still remain unanswered.

This chapter discusses the dynamics and putative role of this poorly understood mRNA modification in eukaryotic systems and the insights that different model organisms have provided in current understanding. This insight occurs on two independent levels: first in terms of the structural components of the putative protein complexes that are necessary for mRNA methylation and second, in terms of the transcripts that become modified. In particular, this chapter focuses on the gene encoding the mRNA methyltransferase, IME4, which is responsible for the formation of m^6^A in budding yeast and has emerged as the model of choice for understanding the role of mRNA methylation in regulating transcript fate. These experiments address the question: how does this gene regulate progression through the diploid-specific nutrient-starvation response of meiosis in yeast?
Central Dogma and the Regulation of Gene Expression

Shortly after the publication of the structure of the DNA double helix in 1953 [1], Francis Crick proposed a model for the transmission of genetic information (i.e., information encoded in genes) in which “once information has got into a protein, it can’t get out again” [2,3]. This model has remained a central paradigm in molecular biology: genetic information encoded in nucleic acids (such as DNA or RNA) is transmitted uni-directionally into the chains of amino acids that from proteins. Crick’s conception of this “central dogma” of molecular biology (outlined in figure 1) recognized the possibility of regulation of this transmission, or gene expression, at any of the nodes of DNA, RNA or protein.

The study of RNA has required the delineation of the various species of the molecule within a cell. Many species of RNA—such as microRNAs or ribosomes—play regulatory or catalytic roles in the cell. Messenger RNAs (mRNAs), in turn, encode the genetic sequence (or, in Crick’s paradigm, “genetic information”) necessary to produce proteins. Many features distinguish mRNA molecules, or transcripts, from other types of RNA molecules. In eukaryotes, mRNAs are transcribed in the nucleus by the RNA polymerase II holocomplex, and then undergo editing and modifications (including splicing, polyadenylation and capping). mRNAs then proceed into the cytoplasm to be translated. In the majority of mRNA molecules, only a portion of the transcribed molecule encodes proteins; features of the 5’ or 3’ untranslated regions (UTRs) often confer modulation to this translation (Figure 2A). Regulation of various aspects of mRNA structure within a cell has been shown to assert much influence over translational efficiency of a single transcript.
In most cases, translation of a single mRNA molecule requires the addition of a 7-methyl-guanylate cap at the 5’ terminus and a poly(A) tract at the 3’ end of the molecule (figure 2A). Both modifications of mRNA occur in the nucleus, simultaneous with the termination of transcription by RNA polymerase II [4,5,6,7]. These modifications both contribute to mRNA stability and enhance translational activity. Eukaryotic caps generally take the form of $m^7GpppN_{1(m)}pN_{2(m)}pN(...)$, where $m^7G$ represents 7-methyl-guanosine. Either of the subsequent nucleotides ($N_1$ or $N_2$) may be any nucleotides, but may be methylated on the 2’-O-position of the ribose ring. In the case that $N_1$ is 2’-O-methyladenosine, further modification of this nucleotidite may result in the methylation at the $N^\text{6}$ group of this base [6,7,8]. In the majority of eukaryotic organisms, many transcripts may undergo splicing, in which introns are excised and exons are spliced together to produce a mature mRNA molecule [4]. Once the mature molecule is produced, mRNA may be transported to various sub-compartments of the cell, where the protein encoded by the mRNA either functions or is processed, to be translated [9,10,11].

**Base modifications of mRNA as a mode of regulating gene expression**

In addition to the structural elements discussed above, the nucleotide sequence of mRNA molecules internal to 5’ methyl cap structure or the 3’ poly(A) tract can undergo four distinct modifications [7] (figure 2B). Deamination of either cytidine or adenosine (to either uridine or inosine, respectively) has been described in metazoan systems. Adenosine deamination has been described in detail for metazoan mRNAs. This reaction is catalyzed by the RNA adenosine deaminases ADAR1 and ADAR2. These genes play essential roles in the
Figure 1. Central Dogma of Biology. A statement of the central dogma of molecular biology by Francis Crick. A diagram of the transfer of genetic information from early draft of Crick’s 1958 *On Protein Synthesis* [2]. In this text, Crick posits that it is possible for DNA to influence DNA and RNA, RNA to influence RNA (due to the presence of RNA viruses), and RNA to influence protein. In this same manuscript, Crick also posits that the reverse—i.e., information in protein influencing DNA sequence—may not happen. This central dogma was formalized in a 1970 publication in *Nature* [2].
The Doctrine of the Triad.

The Central Dogma: "Once information has got into a protein it can't get out again". Information here means the sequence of the amino acid residues, or other sequences related to it.

That is, we may be able to have

\[\text{DNA} \rightarrow \text{RNA} \rightarrow \text{Protein}\]

but never

\[\text{DNA} \leftarrow \text{RNA} \leftarrow \text{Protein}\]

where the arrows show the transfer of information.
**Figure 2. Internal modifications of mRNA.** After, or concurrent with, transcription, multiple structural modifications occur to mRNA. A) Pre-mRNAs (top) are chains of nucleic acids that encode both introns (grey boxes) and exons (blue boxes), prior to processing. Concurrent with transcription, messages become polyadenylated and capped. Subsequently, introns are excised and exons are spliced together. The mature mRNA (bottom) is then exported to the cytoplasm for translation. B) Four modifications have been observed internal to mRNA molecules (i.e., internal to the methyl cap and the poly(A) tail, shown in (A)). Both modifications occur on cytidine and adenosine. In deamination reactions, the $N4$ group on cytidine becomes replaced with a hydroxyl group, forming uridine; this replacement at the $N6$ group of adenosine forms inosine. As in DNA, methylation on cytidine occurs at the 5th position, forming 5-methylcytidine. Methylation of adenosine occurs at the $N6$ group, forming $N6$-methyladenosine.
Figure 2

A

pre-mRNA:

mature mRNA:

protein coding region

5' UTR

3' UTR

B

Deamination reactions: Methylation reactions:

cytosine → uracil

cytosine → 5-methylcytosine

adenine → hypoxanthine (nucleotide form of inosine)
adename → N6-methyladenine
metazoans in which they have been identified: inactivation of ADAR1 results in apoptosis in mouse embryos; inactivation of ADAR2 results in gross neuronal defects in mouse [12,13,14,15]. The specific role of this modification in gene expression remains an active area of study. Deamination of cytidine to uridine causes changes to the transcript sequence and thus translational output of the mRNA molecule [16]. This shift in sequence has been well-characterized for the apolipoprotein B mRNA. This deamination of cytidine to uridine in the transcript results in the creation of an early stop codon in the message, resulting in the expression of a shorter isoform of the transcript (ApoB-48, instead of ApoB-100) [17,18].

Two other modifications arise from methylation of bases internal to mRNA (figure 2B). These modifications also occur on cytidine and adenosine (to form 5-methylcytidine—m^5C—or N6-methyladenosine—m^6A—respectively) [7,19]. Early reports found m^5C in very low levels on mRNA [20,21,22] and subsequent work has characterized the modification as necessary for the tertiary structure of tRNA^{Phe} [23,24]. More recent work has suggested that the initial observation of m^5C in mRNA may have been apocryphal, either resulting from contaminating tRNA in mRNA preparations, or due to residual, ectopic activity of the cytidine methyltransferase [7]. In contrast, m^6A is present in all RNA species but is enriched in mRNA and is unlikely to be an artifact. This modification to mRNA is discussed in depth in the remainder of this chapter.

Methods of studying the target transcripts of these internal modifications are trivial in the case of the deamination reactions, yet significantly more difficult in the case of m^6A. Deamination (of adenosine to inosine or cytidine to uridine) results in a change in the message
sequence. This sequence change is in contrast to m⁶A, which does not appear to affect the base-pairing of the modified nucleoside. Current protocols of detecting m⁶A have relied heavily on high-pressure liquid chromatography (HPLC), mass-spectrometry or thin-layer chromatography (TLC) in order to detect the presence of m⁶A in mRNA [25]. Identification of individual transcripts that carry m⁶A modifications have relied on immunoprecipitation with antibodies raised against m⁶A [26,27]. However, the accuracy of this technique remains questionable. Furthermore, while some modifications of adenosine inhibit RNA-templated DNA ligation reactions, this reaction is significantly more complex for m⁶A and is not easily converted to the whole-genome level [25]. This latter issue is discussed further in chapter 4.

Identification of m⁶A in mRNA of higher eukaryotes

m⁶A has been observed in the transcripts of almost all tested eukaryotic organisms, including plants, mammals and insects [7,8,28,29,30,31,32,33]. Although the modification has been observed in lower eukaryotes as well, specifically S. cerevisiae, there is notable exception of Dictyostelium discoideum and Neurospora crassa [7,34,35]. In addition, viruses that integrate into the host genome as part of their life-cycle (e.g., adenovirus, SV40, herpes viruses, Rous sarcoma virus, influenza) accumulate m⁶A on viral transcripts that are produced from the host nucleus; viruses that do not have a nuclear phase to their life cycle do not accumulate m⁶A [7,36,37,38,39,40]. This latter finding suggests that this modification occurs in the nucleus.

---

1 Inosine base-pairs with cytidine, adenosine or uridine. This is in contrast to adenosine, which base-pairs only with thymidine. Reverse transcription of mRNAs and subsequent sequencing of modified messages would therefore reflect a wobble base pair at inosine bases where the genomic locus would reflect an adenosine/thymidine base pair.

2 It is notable that conversion of 1-methyladenosine—m¹A—is capable of inhibiting RNA-templated DNA ligation. While m¹A is conversion to m⁶A in a Dimroth conversion is favored, the reverse reaction—i.e., m⁶A to m¹A—is not.
Although there is strong evidence to suggest that multiple cellular messages are modified to contain m\(^6\)A, to date the methylation pattern has been mapped in detail on only two transcripts. Work done on bovine prolactin mRNA and Rous sarcoma virus has revealed that the modification occurs in a general context of R(G/A)\(\Delta\)CH (in which A is modified to m\(^6\)A) [32,36]. In each of these instances, m\(^6\)A appears to accumulate near the 3’ terminus of the genes.

Functional significance of adenosyl-methylated RNA

The significance of the m\(^6\)A modification on mRNA remains unclear. Early work from the Rottman group identified and mapped m\(^6\)A sites in bovine prolactin mRNA, raising the possibility that this modification plays a role in mRNA splicing and processing [32,41]. This finding is further reinforced by data from Drosophila, in which mutation of RNA methylation factors resulted in a loss of splicing of various individual transcripts [42,43].

Activation of splicing may only be one of the roles mRNA methylation plays. Many studies have focused on the accumulation of m\(^6\)A on mRNA that do not contain introns and therefore, do not undergo splicing [36,44,45]. Csepany and Beemon attempted to address this function of m\(^6\)A accumulation by mutating the potential methylation sites to synonymous unmethylatable codons on the pol transcript of Rous Sarcoma virus [36]. The authors observed no difference in protein or transcript levels between the unmethylatable and methylatable forms of the transcript. Although these data suggests that m\(^6\)A accumulation on mRNA does not affect its translation, it is possible that this modification affects a transcript’s sub-cellular localization. Alternatively, it is conceivable that the recoded pol transcript failed to mutate a cryptic methylation site that, although lowly methylated, compensates for the lack of
methylation throughout the remaining transcript. Finally, it remains a possibility that RSV pol transcript methylation is a by-product of active mRNA methylation in the nucleus, and therefore, may not have effects on the translation of viral transcripts.

Work by Heilman and colleagues on methylated transcripts that are native to a cell’s expression profile suggests that m\(^6\)A accumulation on transcripts activates translation [44]. These authors utilized an *in vitro* translation assay to measure the translation efficiency of dihydrofolate reductase (DHFR) message that was transcribed *in vitro* either in the presence or absence of m\(^6\)A. This work found a modest increase in protein translated from methylated DHFR transcript as compared to translation of unmethylated transcript. The interpretation of these data must consider many caveats to this experiment: *in vitro* transcription of the DHFR transcript does not necessarily ensure m\(^6\)A incorporation at the positions that are modified *in vivo*. Furthermore, the *in vitro* translation in this experiment utilized a rabbit reticulocyte system and may not reflect the translational output of the transcript *in vivo*.

In a complementary study, Tuck and colleagues inhibited m\(^6\)A accumulation in cultured cells by treatment with the general methyltransferase inhibitor, cycloleucine [45]. This treatment resulted in a modest reduction in the levels of DHFR protein as compared to untreated cells. Taken with the previous result, this finding provides some evidence for the modulation of translation by m\(^6\)A. It is important to note, however, that treatment with cycloleucine inhibits accumulation of methylation on all RNA species, not just the m\(^6\)A mark on mRNA. The reduction of DHFR protein in cycloleucine-treated cells may arise from off-target effects of the drug; the translation of a non-methylated transcript was not monitored in parallel to show that this effect is specific to m\(^6\)A-modified messages.
Despite the significant caveats to both studies, work from the Heilman group suggested for the first time that the $m^6$A mark may activate translation of the transcripts that are modified. It is importantly to note, however, that messages that lack the methyl mark are still translated into protein in both reports from Heilman and colleagues discussed above. Thus, $m^6$A may play a role in modulating translation as opposed to being a necessary component of the translational machinery for the modified messages.

**Structural components of the RNA methyltransferase machinery**

Development of an *in vitro* assay for $m^6$A formation on mRNA permitted the identification of a putative three-component system necessary for catalyzing this modification in HeLa cells by Bokar and colleagues [28,46,47,48]. Fractions of total cellular extract were tested for specific activity in the *in vitro* $m^6$A assay, resulting in the identification of methyltransferase activity in three independent, separable fractions subsequently named MT-A1, -A2 and -B. When combined, these three fractions recapitulated the total methyltransferase activity of total cellular extract [48]. In subsequent studies, MT-A1 was not necessary for maximal activity *in vitro* and was not subsequently cloned. MT-A2 was further isolated and cloned in humans, and is now referred to as *MT-A70*, given its identity as a 70 kDa protein [28].

Although neither MT-B or MT-A, were cloned by Bokar and colleagues, studies of the homologs of *MTA-70* in *Arabidopsis thaliana* provides some clues as to the identity of one of these interacting proteins in humans [30]. Yeast two-hybrid screening of the *Arabidopsis thaliana* homolog, MTA, identified a single interactor, AtFIP37, which bears homology to the human gene, *WTAP-1* (*Wilms Tumour-1 Associated Protein*) and the *Drosophila FL(2)D* (*Female Lethal (2)D*) gene (see Table 2). In addition to the genetic evidence of the two-hybrid screen
<table>
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<th>Organism</th>
<th>Gene name</th>
<th>Gene function/Phenotypes</th>
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<tbody>
<tr>
<td><em>Homo sapiens</em></td>
<td>WTAP-1</td>
<td>IGF-1 signaling; modulates mRNA signaling in smooth muscle cell culture</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Fl(2)d</td>
<td>Pre-mRNA processing, alternative splicing</td>
</tr>
<tr>
<td></td>
<td>CG6315</td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>AtFIP37</td>
<td>Embryonic lethal, involved in endoreduplication. Expressed in various tissues throughout the organism.</td>
</tr>
<tr>
<td></td>
<td>AT3G54170.1</td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>MUM2</td>
<td>Initiation of meiosis, DNA replication, recombination. Expressed during nutrient starvation.</td>
</tr>
<tr>
<td></td>
<td>YBR057c</td>
<td></td>
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</table>
above, co-immuniprecipitation and sub-cellular localization further suggests a physical interaction between the two genes. Furthermore, the T-DNA disruption allele of AtFIP37 results in the same developmental defects as the MTA disruption. As discussed further in chapter 4, work on WTAP-1 in mouse, rats and human cell culture suggests that the gene is involved in multiple developmental processes in various capacities. However, no work has implicated this gene in playing a role in adenosyl methyltransferase activity.

RNA methylation in yeast

Identification of an m^6^A transferase in yeast

Although early work mapping mRNA methylation in eukaryotes found examples in virtually all eukaryotic model organisms, this modification on mRNA was initially thought not to exist in yeast [49]. Early work from Sripati and colleagues analyzed modifications of mRNA undergoing translation (i.e., isolated from polyribosome fractions) in yeast dividing vegetatively in nutrient-rich conditions. This study elucidated numerous modifications of the cap structure that were variant, yet similar to, cap structures in higher eukaryotic organisms. Most strikingly, however, the authors found an absence of m^6^A in the 5' terminus of yeast transcripts. From this finding, it was assumed that S. cerevisiae did not encode an m^6^A-transferase.
<table>
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<th>Organism</th>
<th>Gene name</th>
<th>Gene function/Phenotypes</th>
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<tbody>
<tr>
<td><em>Homo sapiens</em></td>
<td>MT-A70</td>
<td>Methylation of mRNA; knock-down is lethal in cell culture</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Dmime4 CG5933</td>
<td>Regulation of NOTCH signaling. Essential for larval development and gonad development; expressed in gonads.</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>MTA AT4G10760.1</td>
<td>Embryonic lethal, seed formation defects. Expressed in various tissues throughout the organism.</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>IME4 YGL192w</td>
<td>Initiation of meiosis. Expressed in diploid cells upon nutrient starvation.</td>
</tr>
</tbody>
</table>
The yeast-specific methyltransferase was first identified by Bokar and colleagues in studies identifying and cloning the human methyltransferase subunit, MT-A70 [28]. Biochemical isolation and subsequent cloning of the cDNA encoding the methyltransferase from HeLa cells allowed identification of homologs of the gene among other organisms. In yeast, MT-A70 bore homology to IME4, which was known to be important for progression through the meiotic developmental pathway, but whose function was unknown at the time (see Table 2).

Further computational analysis by Bujnicki and colleagues identified structurally conserved motifs in the RNA methyltransferase subunit of the MTA families, specifically, the catalytic site of the protein [50]. This work also identified KAR4 as a potential RNA methyltransferase, although the authors noted the catalytic residues of the gene were sufficiently different that the KAR4 gene product is not likely to have adenosyl methyltransferase activity. However, the IME4 gene was yet again identified as a functional putative N6-adenosyl methyltransferase in yeast with an intact structure and catalytic domain.

**IME4 as a meiotic inducer**

Prior to its identification as an N6-adenosyl methyltransferase, IME4 was independently identified in two independent screens for meiotic inducers in yeast. Starvation for nitrogen in the presence of a non-fermentable carbon source (usually, acetate) induces the meiotic developmental program in diploid yeast cells that are heterozygous at the MAT locus (i.e., MATa/α) (figure 3A, B). Yeast meiosis can be divided into two distinct phases with regard to meiotic

---

3 Following convention, the yeast homolog of MT-A70 will be referred to as IME4 here, although as discussed in the next section, the gene was initially identified as SPO8.
commitment. During the first phase of meiosis, termed meiotic prophase, cells replicate the genome, synapse chromosomes, induce double-strand breaks and engage in recombination. Cells in meiotic prophase are not committed to the developmental program, and shifting cells from starvation conditions into nutrient-rich medium during this time results in a return to vegetative, mitotic growth [51,52]. Cells become committed to meiotic development only after completion of meiotic prophase, when cells undergo two divisions (meiosis I and meiosis II) that segregate the total DNA content of the cell into four distinct masses. These meiotic products become packaged into four spores (two of which encode the MATα mating type locus, two of which encode the MATα mating type locus) encased in a protective ascus. Under favorable conditions, these spores will germinate and form haploid cells. These haploid cells undergo mitotic divisions and will mate with other haploid cells of opposite mating types to form MATa/α diploid cells. This cycle is diagrammed in figure 3A.

Two independent genetic screens implicated IME4 as a regulator of this developmental process. Initial work by Esposito and colleagues took advantage of the ability of a wild haploid spore (i.e., encoding a functional HO endonuclease--HO+) to switch mating type after a single asexual, mitotic division after germination and mate with the resultant daughter cell to form a diploid cell that is homozygous at every locus in the genome (with notable exception, of course, of the MAT locus) (figure 3C, 4A) [53]. Esposito, et al., mutagenized, then germinated spores which were then allowed to self-mate. Thus, individual spores formed clonal populations of diploid homozygous yeast after germination. Because this self-mating results in a homozygous diploid cell, this screen allowed for the identification of recessive alleles of genes that regulate various developmental processes.
**Figure 3. Yeast life cycle.** Brewer’s yeast, *Saccharomyces cerevisiae*, can exist as either a haploid or a diploid. A) Diploid cells (center) of mating type $\text{a}/\alpha$ ($MAT\text{a}/\alpha$) undergo meiosis to form four meiotic products, or spores—two of mating type $\text{a}$ and two of mating type $\alpha$ (bottom). When these spores reach nutrient-rich conditions, they germinate and divide mitotically. When an $\text{a}$ (left) or $\alpha$ (right) cell comes in contact with a cell of the opposite mating type, it will mate (top) to form a zygote, which will produce more diploid cells. B) Meiosis in diploid cells is a specialized division in which the ploidy in resultant cells is ultimately reduced. In this process, diploid, $MAT\text{a}/\alpha$ cells replicate the genome and undergo recombination during meiotic prophase. These cells then undergo two meiotic divisions—meiosis I and II, culminating in the formation of spores in a protective ascus covering. C) Spore germination results in the initiation in budding. After this initiation, wild, HO+ cells (top branch), will switch mating type ($\text{a}$ to $\alpha$, or vice-versa). The resultant pair will then mate to form a diploid, $MAT\text{a}/\alpha$ cell. ho- cells will not switch mating type, and therefore do not form diploid cells—these cells therefore maintain their haploid lineage until introduced to cells of the opposite mating type; the mother cell will re-initiate budding before the daughter (newly-budded) cell.
Figure 3

A

Mating

vegetative growth

Diploid

MAT α/haploid

MAT a/α/haploid

MAT a/α/haploid

MAT a/α/haploid

starvation

meiotic development

B

G1

S

G2

MI & MII

MAT a/α 2C

MAT a/α 4C

MAT a/α 4C

MAT a/α 2C

MAT a/α 2C

(recombined chromosomes)

C

MAT a/α haploid

MAT a/α haploid

MAT a/α haploid

MAT a/α haploid

MAT α haploid

MAT α haploid

MAT a/α haploid

MAT a/α haploid

HO+

ho-

mating
Figure 4. Schemes for meiotic screens. A) Scheme for isolating recessive meiotic regulators in work by Esposito, et al. Diploid, HO+ cells were sporulated, mutagenized and then dissected. Individual spores were allowed to germinate, mate with the daughter cell—and, by that virtue, homozygose--thus allowing for the ability to screen for recessive mutations. The mutagenized diploid population was then tested for the ability to sporulate. B) Scheme for isolating genes capable of overcoming mating-type repression of sporulation in work by Shah, et al. Diploid cells deleted for one of the mating loci (here, MAT\textsuperscript{a}) were transformed with a high-copy two-micro gene library. Cells were subjected to starvation conditions to test for the ability enter and complete meiosis—clones that were able to form spores under nutrient starvation carried plasmids encoding genes necessary for this function.
Figure 4

A

Wild (HO+) diploid yeast

- N, - C sporulate

Mutagenize
Dissect spores

As spores forms colonies, they become diploid (and homozygous) due to HO+

Test for ability to form spores

B

Diploid yeast deleted for MAT a locus *i.e.*, Δ/MAT α

Transform with 2μ library

Test for ability to form spores
Esposito and colleagues subjected the individual clonal populations generated by this protocol to meiosis-inducing starvation. Clones that failed to form spores or were heavily impaired in this process (i.e., carrying sporulation-defective mutations) were back-crossed and subjected to further interrogation. Among other genes involved in meiotic recombination and chromosome segregation, SPO8, isolated from this screen, was involved in induction of meiotic development. A later, alternate screen by Shah and colleagues attempted to identify genes that are involved in bypassing the heterozygous mating type requirement for meiotic induction [29]. In this screen, diploid cells that were deleted for the MATα locus (i.e., a1Δ/MATα) were transformed with high-copy libraries of the entire yeast genome and then subjected to meiotic induction. Transformants able to induce meiosis in the MAT-deficient background (and thus, encoded Inducer of MEiosis genes--IME), were subjected to further analysis (figure 4B). Over-expression of IME4, which subsequently mapped to the SPO8 locus, overcame the heterozygous MAT requirement for induction of meiosis. IME4 transcript accumulated in MATα/α cells and was necessary for transcript accumulation of IME1 and IME2, the earliest-known regulators of meiosis at the time. Furthermore, over-expression of IME1—an early meiosis transcription factor—in a1Δ/MATα cells under starvation conditions was not sufficient to induce transcription of IME4. Together, these data suggested that IME4 acted upstream of IME1, making IME4 the earliest-known regulator of meiosis (see figure 5 for the pathway as inferred from epistasis analysis).

4 It is notable that Shah and colleagues were not the first to perform this type high-copy plasmid screen to overcome mating type repression in yeast—previous work by Kassir and colleagues (54. Kassir Y, Granot D, Simchen G (1988) IME1, a positive regulator gene of meiosis in S. cerevisiae. Cell 52: 853-862.) as well as Mitchell and colleagues (55. Mitchell AP, Driscoll SE, Smith HE (1990) Positive control of sporulation-specific genes by the IME1 and IME2 products in Saccharomyces cerevisiae. Mol Cell Biol 10: 2104-2110.) both utilized a similar technique to identify the meiotic inducers IME1 and IME2.
RNA methylation in yeast upon nutrient starvation

Subsequent work by Clancy and colleagues demonstrated that the m$^6$A-transferase activity of IME4 was necessary for the efficient completion of meiosis [8]. In contrast to vegetative conditions, in which m$^6$A is not detectable, cells that were subjected to nutrient starvation showed high levels of methylated mRNA. Mutation of individual residues of the conserved catalytic domain of Ime4 resulted in the loss of the accumulation of m$^6$A during nutrient starvation. Cells encoding the catalytic mutations of IME4 recapitulated the meiotic phenotypes of the IME4 deletion allele: upon nutrient starvation, cells failed to form spores or enter into the meiotic divisions. These findings emphasize the importance of the post-transcriptional modification of mRNA in regulating meiosis.

Indeed, early meiotic genes are modified with the methyl mark. Work from Bodi and colleagues utilized a technique developed by Bringmann and colleagues to map methylated transcripts [26,27]. In this work, Bodi, *et al.*, developed a monoclonal antibody against m$^6$A and immunoprecipitated modified messages. Individual sequencing of precipitated clones revealed that the transcript of the early meiosis gene IME2 and its transcriptional activator, IME1 are methylated, as well as IME4 itself. Notably, ACT1 transcript also accumulates in the immunoprecipitated fraction. These data suggest two distinct possibilities: if ACT1 mRNA is indeed methylated, such methylation suggests that RNA methylation modifies “house-keeping” genes in addition to genes necessary for meiotic development. Alternatively, it is also possible that ACT1 mRNA is not methylated and that the antibody developed by Bodi and colleagues has affinity for unmodified transcripts in addition to modified transcripts. The report published by these authors did not attempt to differentiate between these two possibilities by
**Figure 5. Early meiotic regulation.** Regulatory network regulating meiotic entry: non-coding RNAs are shown in red, and methylation modifications are shown as blue arrows. A) Regulation of *IME1* and *IME4* by early meiotic genes. While both genes are turned on by nutrient stress (right), these genes are also under regulation of the mating type locus. A heterozygous mating locus in diploid cells (*i.e.*, *MAT a/α*) inhibits *RME1*, which inhibits *IME1* transcription by activating transcription of the non-coding transcript, *IRT1* (F. van Werven, personal communication). *MAT a/α* allows for transcription of *IME4* by inhibiting the inhibitory non-coding transcript transcribed anti-sense to *IME4*, *RME2* [56]. B) *IME1* encodes a master transcriptional activator necessary for transcription of *IME2*, the meiosis-specific cdk-like kinase, as well as other transcriptional activators. These genes are all necessary for progression past meiotic prophase and initiation of the middle meiotic program. *IME4* has been shown to methylate the transcripts of both *IME1* and *IME2* [26] and, in some strain backgrounds, is necessary for the transcript accumulation of these genes [8,29].
Figure 5

A

Mating type locus $\rightarrow$ $RME2$

$RME1 \rightarrow IRT1 \rightarrow IME1$

B

$IME4 \rightarrow IME1$

$IME1 \rightarrow IME2$

$IME2 \rightarrow$ Early Meiosis Genes

Meiosis Genes and Sporulation

Nutrient Signaling
immunoprecipitating non-methylated transcripts (i.e., from an IME4 deletion strains) in parallel to gauge background noise.

The immunoprecipitation of IME2 by Bodi and colleagues led to the mapping of methyl marks on the transcript [26]. This analysis revealed that methylation in yeast reflects the previous data derived from fine-mapping of metazoan transcripts: \( m^6A \) accumulates at the 3’ ends of IME2 and appears to accumulate only in the GAC context\(^5\).

Despite these advances describing the parallels between mRNA methylation in yeast and metazoan systems, there has been no work to address what fraction of messages are modified, or the fate of these methylated messages. Regardless, given that \( m^6A \) accumulates on transcripts necessary for induction of meiosis upon nutrient starvation, it is not unreasonable to hypothesize that this modification activates the expression of these genes. Whether this is through stabilization of mRNA transcripts, through enhanced modifications of the transcripts (as in polyadenylation and capping), or through a higher affinity for the ribosome remain important unanswered questions.

**Regulation of IME4**

IME4 is both sufficient and necessary for meiotic induction upon nutrient starvation. Deletion of IME4, as performed in the screen by Esposito and colleagues, inhibited completion of meiosis, while over-expression of the gene, as demonstrated by Shah and colleagues,

\(^5\) It is notable that the technique employed by Bodi and colleagues utilizes digestion with RNase T1, which digests only after G. The resultant molecule is 5’ radio-labeled with \(^{32}P\), is digested to single nucleotides and analyzed on TLC. Thus, this technique only identifies the presence of \( m^6A \) after G (i.e., in the Gpm\(^6A\) context). Testing digesting with RNase A, which digests after C or U revealed that there is no detectable levels of Cpm\(^6A\) or Upm\(^6A\). The authors, did not test whether \( m^6A \) appears in the Apm\(^6A\) context, which is a minor methylation motif for the methyltransferase 26. Bodi Z, Button JD, Grierson D, Fray RG (2010) Yeast targets for mRNA methylation. Nucleic Acids Res 38: 5327-5335.
resulted in ectopic meiosis under nutrient limiting conditions [29,53]. Work by Hongay, et al., reinforced this latter finding: over-expression of IME4 in haploid cells (which do not undergo meiosis under starvation conditions) resulted in expression of the early meiosis genes IME1 and IME2 when these cells were placed under nutrient-limiting conditions⁶ [56] (figure 6). This expression results in aberrant meiosis and the formation of four spores—a developmental transition that results in lethality⁷. Given the energy cost to a diploid cell if meiosis is induced under inappropriate conditions, as well as potential lethality if meiosis is inappropriately initiated in the wrong cell type, tight regulation of the IME4 is necessary to ensure cells do not undergo meiosis ectopically. Recent work has elucidated the mechanisms of this regulation to ensure the expression of IME4 under appropriate conditions [56].

Regulation of IME4 in haploid cells

Regulation of IME4 expression is strongly dependent on cell type. Haploid cells transcribe a non-coding antisense transcript, termed Repressor of MEiosis 2—RME2—at the IME4 locus that proceeds 3’ to 5’ of the sense (i.e., protein-coding) strand of IME4 [56,57]. This transcription of RME2 prevents transcription of sense IME4 in haploid cells. In diploid cells, RME2 is inhibited by the binding of the diploid-specific a1/alpha2 protein heterodimer downstream of the IME4. Work by Hongay and

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⁶ This expression may arise from direct action at the promoters of these genes. Alternatively, this expression may occur through inhibition of Sir2. In haploid cells, Sir2 activity silences HMR and HML; deletion of SIR2 results in expression of the a1/a2 heterodimer in haploid cells, which results in meiosis upon nutrient starvation. An alternative hypothesis to the expression of IME1 and IME2 in haploid cells upon over-expression of IME4 is that IME4 inhibits expression SIR2 and silencing at the mating-type locus. This hypothesis has yet to be tested.

⁷ In diploid cells, meiosis results in a duplication of the genome from 2C to 4C followed by two reductive divisions into four spores, each of which have a haploid complement of 1C DNA content. In haploids, the same developmental program results in a duplication of the genome from 1C to 2C. However, the resultant two reductive divisions result in spores that do not carry the full haploid genetic complement and are thus inviable.
**Figure 6. Cell-type regulation of IME4.** IME4 is tightly regulated by cell type in budding yeast. In haploid MAT a or α cells, an antisense transcript, RME2 is transcribed against (i.e., in the opposite direction of the protein-coding transcript) the IME4 ORF (top panel). The antisense transcript inhibits IME4 expression in both vegetative as well as nutrient-stress conditions. In diploid, MAT a/α cells, this antisense transcript is silenced (middle panel), thus permitting induction of the locus. When diploid cells are placed under nutrient-stress conditions, sense (i.e., protein-coding IME4) transcript is induced and the meiotic program is initiated.
Figure 6

Haploid cells (MAT a or $\alpha$):

Transcription of non-coding regulatory transcript, *RME2*.

Diploid cells (MAT a/$\alpha$),
Vegetative growth:

Diploid cells (MAT a/$\alpha$),
Nutrient stress:

Transcription of *IME4* protein-coding transcript
colleagues demonstrated that mutation of the \(a1/\alpha2\) binding site downstream of the \(IME4\) ORF (such that the protein complex did not bind) results in the transcription of \(RME2\) in diploid cells. These diploid cells transcribed \(RME2\), unable to induce transcription of sense (protein-coding) \(IME4\). Correspondingly, these diploid cells also failed to undergo meiosis. This work demonstrated that transcription of \(RME2\) is a potent inhibitor of sense \(IME4\); inhibition of \(RME2\) in diploid cells allows for the transcription and subsequent expression of Ime4 in diploid cells.

Mechanistically, antisense regulation of \(IME4\) by \(RME2\) invokes a model of transcriptional interference as previously posited by Proudfoot and colleagues [58,59,60,61]. In this model, convergent (head-to-head) transcription of two neighboring genes inhibits transcription of both genes either by preventing transcriptional elongation from read-through from either promoter, or by stripping transcriptional activators from the promoters of either gene. This latter scenario appears to be relevant for the \(IME4\) locus. Inhibition of sense \(IME4\) by \(RME2\) occurs via \(cis\)-regulatory mechanisms: expression of \(RME2\) from an ectopic locus (i.e., in \(trans\)-) in diploid cells failed to inhibit sense \(IME4\) expression in diploid cells, and these cells were capable of undergoing meiosis, comparable to wild-type cells [56]. Furthermore, chromatin immunoprecipitation in these cells with an antibody against Pol II suggested that the polymerase assembled at the \(IME4\) locus, and that \(RME2\) inhibits sense \(IME4\) expression at a post-initiation step of antisense transcription. Subsequent studies by Gelfand and colleagues also showed that transcription factors also assembled at the \(IME4\) locus, suggesting that antisense transcription at the \(IME4\) locus inhibits sense transcription by modifying the chromatin state of the locus, making it less favorable for transcription [57]. These authors
showed that this form of regulation by an antisense transcript appears to occur at other early meiotic genes, particularly, ZIP2 and HOP1.

Regulation of IME4 in diploid cells

IME4 is inhibited in haploid cells and expressed only in diploid cells upon nutrient stress [8,29,56]. Diploid cells growing vegetatively on rich medium do not express detectable levels of IME4—notably, these cells also do not transcribe RME2 [8,26,29,31]. Although there is no active inhibition at the locus in diploids by antisense transcription as there is in haploid cells, the locus appears to remain silent during vegetative growth—capable of inducing expression of IME4 upon nutrient limitation. Analysis of IME4 regulation in diploid cells may shed light on the role of the gene in regulating meiotic entry.

Previous work from the Rose group suggests that the catalytically inactive ohnolog of IME4, KAR4, encodes a transcriptional activator of the IME4 gene [62,63,64]. This revealed that deletion of KAR4 in diploid cells results in a defect in progression into the meiotic divisions, similar to that of the ime4Δ/Δ phenotype. The KAR4 transcript accumulates in G1 of the cell cycle, during which cells are capable of entering into meiosis [62]. Finally, consistent with all these data, ChIP-Chip analysis by Lahav and colleagues from the Rose group revealed that Kar4 binds to the promoter of IME4, which also contains a strong consensus motif for Kar4 binding [63]. Thus, these data suggest a model in which, as cells accumulate in G1 upon nutrient starvation, Kar4 activates expression of IME4, which then progresses to activate transcription and expression of early meiosis factors and the ensuing meiotic developmental program.
Alternate roles for IME4

IME4 in pseudohyphal development

Work by Hongay and colleagues revealed that IME4, in addition to its role in meiosis, played a role in the adhesive properties of yeast [56]. Adhesion in yeast (both cell-cell and cell-surface) is mediated by cell-surface glycoproteins of the FLOcculin—FLO—gene family. Although members of the FLO family are expressed highly in haploid (either MAT a or MAT α) cells, these genes are down-regulated in diploid cells (Mat a/α) [65]. Members of this family of cell surface molecules are involved in many haploid-specific properties. A subset of these genes is involved in cell-cell adhesion and flocculation in liquid cultures (i.e., FLO1, FLO5) [66,67]; other genes (i.e., FLO11, FLO10) are necessary for binding to plastics and agar, as well as biofilm formation [68,69,70]. FLO8 encodes a transcriptional activator for all of these cell surface molecules; genetic backgrounds that encode a non-functional allele of this gene (e.g., S288c or W303) have not only severely reduced levels of these transcripts, but also are much less adherent than cells with functional copies of FLO8 [71].

Although expressed in diploid cells at low levels, the cell surface molecules FLO11 and FLO10 have also been shown to play a role in the diploid-specific program of pseudohyphal (PH) development [69,70]. When plated on solid nitrogen starvation medium (synthetic low ammonium dextrose—SLAD medium), diploid cells form elongated daughter cells termed pseudohypal (PH) cells [72] (figure 7). The cell cycle during PH development differs from mitotic budding in many respects: whereas diploid cells vegetatively dividing under rich conditions initiate S phase synchronously with bud emergence, PH cells initiate budding only after the completion of DNA replication [73]. In vegetative, yeast-form growth, mother cells initiate re-
budding earlier than daughter cells upon completion of M phase and septation; in PH
development, mother and daughter cells synchronously re-bud after cell division [73].

Furthermore, diploid cells usually engage in bi-polar budding, in which daughter cells emerge
from either pole of the cell; PH cells exhibit uni-polar budding, in which daughter cells only
emerge from one designated pole of the cell [72]. Repeated division under this program results
in chains of cells, or filaments, that protrude from a central mass of largely vegetative cells
grown on this starvation medium and resemble the hyphal cells of true hyphal fungi (hence the
term “pseudohypal”). Cells that do not express \textit{FLO11} (or, in its stead, \textit{FLO10}) are incapable of
forming these protrusions or these filamentous colonies on nitrogen-starvation medium. Over-
expression of \textit{FLO11} results in hyper-filamentation on solid nitrogen-starvation medium (SLAD),
but does not overcome the diploid cell-type requirement necessary for this form of growth
[74,75,76].

Although initial work isolated \textit{IME4} as a regulator of meiosis, subsequent work revealed
that diploid cells that do not express \textit{IME4} (either because of inhibition by \textit{RME2} or because of a
homozygous deletion of the gene) display increased agar adhesion in a \textit{FLO11}-dependent
manner [56]. Furthermore, diploid strains that were incapable of \textit{IME4} expression also
displayed increased levels of \textit{FLO11} transcript. These findings suggest that \textit{IME4} may play a role
in regulating PH development in diploid yeast.

\textit{IME4} is not the first early-meiosis gene implicated in filamentous development. When
grown on low-nitrogen medium with a non-fermentable carbon source (\textit{e.g.}, acetate, glycerol)
yeast engage in a hybrid development in which PH cells of filamentous colonies form
ascospores in an early meiosis gene-dependent (\textit{i.e.}, \textit{IME1, IME2}), yet \textit{FLO11}-independent,
Figure 7. Budding pattern in yeast. Mitotic division in budding yeast results in the formation of a daughter cell that buds off of the mother cell; placement of this daughter cell is regulated by both cell type and nutrient conditions. A) Haploid cells engage in axial budding. Under this program, daughter cells bud off from a mother cell; after septation, the mother cell re-engages in budding proximal to the bud site. After a short pause, the daughter cell buds proximal to the site of budding. This is in contrast to budding in diploid, \( MAT^a/\alpha \) cells, which engage in bipolar budding. Under this program, daughter cells again bud off from the mother cell, which re-engages in budding either proximal or distal to the bud site after septation. After a short pause, the daughter cell engages in budding distal to the mother cell. C) Under nitrogen starvation, diploid, \( MAT^a/\alpha \) cells engage in unipolar, pseudo-hyphal budding. Mother cells form an elongated bud and daughter cells. After septation, the mother and daughter cells re-initiate budding synchronously. The mother cell re-buds proximal to the bud site, while the daughter cell re-buds distal to the bud site.
Figure 7

A  Haploid cells--axial budding.

B  Diploid cells--bipolar budding.

C  Diploid cells--unipolar, pseudohyphal budding.
manner [77]. In addition, previous work has shown that early meiotic transcriptional regulators regulate agar invasion and pseudohyphal development [78,79,80].

**IME4 in mating**

In addition to its role in meiosis, work by Roberts and colleagues suggests a role for *IME4* in haploid mating [81]. Despite its inhibition in haploid cells due to antisense transcription, microarray analysis of gene expression during pheromone induction showed a sharp increase in *IME4* transcript accumulation upon treatment with the pheromone α-factor. As in the ectopic expression of *IME4* in haploid cells exposed to starvation conditions by Hongay and colleagues, this work also showed an accumulation of *IME1* and *IME2*.

Similarly, as in diploid cells, this expression of *IME4* may be mediated by *KAR4*. *KAR4* is expressed in two isoforms—a high- and low-molecular-weight species [62,64]. The former accumulates upon treatment with pheromone and activates transcription of its target genes (among which is *IME4*) in haploid cells. As Kar4 does not appear to target either *IME1* or *IME2* [63], these data suggest that Ime4 may be—in part—functional in response to pheromone induction, in order to induce transcription of these genes.
Summary

This thesis investigates the process and consequences of mRNA methylation using the model system, *Saccharomyces cerevisiae*. In chapter 2, I investigate the role mRNA methylation plays in negotiating a decision between the two diploid-specific developmental pathways initiated by nutrient starvation: meiosis and PH development. In that chapter, I find that components implicated in mRNA methylation activate progression through meiosis, but inhibit progression into PH development. This finding reveals a novel method of inducing PH development, by shifting cells from pre-meiotic G2 into nutrient-rich medium: cells returned to mitotic division, however, produce an elongated, PH daughter cell. Development in this nutrient-shift condition results in a loss of m\(^6\)A from mRNA and a sharp reduction of methyltransferase transcript. Cells that over-express methyltransferase do not engage in PH development, suggesting that the components involved in mRNA methylation are inhibitory to this development. In addition to providing insights into the consequences of mRNA on developmental programs, this form of inducing PH development provides a novel method of studying PH development—one which, for the first time—allows for the biochemical analysis of PH development at high temporal resolution.

Chapter 3 identifies a putative complex that modifies mRNA molecules in yeast. I identify a three-component system comprised of Ime4, Mum2 (both of which have homologues in other organisms) and Slz1 (which does not have any easily-identifiable homologs in other organisms). These three components are involved in catalyzing mRNA methylation; deletion of any one of these components results in the meiotic and mitotic defects discussed in chapter 2. Finally, I discuss the role of these genes in regulating translation of *IME2* mRNA, which has been
previously characterized as a target of the methylation complex in yeast upon nutrient starvation. I find that deleting IME4, such that methylated mRNA no longer accumulates during starvation, results in a significant reduction in translation of IME2 mRNA. Together, this work demonstrates the power of using the genetically-tractable yeast, S. cerevisiae as a system that permits an understanding of mRNA both at a mechanistic level, as well as understanding its regulation of development in the broader context of cellular decision-making.
References


Chapter II: RNA Methylation Regulates Cell Fate in Yeast

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ABSTRACT:

Developmental potential becomes restricted during cellular differentiation. Upon nutrient-deprivation, diploid *Saccharomyces cerevisiae* cells switch from yeast-form budding into one of two differentiation programs: foraging pseudohyphal (PH) growth or formation of protective spores by meiosis. We show here that after meiotic entry, cells become committed to differentiation. Even when returned into nutrient-rich conditions from meiotic prophase, cells retained the memory of starvation and initiated synchronous PH development. This return-to-growth PH response was indistinguishable from starvation-induced PH growth and required *FLO8, FLO11* and the inducers of meiosis *IME1* and *IME2*, all of which are known activators of PH growth. By contrast, the inducer of meiosis, *IME4*, which encodes an RNA methyltransferase that regulates *IME1* and *IME2*, functioned as a potent inhibitor of the PH program. Both meiotic induction and PH suppression required the methyltransferase activity of *IME4*. Strikingly, in the absence of *IME4*, PH growth no longer required *IME2*, revealing an alternative pathway toward this process. Together, our results support the notion that the yeast starvation response is an extended process that progressively restricts cell fate and reveal a broad role of post-transcriptional RNA methylation in these decisions.
INTRODUCTION:

Upon nutrient limitation, diploid cells of the yeast *Saccharomyces cerevisiae* can undergo two distinct developmental responses. Starvation for nitrogen in the presence of a fermentable carbon source (e.g., dextrose or galactose) induces a modified mitotic division that results in elongated diploid daughter cells called pseudohyphae (PH). Reiterated cell division under this program forms chains of elongated cells that form protruding filaments on solid agar, ultimately allowing yeast to forage for nutrients [1,2]. In contrast, starvation for nitrogen in the presence of a non-fermentable carbon source (e.g., acetate or glycerol) induces meiotic development and sporulation. Under this program, the diploid genome is duplicated (2C to 4C), then segregated into four haploid (1C) meiotic products encased in a spore wall. This spore structure protects haploid progeny until favorable nutrient conditions are available.

The two developmental responses to nitrogen deprivation, PH development and sporulation, share a set of regulatory factors. It was shown recently that PH cells that are grown on a non-fermentable carbon source undergo sporulation [3]. Genes that are necessary for meiotic induction, *IME1* and *IME2* (Initiator of Meiosis 1 and 2, respectively), are necessary not only for spore development under these conditions, but also for PH development and the subsequent formation of filaments on solid agar [3,4,5]. Furthermore, strains lacking the early meiotic gene *IME4* display both meiotic defects and an increased ability to adhere to agar, a phenotype associated with PH development and the function of the key PH effector, the cell surface protein, *FLO11* [6,7].

In yeast, *IME4* encodes the sole functional member of a class of RNA-modifying enzymes conserved throughout eukaryotes. These enzymes, identified by homology to the \(N^\delta\)-adenosyl
methyltransferase in humans, MT-A70, catalyze the post-transcriptional methylation of adenine (to form $N^6$-methyladenine—m$^6$A) in RNA [8,9,10]. The function of this modification on mRNA is as yet unclear. In vitro experiments suggest that m$^6$A enhances the translational activity of modified messages [11], whereas in vivo experiments suggest that this modification may play an additional role in message stability and processing [12,13,14]. Although this form of RNA methylation is barely detectable in yeast undergoing vegetative (mitotic) growth, m$^6$A accumulates in mRNA isolated from yeast cells undergoing meiosis [8,15,16]. Strains encoding a catalytically-inactive allele of IME4 do not accumulate m$^6$A and display defects in meiotic entry [8]. Specifically, Ime4 modifies the transcripts of the early meiosis genes IME1 and IME2 under these conditions, which may explain these defects upon nutrient starvation [16].

To better understand the response of yeast cells to nitrogen deprivation, we determined the roles of meiotic initiators in both meiosis and PH development. We show here that cells initiating the meiotic program through IME1 and IME2 lose the ability for vegetative proliferation, but maintain the potential for PH growth until pre-meiotic G2. When returned to rich medium at that stage, cells initiated PH growth in a highly synchronous manner. In contrast to IME1 and IME2, which were required for this process, complete loss of their putative regulator Ime4 or its methyltransferase activity resulted in hyper-filamentous PH development, suggesting that Ime4 has additional roles in regulating the starvation response. Consistent with this possibility, the induction of PH development in ime4Δ/Δ was independent of Ime2. These data indicate that the inhibitory role of Ime4 in PH development is separable from its role as a meiotic inducer and point to a broader role of post-transcriptional RNA methylation in regulating a decision between meiosis and PH development.
RESULTS:

Return to mitotic growth from pre-meiotic S or G2 results in PH development.

Given that PH development and meiosis share a common set of regulators, we investigated the relationship between meiotic induction and PH growth in SK1, a strain that efficiently undergoes both developmental programs [3,17]. Meiosis is normally induced by incubating cells in a medium lacking nitrogen and fermentable carbon (sporulation medium--SPO). Induction of the meiotic program is reversible prior to meiotic commitment, as cells that are transferred back to nutrient-rich medium (yeast peptone dextrose--YPD) during pre-meiotic S or G2 phases return to mitotic growth instead of undergoing the meiotic divisions [18,19]. The shift from SPO (starvation conditions) to YPD (nutrient-rich conditions) is often referred as a Return-To-Growth assay, here abbreviated as RTG. Using this assay, we observed that RTG of cells after 3 hours in SPO (RTG3) exhibited elongated bud morphology characteristic of PH cells (Figure 1A). This elongated morphology required prolonged exposure to SPO conditions, as it did not occur in cells that were returned to growth at 0 hours (RTG0; Figure 1A). The effect of RTG on bud morphology was quantified by measuring the axial ratio (length-to-width quotient) of the newly formed daughter cells as a function of the time the cells spent in SPO. RTG0 daughter cells had a morphology similar to yeast-form, vegetatively growing cells and had an axial ratio of 1.6 (figure 1A, B). RTG3 cells, the majority of which, under our conditions, had reproducibly completed pre-meiotic S phase but had not yet initiated the meiotic divisions (i.e., cells were in the pre-meiotic G2 phase; figure 1B), produced buds with significantly larger axial ratio of approximately 2.0 (figure 1A, B), similar to that of pseudohyphal (PH) cells grown on
solid media (figure 1D). RTG after pre-meiotic G2, when cells induce meiotic divisions and are committed to meiosis, resulted in spore formation even after the shift to rich medium, as previously reported (figure 1A, B) [18]. Thus, cells that were shifted to rich medium during pre-meiotic G2 divide mitotically to produce an elongated daughter cell morphologically similar to a PH cell formed on solid nitrogen starvation medium.

The formation of the elongated bud upon RTG in pre-meiotic G2 (RTG-PH development) paralleled PH development on solid nitrogen starvation medium (Synthetic Low Ammonium Dextrose—SLAD) in all aspects tested. RTG₃ bud elongation occurred only in diploid cells and was dependent on FLO11 and its transcriptional regulator, FLO8 (figure 1C, D, supplemental figure 1A) [20,21,22]. Furthermore, bud formation occurred after DNA replication, and mother and daughter pairs re-budded synchronously after cytokinesis (supplemental figure 1) [2]. In addition, similar to SLAD filaments, RTG filaments required the function of the early meiosis genes IME1 and IME2 (supplemental figure 2) [3]. Cells lacking either IME1 or IME2 formed yeast-form cells instead of the elongated cells formed by wild-type upon RTG₃ (figure 2A, B). These results indicate that meiotic initiation is necessary for RTG-PH development in wild-type cells. Moreover, the fact that cells returned to growth in the early meiotic program enter PH growth even in the presence of nitrogen-rich medium indicates that cells are committed to PH growth under these conditions.

We determined which aspect of meiotic initiation potentiates PH development. IME1 encodes a transcriptional activator of many early meiotic genes, including IME2, a meiosis-specific CDK-like kinase. Both ime1Δ/Δ and ime2Δ/Δ cells fail to enter into pre-meiotic S phase
Figure 1. RTG cells result in three distinct morphologies.

(A) Representative morphologies of daughter cell after development in YPD upon RTG from SPO throughout a meiotic time-course as indicated in panels: RTG at 0 hours (RTG₀), RTG at 3 hours (RTG₃)—which is comparable to PH cells from solid nitrogen medium—or RTG₆ hours. Arrows indicate primary buds.

B) Quantification of axial ratio in wild-type (SAy821) (red bars, left axis n=200 cells/time-point) relative to % of 4C cells (blue diamonds, right axis) and % cells undergoing meiotic divisions as assayed by DNA segregation (green triangles, right axis).

C) Wild-type (SAy821), flo11Δ/Δ (SAy789) and flo8Δ/Δ (SAy905) daughter cell morphology upon RTG₃ (top panels). Arrows indicate primary buds. The same strains were photographed after growth on SLAD for 6 days (bottom panels).

D) Distribution of axial ratios of primary buds upon RTG₃ for strains in (F): wild-type (blue bars), flo11Δ/Δ (red bars) and flo8Δ/Δ (green bars) (n=200 cells/strain).
Figure 1

A

RTG₀

PH cells

RTG₃

Spores

B

Axial Ratio

% 4C cells

% bi-/tri-/tetranucleate

% meio
tic

Time in SPO (hours)

Percent Cells

Axial Ratio

wt rtg=3

flo11Δ/Δ rtg=3

flo8Δ/Δ

C

RTG-filaments

flo11Δ/Δ

flo8Δ/Δ

SLAD

D

Frequency

Axial Ratio

wt

flo11Δ/Δ

flo8Δ/Δ
**Figure 2.** Early meiosis genes regulate PH development.

A) Representative bud morphology after RTG$_3$ in wild-type (SAy821), *ime1Δ/Δ* (SAy834), *ime2Δ/Δ* (SAy859) and *ime4Δ/Δ* (SAy771). Arrows indicate primary buds. Axial ratios of RTG$_3$ cells were quantified in (B) (n=200 cells/strain).

C) Representative images of RTG$_3$ cells of the *ime2-as1* strain (SAy1126) that were treated with solvent (DMSO) (1 & 2) or with 20 μM 1-NA-PP1 (3 & 4) during sporulation conditions followed by either solvent (1 & 3) or 1-NA-PP1 (2 & 4) treatment upon return to growth. Axial ratios of RTG$_3$ cells are quantified in (D) (n=200 cells/strain).
Figure 2

A

wt
ime1Δ/Δ
ime2Δ/Δ
ime4Δ/Δ

B

Frequency

Axial Ratio

wt
ime1Δ/Δ
ime2Δ/Δ
ime4Δ/Δ

C

1) SPO: -
YPD: -

2) SPO: -
YPD: inhibitor

3) SPO: inhibitor
YPD: -

4) SPO: inhibitor
YPD: inhibitor

D

Frequency

Axial Ratio

1 1.2 1.4 1.6 1.8 2 2.2 2.4 2.6 2.8 3 3.2 3.4 >3.4

1 2 3 4
[5,23,24], suggesting that pre-meiotic DNA replication may contribute to RTG-PH development. We therefore determined whether DNA replication was required to form PH daughter cells. However, inhibition of both pre-meiotic DNA synthesis and homologous recombination, either chemically (using hydroxyurea) or genetically (by deleting the S phase cyclins CLB5 and CLB6), did not inhibit PH development in either the RTG or solid medium context (supplemental figure 3), indicating that pre-meiotic S phase is not itself necessary for this RTG3 PH development. To test the contribution of recombination on RTG-PH development directly, we tested whether the formation of DNA double-strand breaks (a process catalyzed in meiosis by Spo11) resulted in a defect in PH development. However, neither RTG, nor colony development on SLAD was affected in the spo11Δ/Δ strain, suggesting that initiation of double-strand breaks is not necessary for PH development (supplemental figure 3G, H).

We tested whether the observed effects of IME2 deletion is the result of a replication-independent role of Ime2 kinase during meiosis or due to a role of IME2 during RTG. We employed a diploid strain encoding an analogue-sensitive allele of IME2, ime2-as1, whose kinase activity is inhibited by the addition of the drug 1-NA-PP1 [24]. Cells grown in the absence of inhibitor in both meiosis and upon RTG3 formed wild-type filaments, confirming that cells encoding the ime2-as1 allele exhibit wild-type behavior upon RTG3 (figure 2C, D, condition 1). Cells grown in the presence of inhibitor in both meiosis and RTG3 failed to form RTG-filaments, much like ime2Δ/Δ cells, confirming that 1-NA-PP1 effectively inhibited the ime2-as1 allele and that IME2 kinase activity is necessary for RTG PH development (figure 2C, D, condition 4). Similarly, cells grown in the presence of inhibitor on SPO and then in the absence of inhibitor upon RTG3—after DNA replication from 2C to 4C—failed to form filamentous daughter cells
(figure 2C, D, condition 3) indicating that Ime2 kinase has a replication-independent role in priming cells for PH growth during meiotic pre-meiotic G2. To test whether Ime2 activity also contributes to PH growth upon RTG₃, meiosis was initiated in the ime2-as1 strain in the absence of inhibitor and cells were then returned to growth in the presence of inhibitor. These cells exhibited wild-type PH development, suggesting that Ime2 kinase activity does not play a role in PH development after RTG₃ and subsequent bud formation (figure 2C, D, condition 2). Together, these data indicate that meiotic IME2 kinase activity is necessary for enabling RTG cells to form PH cells.

**IME4 mRNA methylation activity inhibits formation of PH cells.**

Given that IME1 and IME2 are regulated by Ime4, we investigated the role of IME4 in the formation of PH cells upon RTG₃ [8,16,25]. In contrast to the phenotypes observed for ime1Δ/Δ or ime2Δ/Δ mutant strains, deletion of IME4 resulted in hyper-elongation upon RTG₃ (figure 3A). Similarly, ime4Δ/Δ cells formed hyper-filamentous colonies on SLAD medium (figure 3B). The same results were also observed using diploid strains encoding a catalytically inactive allele of IME4 (IME4-D349A, W351A, referred to as ime4-cat) that is deficient in the methyltransferase activity [8] (figure 3A, B, supplemental figure 4). These data suggest that the methyltransferase activity of Ime4 is necessary for the inhibition of PH development upon RTG and filamentation on SLAD medium.

The hyper-elongation phenotype of N⁶-methyltransferase-deficient cells (i.e., ime4-cat/-cat or ime4Δ/Δ) upon RTG₃ depended upon the function of the same genes necessary for filamentous development. Deletion of either FLO11 or FLO8, in an ime4Δ/Δ strain resulted in a
Figure 3. IME4-mediated RNA methylation regulates filamentation.

A) Representative images of cells from wild-type (SAy821), ime4-cat/-cat (SAy1086) and ime4Δ/Δ (SAy771) after RTG3 (top panels—arrows indicate primary buds) and colonies grown on SLAD (bottom panels). Axial ratios of RTG3 cells are quantified in (B) (n=200 cells/strain).

C) Representative images of cells from wild-type (SAy821), ime4Δ/Δ (SAy771), ime4Δ/Δ flo11Δ/Δ (SAy890) and ime4Δ/Δ flo8Δ/Δ (SAy938) after RTG3 (top panels). Arrows indicate primary buds. Axial ratios of RTG3 cells are quantified in (D) (n=200 cells/strain).

E) Representative images from colonies from (C) grown on SLAD.
Figure 3
failure to form hyper-elongated daughter cells upon RTG3 (figure 3C, D) or on SLAD medium (figure 3E). It is notable that, as in RTG-PH development, the constituent individual cells of \textit{ime4Δ/Δ} isolated from filamentous colonies grown on SLAD were hyper-elongated compared to wild-type cells grown under the same conditions (supplemental figure 5), further reinforcing the parallels between SLAD- and RTG-PH development. These data suggest that the m\textsuperscript{6}A transferase function of \textit{IME4} inhibits PH development both upon RTG and during nitrogen starvation on solid medium.

\textbf{IME4} mRNA methylation activity promotes progression through meiosis.

Given the opposing PH growth phenotypes of the \textit{ime4Δ/Δ} mutant compared to \textit{ime1Δ/Δ} and \textit{ime2Δ/Δ} mutant cells, we investigated the role of \textit{IME4} in meiotic entry and progression in the SK1 background. Cells of m\textsuperscript{6}A-transferase-deficient strains did not form meiotic products—spores—as efficiently as wild-type upon meiotic starvation (figure 4A) [6,7]. However, the viability of spores from m\textsuperscript{6}A-transferase-deficient strains was comparable to that of wild-type, suggesting that chromosome segregation during the meiotic divisions was not affected in these strains (figure 4B). Strains mutated for \textit{IME4} progressed through pre-meiotic DNA synthesis nearly as well as wild-type cells (figure 4C) but showed a significant delay in induction of the meiotic divisions (figure 4E). To understand the mechanism of this delay, we monitored the induction of \textit{NDT80}, which encodes a transcription factor that initiates the middle-meiotic program, progression from G2 into meiosis I (MI), and commitment to meiosis [24,26,27]. We found that m\textsuperscript{6}A-transferase-deficient strains showed a delay in the induction of \textit{NDT80} (figure 4D). These data
show that, in the SK1 strain background, IME4 and m$^6$A-methyl-transferase activity promote meiotic progression by activating NDT80.

Consistent with the function of Ime4 in early meiosis, we observed increased IME4 expression and activity during this period. Measurement of m$^6$A levels throughout a synchronous meiosis revealed that m$^6$A accumulated in early meiotic prophase, peaking during pre-meiotic G2 (figure 5A). The accumulation of m$^6$A correlated in time with the presence of Ime4-encoding sense transcript and Ime4 protein (figure 5B, C). After entry into the meiotic divisions, m$^6$A levels dropped (figure 5A). Consistent with this decrease, there was a concomitant decrease in IME4 sense transcript accompanied by an accumulation of the IME4 regulatory antisense transcript, RME2 [6,28] (figure 5B). The decrease in full-length Ime4 protein levels was associated with the accumulation of a lower molecular weight band that may represent a degraded form of Ime4 protein (figure 5C).

Changes in IME4 expression—a decrease in IME4 transcript, increase in the anti-sense RME2 transcript and disappearance of m$^6$A on mRNA—were dependent on progression from pre-meiotic G2 into the meiotic divisions. Diploid cells encoding an estradiol-inducible allele of NDT80 continued to produce sense IME4 transcript after 9 hours in sporulation conditions in the absence of induction with estradiol [24,29] (Figure 5B), whereas cells that were induced to express NDT80 produced RME2, but no sense IME4 transcript, at this time point (figure 5D). m$^6$A levels from these cells paralleled this regulation of IME4: cells induced for NDT80 expression displayed reduced levels of m$^6$A, whereas un-induced cells did not display a reduction of m$^6$A (figure 5E). Thus, IME4 expression and concomitant
Figure 4. m^6A-transferase mutants show meiotic defects.

A) Number of asci with one, two, three, four, or no spores (i.e., unsporulated) in the wild-type (SAy 821), ime4-cat/cat (SAy1086) and ime4Δ/Δ (SAy771) after 24 hours in SPO medium (n=200 cell/strain).

B) Spore viability in the strains from (A); legend indicated number of surviving spores upon dissection (n=187 tetrads/strain).

C) FACS analysis of DNA synthesis in strains from (A) throughout the meiotic time course (n=3x10^4 cells/strain/timepoint). DNA content of diploid cells in G1 (2C) and G2 (4C) is indicated.

D) qPCR of accumulation of NDT80 transcript relative to ACT1 throughout meiosis in strains from (A). Error bars represent standard deviation of the average of three quantifications.

E) Accumulation of cells undergoing either MI or MII (as assayed by DNA staining by DAPI) in the strains from (A) (n=200 cells/strain).
Figure 4

A) Bar graph showing the percentage of spores for different conditions: wt, ime4-cat/ime4-cat, ime4Δ/Δ. The bars are color-coded to represent different stages of sporulation: Tri-/Tetra, Dy-, Mono-, and Unsporulated.

B) Bar graph showing the percentage of survival for different conditions: wt, ime4-cat/ime4-cat, ime4Δ/Δ. The bars are color-coded to represent different stages of survival: 4, 3, 2, 1, and 0.

C) Time-course graph showing the cell cycle progression for different conditions: wt, ime4-cat/ime4-cat, ime4Δ/Δ. The graph includes time points from 0 to 9 hours and indicates cell cycle phases 2C and 4C.

D) Line graph showing the NDT80 rel. ACT1 expression over time for different conditions: wt, ime4-cat/ime4-cat, ime4Δ/Δ. The x-axis represents time in SPO (hours), and the y-axis represents NDT80 rel. ACT1 expression levels.

E) Line graph showing the percentage of cells over time for different conditions: wt, ime4-cat/ime4-cat, ime4Δ/Δ. The x-axis represents time in SPO (hours), and the y-axis represents the percentage of cells.
**Figure 5.** m^6^A accumulates prior to meiotic divisions.

A) Quantification of m^6^A abundance relative to cytidine throughout meiosis (green triangles, left axis) in a wild-type strain (SAy821). % cells 4C (blue diamonds, right axis) and % cells undergoing nuclear divisions as assayed by DAPI staining (red squares, right axis) are shown as references for meiotic progression.

B) Strand-specific qPCR for sense *IME4* (red squares, left axis) and antisense *RME2* (blue diamonds, right axis) transcript throughout meiosis in (A).

C) Western analysis for 3xmyc-tagged Ime4 protein (SAy914) throughout meiosis (top panel); Pgk1 protein (bottom panel) serves as loading control.

D) Strand specific Northern analysis for meiotic cells that were either induced with 1μM β-estradiol (lanes 1 & 2) or un-treated (lanes 3&4) for *NDT80* in SAy995 at either 5 hours (lanes 1&3) or 9 hours (lanes 2&4) in SPO.

E) m^6^A relative to cytidine quantification in SAy995 with (+induction) or without (-induction) of *NDT80* induction with β-estradiol.
Figure 5

A

B

C

D

E

[Image of a graph showing the percentage of cells in different phases of the SPO process over time, with markers indicating the induction of NDT80 and the corresponding changes in [m6A]/[C].]

[Image of a graph showing the relative expression levels of IME4 and RME2 over time in the SPO process, with error bars indicating variability.]
m$^6$A accumulation in mRNA are restricted to a period prior to meiotic commitment, accumulating in pre-meiotic G2.

**IME4 down-regulation is necessary for RTG PH development.**

To determine how cells expressing high levels of the PH inhibitor Ime4 in pre-meiotic G2 are able to induce PH growth upon return to rich medium, we monitored Ime4 expression and m$^6$A levels during RTG. Upon RTG$_3$, we observed a sudden decrease in Ime4-encoding sense transcript: within fifteen minutes after the shift, the level of *IME4* sense message was reduced to the low levels found in vegetative cells (figure 6A). Moreover, although the level of m$^6$A remained somewhat elevated at 30 minutes after RTG$_3$, these levels continued to decrease to that of vegetative cells at 75 minutes after RTG$_3$, shortly before bud formation was initiated in RTG cultures. Such a decrease in *IME4* expression is necessary for the formation of PH cells upon RTG. By contrast, cells that over-expressed *IME4* failed to form PH cells and instead developed buds during RTG, whose dimensions were comparable to those of yeast form cells (figure 6B, C). These data suggest that the m$^6$A mark on mRNA is inhibitory to PH development and therefore that the decrease in *IME4* expression upon RTG is necessary for PH cell development.
The role of Ime4 in PH development is separable from its role in meiosis.

We investigated whether the role of IME4 in PH growth is independent of its role in promoting meiotic progression. To understand the relationship of the two developmental programs, we tested the PH formation of cells lacking both IME4 and IME2 in both RTG and on solid SLAD medium. We found that ime4Δ/Δ cells were hyper-elongated in both the RTG and SLAD contexts, irrespective of the presence or absence of IME2 (figure 7A-D), indicating that the removal of IME4 bypasses the necessity of IME2 in PH growth. This result is in contrast to their epistatic roles in promoting meiotic development and suggests that Ime4 regulates PH development in part independently of meiotic initiation. Importantly, quantification of bud axial ratios in the ime4Δ/Δ ime2Δ/Δ double mutant at RTG3 revealed that although ime4Δ/Δ ime2Δ/Δ cells were hyper-filamentous compared to wild-type and ime2Δ/Δ alone, double mutant cells were not as filamentous as ime4Δ/Δ alone (figure 7B). The intermediate cell elongation phenotype of the ime4Δ/Δ ime2Δ/Δ double mutant upon RTG3 was also observed on solid media in colony morphology (figure 7C, D). Thus, IME4 and IME2 contribute separately to PH growth.

To investigate the roles of IME2 and IME4 in regulating PH growth further, we analyzed FLO11 expression. FLO11 transcript levels were reduced in the ime2Δ/Δ strain compared to wild-type (figure 7E), suggesting that IME2 regulation of PH development occurs through control of FLO11 transcript. Similarly, introducing the ime2Δ/Δ mutation into the ime4Δ/Δ
**Figure 6.** m$^6$A formation inhibits filamentation.

A) Quantification of m$^6$A relative to cytidine (blue bars, left axis), IME4 transcript relative to ACT1 (red bars, left axis—error bars represent standard deviation of four experiments) and budding index (green triangles, right axis) upon RTG$_3$ or in mid-log vegetative growth (“Veg.”).

B) Representative images of cells from wild-type (SAy821), ime4Δ/Δ (SAy771) and P$_{GPD1}$-IME4 (SAy1151) after RTG$_3$. Arrows indicate primary buds. Axial ratios of RTG$_3$ cells are quantified in (C) (n=200 cells/strain).
Figure 6

A

B

C

[Graph A: Enrichment Ratio vs. Budding Index (% cells)

[Graph B: Images of fungal cells labeled wt, ime4Δ/Δ, and P_gpd1::IME4]

[Graph C: Frequency vs. Axial Ratio]
Figure 7. IME2 and IME4 interact to regulate filamentation upon RTG.

A) Representative images of RTG3 cells of wild-type (SAy821), ime2Δ/Δ (SAy859), ime4Δ/Δ (SAy771) or ime2Δ/Δ ime4Δ/Δ (SAy1123) strains. Arrows indicate primary buds. Axial ratios of RTG3 cells are quantified in (B) (n=200 cells/strain).

C) Representative colony morphologies of strains from (C) grown on SLAD.

D) Axial ratio quantification of cells grown in suspension in low nitrogen medium—strains as in (A) (n=200 cells/strain).

E) qPCR of FLO11 transcript accumulation relative to ACT1 message sixty minutes after RTG3 for strains in (A).
Figure 7

A

wt  ime2Δ/Δ  ime4Δ/Δ  ime2Δ/Δ

B

wt  ime2Δ/Δ  ime4Δ/Δ  ime2Δ/Δ ime4Δ/Δ

C

wt  ime2Δ/Δ  ime4Δ/Δ  ime2Δ/Δ

D

wt  ime2Δ/Δ  ime4Δ/Δ  ime2Δ/Δ ime4Δ/Δ

E

wt  ime2Δ/Δ  ime4Δ/Δ  ime2Δ/Δ ime4Δ/Δ
strain significantly reduced *FLO11* transcript levels from the elevated levels found in the *ime4Δ/Δ* alone (Figure 7E). These data support a model in which Ime4-catalyzed RNA methylation acts in parallel with, not downstream of, Ime2-catalyzed protein phosphorylation to regulate PH development. These two pathways converge on control of *FLO11* to regulate PH development.

Despite the vast differences in strain background, we found that the early meiotic network that regulates PH development in SK1 is conserved in Σ1278b [30], the strain in which PH development and filamentous colony morphology were first observed and have been extensively characterized [1,2,31]. As in SK1, we found that both *IME1* and *IME2* are necessary for PH development in the Σ1278b background and that *IME4* encodes an inhibitor of PH development (supplemental figure 6A). Additionally, as in SK1, the hyper-filamentous colony morphology of *ime4Δ/Δ* mutant was also independent of *IME2* in the Σ1278b background (supplemental figure 6B), indicating that RNA methylation is a general mediator of the starvation response in yeast.
CONCLUSIONS:

An experimental protocol to study synchronous PH development

We describe a new procedure—the RTG-PH protocol—in which cells form PH daughter cells synchronously in liquid culture, while exhibiting genetic and cytological characteristics identical to those of PH development on solid nitrogen starvation media. Understanding the genetic requirements for PH development—specifically in terms of gene expression—has proven extremely difficult. Previous assays analyzed PH development in *S. cerevisiae* on solid medium or liquid suspension [2,31,32]. Under these conditions, PH cells form colonies comprised of a mixture of yeast form and PH cells that divide asynchronously with respect to each other and invade into agar. The asynchronous population of multiple cell types prohibited a biochemical analysis of gene expression in PH cells. Other studies have utilized butanol in liquid culture to study PH development [33,34]. Although that method results in elongated cells, formation of those cells does not require *FLO11* or its regulators. The RTG-PH method therefore provides a novel opportunity to study PH development in homogeneous and synchronous cultures.

RNA methylation restricts cell fate

Our ability to follow cells in liquid medium progressing through either the meiotic or PH developmental programs enabled us to identify several roles for Ime4-dependent RNA methylation in modulating the cell fate decisions during starvation. Ime4 RNA methyltransferase activity had previously been shown to promote *IME1* expression and entry into the meiotic program, although the importance of this role varied according to strain
background [3]. We now show that $IME4$ also functions later in the meiotic program to promote the expression of the middle-meiosis transcription factor $NDT80$ and the initiation of the meiotic divisions. In addition, $IME4$ suppresses PH development by reducing the mRNA levels of the cell wall glycoproteins Flo11 and Flo10. These observations indicate that mRNA methylation plays a central role in regulating the yeast starvation response. Previous data suggested that the $m^6A$ mark on mRNA enhances the translational activity [11,14] or regulates message processing and stability [12,13]. Ime4 may activate $NDT80$ expression either directly, by methylating the $NDT80$ transcript, or indirectly, through the previously characterized modification of $IME2$ transcript [16]—a gene that is necessary for expression of Ndt80 protein [24]. Conversely, inhibition of $FLO11$ expression may occur through methylation (and hence, activation) of $SFL1$, a well-characterized inhibitor of $FLO11$ and the PH program [35]. Identification of specific substrates for $m^6A$ methylation will be necessary to determine how Ime4 regulates these developmental decisions.

**Developmental regulation of the RNA methylation program**

$IME4$ expression is highly dynamic during starvation, as indicated by the initial strong induction of $IME4$ expression in response to nutrient limitation, followed by a rapid down-regulation of $IME4$ and $m^6A$ methylation as cells progress to MI or upon shift to nutrient-rich medium. Down-regulation of $IME4$ upon entry into the meiotic divisions depends on $NDT80$, revealing a negative feedback loop, in which $IME4$-promoted $NDT80$ expression suppresses further $IME4$ expression. Ndt80 may down-regulate $IME4$ by inducing the regulatory $RME2$ antisense transcript, which accumulates concomitant with the loss of $IME4$. Alternatively, transcription of the haploid-specific $RME2$ transcript may arise from a loss of heterozygosity at
the MAT locus once homologous chromosomes segregate in MI and the diploid-specific a1-α2 repressor of RME2 is lost. In addition, Ime4 may also be regulated post-translationally, as the full-length Ime4 is converted into a shorter peptide upon entry into the meiotic divisions. The functional importance of IME4 down-regulation and processing during meiotic progression remains unclear, but the rapid loss of IME4 transcript and subsequent loss of m^6^A-mRNA are important when cells return to growth from pre-meiotic G2. Failure to down-regulate IME4 at this point blocks PH development and causes cells to form yeast-form buds, thereby abrogating the PH developmental program.

**Control of cell fate during starvation**

Our results suggest that in response to starvation, the fate of diploid cells becomes restricted in two distinct stages. In an initial response to severe nutrient deprivation, cells lose the ability to produce yeast-form offspring. At this point, cells are committed to differentiate but remain bipotential, as they can either form spores or engage in PH development, depending on nutrient availability. Only after starvation conditions have persisted long enough to initiate the middle-meiotic program do cells become committed to meiosis and sporulation [18,19].

We propose that the initial cell fate restriction into a bipotential state is geared toward balancing the need to proliferate with survival under nutrient deprivation. Although the formation of protective spores provides better odds of survival for individual cells (or their meiotic offspring) in a harsh environment, sporulation is very time-intensive, which represents a competitive disadvantage if neighboring cells continue to proliferate. By maintaining the option to enter PH development while completing the early stages of sporulation, cells are
primed to forage the environment and proliferate should nutrient deprivation prove to be transient. Our results indicate that Ime4-dependent RNA methylation plays a central role in optimizing these developmental decisions.
**METHODS:**

**Strains and growth conditions.** Strain genotypes are shown in Supplemental Table 1. To induce synchronous meiotic entry, cells were pre-selected on 1% yeast extract, 2% peptone, 2% glycerol, 2% agar for 24 hours at 30°C, grown for 24 hr in 1% yeast extract, 2% peptone, 4% dextrose at 30°C, diluted in BYTA (1% yeast extract, 2% tryptone, 1% potassium acetate, 50 mM potassium phthalate) to OD$_{600}$ = 0.2 and grown for another 16 hr at 30°C, 300 rpm. Cells were then washed once with water and re-suspended in SPO (0.3% potassium acetate) at OD$_{600}$ = 2.0 and incubated at 30°C at 190 rpm. For RTG experiments, cells were removed from SPO at the indicated times, pelleted by centrifugation, re-suspended in pre-warmed 1% yeast extract, 2% peptone, 2% dextrose and incubated at 30°C at 190 rpm. Pseudohyphal growth was assayed after 6 days of growth on synthetic low-ammonium dextrose (SLAD) medium described in [1] containing 0.5% glucose for the SK1 background, 2% glucose for the Σ1278b background. To assay individual cell morphology on SLAD medium, cells were grown in liquid suspension as reported in [2].

**Cell Morphology Quantification.** Cells were photographed under 40X magnification and primary bud morphology was quantified using ImageJ (Rasband W., National Institutes of Health, [http://rsb.info.nih.gov/ij/index.html](http://rsb.info.nih.gov/ij/index.html)).

**Quantitative PCR.** Total RNA was obtained by standard phenol:chloroform:isoamyl alcohol extraction. cDNA was generated using random hexamers or strand-specific primers and the Qiagen QuantiTect Reverse Transcription Kit. Transcript abundance was quantified using reagents from Applied Biosystems and the ABI 7500 real-time PCR system. Primer sequences are provided in Supplemental Table 2.
**Other Techniques.** Flow cytometric analysis of DNA content, 4',6-diamidino-2-phenylindole (DAPI) staining for DNA segregation analysis and cell staging by spindle morphology using tubulin indirect immunofluorescence were performed as described in [36]. Northern blots were performed against total RNA as described in [6] with a strand-specific ribo-probe against either *IME4* or *RME2* as generated by the Ambion MaxiScript T7 kit. Western analyses were performed as described in [37], with anti-c-myc (9E11, Covance) at a concentration of 1:1000. TLC analysis was carried out as in [12]; mRNA was purified with the Dynabeads mRNA purification system (Invitrogen) and analyzed on cellulose plates (20cm x 20cm) from EMD.
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REFERENCES:


SUPPLEMENTAL MATERIALS

Supplemental Figure S1. RTG filamentation reflects filamentation on SLAD.

A) MATa/α diploids (SAy821) (top left panel) or MAT α haploids (H224) (top right panel) were returned to growth in rich medium after meiotic induction for 3 hours. Arrows indicate primary buds. Bottom panels represent colony morphology after growth on SLAD for 6 days. Axial ratios are quantified in (B).

C) Comparison of cell cycles of RTG0 (top panel) and RTG3 cells (bottom panel) in budding index (n=200 cells/time-point, blue diamonds), DNA content (n=3x10⁴ cells/time-point, red squares), % metaphase spindles (green triangles) and % anaphase spindles (purple crosses) (n=200 cells/time-point, purple crosses) in wild-type cells (SAy821). Dashed vertical line in bottom graph represents time of shift to rich medium (YPD) from meiosis-inducing medium (SPO) for RTG3 cells in bottom panel.

D) Measurement of nascent bud length in wild-type (SAy821) mother (x-axis) and daughter cells (y-axis) for RTG0 (red diamonds) and RTG3 (blue squares) (n=50 cells/condition).

Supplemental Figure S2. Early meiosis genes regulate SLAD filamentation. Representative colony morphologies of wild-type (SAy821), ime1Δ/Δ (SAy834), ime2Δ/Δ (SAy859) and ime4Δ/Δ (SAy771).

Supplemental Figure S3. DNA synthesis is not necessary for filamentation.
A) DNA synthesis profiles of wild-type cells (SAy821) (left panel) or wild-type cells treated with
20mM hydroxyurea (right panel) in meiosis (n=3x10^4 cells/time-point).

B) Cells from (A) were returned to growth at three hours after meiotic initiation and were
allowed to develop initial buds. Axial ratios (n=200 cells/condition) are quantified in (C).

D) DNA synthesis of wild-type (SAy821) (left panel) and clb5Δ/Δ clb6Δ/Δ (SAy1087) cells (right
panel) (n=3x10^4 cells/strain/time-point). RTG3 axial ratios were quantified in (E) (n=200
cells/strain). Colony morphology after 6 days on SLAD is represented in (F).

G) Wild-type (SAy821) and spo11Δ/Δ (SAy1013) daughter cell morphology upon RTG3 (top
panels). The same strains were photographed after growth on SLAD for 6 days (bottom panels).

H) Distribution of axial ratios of primary buds upon RTG3 for strains in (F): wild-type (red bars),
spo11Δ/Δ (green bars) (n=200 cells/strain).

Supplemental Figure S4. 2-dimensional TLC for m6A on mRNA isolated from wild-type (SAy821)
(top panel) or ime4-cat/-cat (SAy1086) (bottom panel) three hours after shift to meiosis-
inducing medium (SPO). m6A is circled.

Supplemental Figure S5. m6A-transferase hyperfilamentation extends to SLAD conditions.

(A) Representative morphology of wild-type (SAy821) and ime4Δ/Δ (SAy771) cells grown in
suspension in low nitrogen medium. Axial ratios were quantified in (B) (n=200 cells/strain).

Supplemental Figure S6. Regulation of filamentation by early meiosis genes is conserved in the
Σ1278b background.
A) wild-type (SAy355), ime1Δ/Δ (SAy634), ime2Δ/Δ (SAy610) and ime4Δ/Δ (SAy446)

B) wild-type (SAy335), ime2Δ/Δ (SAy610), ime4Δ/Δ (SAy446), ime2Δ/Δ ime4Δ/Δ (SAy613) in the Σ1278b backgrounds. Colonies were photographed after six days of growth on SLAD from comparable regions of colony density.
**Supplemental Table 1.** Strains and Genotypes. All strains are of the SK1 background unless otherwise specified.

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Supplemental Table 2. Primer sequences:

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*T7 promoter sequence is underlined
Agarwala_Supplemental_Figure_1

A

MAT α/α diploid  MAT α haploid

3h SPO, 220 min YPD

SLAD 6 days

B

MAT α/α Diploid

MAT α Haploid

Frequency vs. Axial Ratio

C

RTG=0 hours

RTG=3 hours

% Budded

% 4C

% Metaphase

% Anaphase

D

Mother 2’ Bud vs. Daughter 1’ Bud Length

0h SPO, 3h YPD

3h SPO, 3h YPD

y = 0.8594x + 1.1213

R² = 0.9182

y = 0.8876x - 5.0278

R² = 0.8142
Agarwala_Supplemental_Figure_2

wt  ime1Δ/Δ  ime2Δ/Δ  ime4Δ/Δ
A

wt  \( \text{ime4Δ/Δ} \)

B

An Axial Ratio frequency plot comparing wt and ime4Δ/Δ strains.
Chapter III: Defining a Yeast mRNA Methylation Complex

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Whitehead Institute and Department of Biology, Massachusetts Institute of Technology

Cambridge, MA 02142

SDA designed and executed experiments

GRF designed experiments
ABSTRACT:

The internal methylation of mRNA is a post-transcriptional process that is conserved in virtually all eukaryotes. In the brewer’s yeast, *Saccharomyces cerevisiae*, mRNA methylation is induced in cells undergoing meiosis—the diploid response to nutrient deprivation. Here, we identify Ime4, Mum2 and Slz1 as the components of a protein complex that catalyzes mRNA methylation in yeast. These components are necessary for m$^\text{6}$A accumulation during nutrient starvation; mutation of any one of these components results in defects in meiotic and PH development. Furthermore, we find that ectopic expression of these components under nutrient-rich conditions is sufficient to catalyze this methylation of mRNA. Finally, we provide evidence that this modification is necessary for the activation of translation of genes under starvation conditions. These findings provide evidence for a method of fine-tuning translation under nutrient-stress conditions.
INTRODUCTION

Although the presence of methylated adenosine in mRNA was first reported by Perry and colleagues in 1974, the function of this modification in transcript processing and translational fate has remained elusive [1]. The internal methylation of mRNA (i.e., internal to the methyl cap structure and poly(A) tail) at the $N^6$ group of adenosine, forming $N^6$-methyladenosine—m^6A—has been subsequently reported in almost all eukaryotic systems studied, including plants, metazoans (insects and mammals), viral genomes that undergo nuclear replication and—despite initial reports suggesting otherwise—fungi, specifically, brewer’s yeast, *Saccharomyces cerevisiae* [2,3,4,5,6,7,8,9,10].

Despite the wide conservation of the process, the components involved in this process have only been partially defined. Initial work in human cell culture identified a potentially three-component system necessary for recapitulating this process *in vitro* [4,5,11,12]. One of these components appeared to be of small consequence, and appeared unnecessary in subsequent assays [5]. Of this two- or three-component system, only one has been cloned in humans: MT-A70, encoding the methyltransferase component of the complex. Computational analysis revealed that the human methyltransferase has homologs in a vast variety of eukaryotic organisms, including *Arabidopsis thaliana* gene MTA (which subsequent work has revealed a two-component system involved in catalyzing adenosyl-methylation) as well as the *S. cerevisiae* gene, IME4, which is expressed in diploid cells upon nutrient stress [2,5,13,14].

IME4 has previously been characterized in regulating meiosis in yeast (hence, *Initiator of Meiosis 4*) [15]. Starvation for nitrogen in the presence of a non-fermentable carbon source
(e.g., acetate or glycerol) induces meiotic development and sporulation. Under this program, the diploid genome is duplicated (2C to 4C), then segregated into four haploid (1C) meiotic products encased in a spore wall. This spore structure protects haploid progeny until favorable nutrient conditions are available. Early in response to nutrient starvation in diploids, IME4 is induced and catalyzes the methylation of early meiosis transcripts, specifically, IME1, IME2 and IME4 itself [14,15,16].

Both the mechanism and the consequence of this modification remain unclear in yeast. Although IME4 appears to be homologous to the human methyltransferase, other components of this mRNA methyltransferase complex have not yet been defined in yeast. Furthermore, although work by Tuck and colleagues on the dihydrofolate reductase (DHFR) transcript suggests that this modification activates these transcripts towards translation [17], no such evidence has been provided in yeast. Although cells that are incapable of modifying transcripts (either due to a deletion of IME4 or mutation of the catalytic site) are incapable of forming spores in response nutrient depletion, the purpose of this modification is as yet unknown [14,16,17,18].

We address both the function of IME4 and the m^6A modification. We conducted a screen that identified three proteins, Ime4, Mum2 and Slz1, which appear to interact to catalyze the methylation of adenosine on mRNA. These genes not only regulate progression through meiosis, but also play a role in inhibiting pseudohyphal development—an alternate diploid-specific response to nutrient starvation in which a mother cells gives rise to elongated daughter cells that ultimately allow yeast to forage for nutrients. Furthermore, we find that ectopic expression of these genes under nutrient-rich conditions results in adenosyl
methyltransferase on mRNA, suggesting that expression of these genes is sufficient for this activity. Finally, we provide evidence that the destabilization of this three-component complex results in the reduction of the translational activity of messages that are targeted by this complex. These data not only support a three component model in humans and perhaps other higher eukaryotes, but also provide evidence suggesting that methylation of adenosine results in fine-tuning of the translational machinery to enhance the amount of protein produced from a single transcript.
RESULTS

Yeast two-hybrid reveals Mum2 and Slz1 interact with Ime4

To determine whether there were other proteins that interact with IME4, we screened for Ime4 these putative proteins using a yeast two-hybrid system [19]. A haploid bait strain encoding Ime4 fused to the Gal4 DNA-binding domain was mated to a haploid prey library transformed with yeast 2-micron plasmids containing genomic fragments fused to the Gal4 activation domain. Diploid Gal+ candidates were selected and individual library plasmids were sequenced and re-transformed. We screened the library at 21-fold complexity and isolated plasmids that identified 13 unique genes. Of these, only two genes, **MUM2** (MUddened Meiosis 2) and **SLZ1** (Sporulation-specific Leucine Zipper 1) accumulated both transcript (figure 1A) and protein (figure 1B) during meiosis; thus, these are viable candidates for acting in complex with Ime4 upon nutrient starvation [20]. We found that immunoprecipitation of Ime4 resulted in co-immunoprecipitation of Mum2 and Slz1, suggesting that these proteins physically interact during meiosis. It is notable that although SLZ1 has been isolated from high-throughput screens for genes that are incapable of meiotic development and spore formation, the function has not been extensively characterized in meiotic development [21,22]. Previous work on MUM2 has shown it is necessary for progression past pre-meiotic DNA synthesis, and plays a role in DNA recombination during meiosis [23,24].

This two-hybrid screen helped to identify a sufficient interaction domain for Mum2 and Slz1 interaction with Ime4. Our screen identified 28 independent clones of **MUM2** and 8
**Figure 1.** Yeast two-hybrid reveals Mum2 and Slz1 as potential integrators with Ime4.

A) Northern analysis analysis for *MUM2* and *SLZ1* expression in haploid (*MAT* α—AH224) and
*MAT* a/α (SAy821) diploid cells growing in mid-log conditions (first and second columns), as well
in sporulation conditions. Numbers above columns represent hours in meiosis medium. *ACT1*
and ethidium-bromide-stained ribosomal bands are shown as loading controls.

B) Western analysis for HA-tagged Mum2 protein (SAy1235) or HA-tagged Slz1 protein
(SAy1254) throughout meiosis; Pgk1 protein serves as loading control.

C) Western analysis for co-immunoprecipitation of Mum2 (left panels) and Slz1 (right panels)
with Ime4. HA-tagged Mum2 or Slz1 was immunoprecipitated from cellular extracts and probed
for interaction with myc-tagged Ime4 (SAy1232, SAy1253, respectively). A MYC-Ime4 (SAy914)
strain without HA-tags served as a control. Arrows in the IP lanes indicate IgG bands.

D) Map of clones isolated for *MUM2*. X-axis represents positions on chromosome II.
Independent clones are represented on the y-axis. *MUM2* coordinates are represented at y=0
in dark blue. Sequencing of clone 13 and 19 revealed the 5’ terminus overlaps with the
neighboring gene, *UBP17*; fusion of a Mum2 domain to the Gal4 domain is unlikely to occur in
these instances.

E) Map of clones isolated for *SLZ1*, as in (D).
Figure 1

A

MUM2

SLZ1

ACT1
rRNA

B

Clone # vs. Chromosome II position

C

Clone # vs. Chromosome XIV position
independent clones of SLZ1. The 28 independent clones of MUM2 all spanned a 3’ region of the
gene (figure 1B), suggesting the N-terminal region of Mum2 is sufficient for interaction with
Ime4. Notably, this region bears homology to an ATPase-domain. Similarly, the 8 independent
clones of SLZ1 all spanned the 3’ region of the gene, suggesting this region is sufficient for
interaction with Ime4 (figure 1C). Notably, this region excludes the highly-structured leucine
zipper motif at the N-terminus of the protein.

**mum2Δ/Δ and slz1Δ/Δ exhibit ime4Δ/Δ phenotypes**

If Mum2 and Slz1 act in complex with Ime4 and are essential for m6A formation during
starvation, deletion of either Mum2 or Slz1 should exhibit phenotypes associated with cells that
are incapable of modifying transcripts (i.e., ime4Δ/Δ). To test this hypothesis, we first tested
whether mum2Δ/Δ and slz1Δ/Δ exhibited the same meiotic phenotypes as ime4Δ/Δ. As
previously established, ime4Δ/Δ is defective in spore formation as compared to wild-type
diploid cells when placed under starvation conditions. Deletion of MUM2 results in a similar
defect in spore formation, whereas loss of SLZ1 has a reduced but not as severe a spore-
formation defect (figure 2A).

The spore defect in mum2Δ/Δ and ime4Δ/Δ both appear to arise from a defect in exiting
meiotic prophase. As in ime4Δ/Δ, both mum2Δ/Δ and slz1Δ/Δ replicate DNA under starvation
conditions with wild-type kinetics (figure 2B). However, staining for meiotic divisions using 4’,6-
diamidino-2-phenylindole (DAPI) revealed a delay in progressing into the meiotic divisions. Like
ime4Δ/Δ, mum2Δ/Δ showed a severe defect in inducing these divisions. Compared to wild type,
slz1Δ/Δ is also slightly delayed; however, these strains are not nearly as delayed as either ime4Δ/Δ or mum2Δ/Δ (figure 2C).

Like ime4Δ/Δ, mum2Δ/Δ is also hyper-filamentous. When plated on solid low-nitrogen medium (Synthetic Low-Ammonium Dextrose Medium—SLAD), wild-type cells will form colonies of largely vegetatively-dividing cells with chains of elongated cells that protrude out from a central mass of vegetatively dividing cells. Deletion of IME4 results in cells that are not only hyper-elongated, but also form more chains, termed filaments or pseudohyphae. Deletion of MUM2 results in this hyper-filamentation phenotype; notably, deletion of SLZ1 does not appear to affect the morphology of colonies on nitrogen starvation medium (figure 3A).

This development is paralleled in cells that are shifted to rich medium (YPD) from liquid meiosis-inducing medium during pre-meiotic G2. These return-to-growth (RTG) cells are elongated as compared to vegetatively dividing cells and parallel pseudohyphal (PH) development. As in SLAD-PH cells, deletion of IME4 results in hyper-elongation of the daughter cell during RTG-PH development. Consistent with the SLAD colony morphology, deletion of MUM2 also results in hyper-elongation during RTG-PH. Although slz1Δ/Δ does not appear to form hyper-filamentous colonies on SLAD, cells of this strain appear to form slightly hyper-elongated PH daughter cells upon RTG (figure 3A, B).

**MUM2, IME4 and SLZ1 are necessary for m6A accumulation in meiosis**

Taken together, the meiotic and PH phenotypes suggest a model in which both MUM2 and IME4 are essential components of the methyltransferase complex, which we term the MIS (MUM2, IME4, SLZ1) complex. Deletion of any of these components results in phenotypes
Figure 2. Deletion of MUM2 or SLZ1 results in meiotic defects comparable to IME4 deletion.

A) Number of asci with one, two, three, four, or no spores (i.e., unsporulated) in the wild-type (SAy 821), ime4Δ/Δ (SAy771) and mum2Δ/Δ (SAy1196) and slz1Δ/Δ (SAy1206) after 24 hours in SPO medium (n=200 cell/strain).

B) FACS analysis of DNA synthesis in strains from (A) throughout the meiotic time course (n=3x10^4 cells/strain/timepoint). DNA content of diploid cells in G1 (2C) and G2 (4C) is indicated.

C) Accumulation of cells undergoing either MI or MII (as assayed by DNA staining by DAPI) in the strains from (A) (n=200 cells/strain).
Figure 2

A

![Bar chart showing percent cells relative to time in SPO (hours) for different genotypes: wt, ime4Δ/Δ, mum2Δ/Δ, slz1Δ/Δ. The bars are color-coded for tri-/tetra-, dy-, mono-, and unsporulated cells.](image)

B

![Graph showing meiotic cell cycles for different genotypes: wt, ime4Δ/Δ, mum2Δ/Δ, slz1Δ/Δ.](image)

C

![Graph showing the percentage of meiotic cells over time in SPO for different genotypes: wt, ime4Δ/Δ, mum2Δ/Δ, slz1Δ/Δ.](image)
**Figure 3.** Deletion of *MUM2* or *SLZ1* results in PH-developmental defects comparable to *IME4* deletion.

A) Wild-type (SAy821), *ime4Δ/Δ* (SAy771), *mum2Δ/Δ* (SAy1196) and *slz1Δ/Δ* (SAy1206) daughter cell morphology upon RTG₃ (top panels). Arrows indicate primary buds. The same strains were photographed after growth on SLAD for 6 days (bottom panels).

B) Distribution of axial ratios of primary buds upon RTG₃ for strains in (A).
Figure 3

A

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B

- wt
- slz1Δ/Δ
- mum2Δ/Δ
- ime4Δ/Δ

Frequency vs. Axial Ratio
associated with loss of m\textsuperscript{6}A in cells (i.e., catalytic mutation of IME4 discussed in chapter 2). In contrast, SLZ1 does not appear to be an essential component of the RNA methylosome. Although deletion of SLZ1 appears to display minor defects in meiosis and low levels of hyper-filamentation, neither of these phenotypes appear to be as severe as the MUM2 or IME4 deletion alleles. Both meiotic and filamentous defects suggest that SLZ1 is not an essential component of the RNA methylosome and may play a lesser role in RNA methylation.

To test whether SLZ1 or MUM2 affect m\textsuperscript{6}A accumulation on mRNA, we quantified m\textsuperscript{6}A by thin-layer chromatography in mRNA isolated from cells exposed to starvation conditions for three hours. At this time-point, m\textsuperscript{6}A is at a maximum in wild-type cells. As previously reported, ime4\textsuperscript{Δ/Δ} did not accumulate m\textsuperscript{6}A in meiosis. Corresponding with its essential role in the RNA methylosome, mum2\textsuperscript{Δ/Δ} also failed to accumulate m\textsuperscript{6}A in meiosis. slz1\textsuperscript{Δ/Δ}, in contrast, accumulated low levels of m\textsuperscript{6}A that were not comparable to wild-type levels, consistent with its role as a non-essential role in RNA methylation (figure 4A).

\textit{MUM2, IME4 and SLZ1 are sufficient for m\textsuperscript{6}A accumulation}

Given the requirement for IME4, MUM2 and SLZ1 function for m\textsuperscript{6}A accumulation in mRNA during meiosis, we tested whether these genes were sufficient to induce m\textsuperscript{6}A on RNA in rich conditions, where m6A is not typically present. These three genes were each placed under control of the inducible \textit{CUP1} promoter in diploid cells. Expression of these genes was induced in rich medium, under which condition m\textsuperscript{6}A does not usually accumulate in mRNA. We found that ectopic expression of IME4 alone was not sufficient to induce m\textsuperscript{6}A accumulation on mRNA. However, induction of both IME4 and MUM2 in rich conditions resulted in an accumulation of
m$^6$A above background. Induction of all three of the components (*i.e.*, IME4, MUM and SLZ1) resulted in comparable accumulation of m$^6$A as IME4 and MUM2 alone, consistent with the role of SLZ1 as a non-essential component of the RNA methylosome (figure 4B). Notably, neither one of the strains that express m$^6$A in rich conditions exhibits any obvious morphological or growth differences as compared to strains that do not express m$^6$A (data not shown).

**Ime2 accumulates at low levels in the absence of Ime4**

Immunoprecipitation of methylated transcripts has revealed that IME2 is heavily methylated near the 3' terminus of the transcript [16]. To test if the increased accumulation of m$^6$A at three hours in meiosis in wild-type cells can be attributed to the accumulation of IME2 alone, we compared m$^6$A accumulation in the ime2Δ/Δ background to wild-type cells under the same conditions. Wild-type cells accumulated high levels of m$^6$A under these conditions; cells deleted for IME4, the putative catalytic subunit of the RNA methylosome, failed to accumulate m$^6$A under these conditions, as previously described (figure 5). We observed near-wild-type levels of m$^6$A in the ime2Δ/Δ background, suggesting that not only is IME2 not the major contributor to m$^6$A accumulation three hours into meiosis, but also that the m$^6$A accumulation on IME2 mRNA is negligible when compared to the total m$^6$A accumulation on mRNA during meiosis (figure 5). Deletion of IME1, the master transcriptional activator for early meiosis genes [25], resulted in a significant decrease in m$^6$A levels three hours into meiosis; although this level of m$^6$A was lower than wild-type at the same time point, m$^6$A levels were higher than background (*i.e.*, levels observed in ime4Δ/Δ) (figure 5). This finding suggests that either m$^6$A
Figure 4. IME4, MUM2 and SLZ1 are necessary and sufficient for m$^6$A transferase activity.

A) Quantification of m$^6$A on mRNA three hours after meiotic starvation, when m$^6$A accumulation is maximal in wt cells (SAy821). Deleting any one of ime4Δ/Δ (SAy771), mum2Δ/Δ (SAy1196) and slz1Δ/Δ (SAy1206) results in a reduction in m$^6$A levels.

B) MIS complex expression is sufficient to induce m$^6$A accumulation on mRNA. m$^6$A accumulation on mRNA was quantified in rich conditions in wild-type (SAy821), ime4Δ/Δ (SAy771), P_CUP1-IME4 (SAy1249), P_CUP1-MUM2 (SAy1251), P_CUP1-SLZ1 (SAy1250), P_CUP1-IME4 P_CUP1-MUM2 P_CUP1-SLZ1 (SAy1248) and P_CUP1-IME4 P_CUP1-IME4 (SAy1252) after 150 minutes of mitotic growth in the presence of cupric sulfate.
Figure 4

A

B

$\frac{[m6A]/[C]}{\text{wt}}$  ime4Δ/Δ  mum2Δ/Δ  slz1Δ/Δ

$\frac{[m6A]/[C]}{1225}$  $1209$  $1212$  $1227$
Figure 5. m$^6$A quantification in wt (SAy821), ime4Δ/Δ (SAy771), ime1Δ/Δ (SAy834), and ime2Δ/Δ (SAy856) three hours in meiotic starvation.
Figure 5

![Bar chart showing the comparison of $\frac{[\text{m6A}]}{[\text{C}]}$ across different conditions: wt, ime4Δ/Δ, ime1Δ/Δ, ime2Δ/Δ. The y-axis represents the ratio $\frac{[\text{m6A}]}{[\text{C}]}$, and the x-axis represents different genotypes. The chart indicates higher values for wt and ime1Δ/Δ compared to ime4Δ/Δ and ime2Δ/Δ.]
accumulates specifically on transcriptional targets (or a particular individual target) of Ime1, or that Ime1 is necessary for total activity of the RNA methylosome.

We next tested the translational fate of methylated and unmethylated IME2 transcript during meiosis by comparing epitope-tagged Ime2 in wild-type and ime4Δ/Δ cells. Quantification of IME2 transcript throughout meiosis revealed that the transcript levels are roughly comparable between wild type and ime4Δ/Δ 0 hours in meiosis, in pre-sporulation medium, prior to induction of the meiotic program (figure 6A). Thus, we chose the 0 hour time-point to analyze the translational capacity of the transcript by Western analysis. We observed lower levels of protein levels of the epitope tagged IME2 transcript in the ime4Δ/Δ background as compared to wild type at steady state, despite the comparable levels of transcript (figure 6B). It is notable that, despite the significant down-regulation of protein, low levels of Ime2 protein were still observed in the ime4Δ/Δ background: unmethylated transcript does not appear to lose its translational capacity altogether. Instead, methylation of transcripts appears to enhance translation of a given transcript.
CONCLUSIONS:

Defining an RNA methylation complex

Consistent with a three-component model for the methyltransferase in humans, our results define the components of an mRNA methylation complex in yeast, composed of Mum2, Ime4 and Slz1, which we term the MIS complex. Deletion of either IME4 or MUM2 results in a loss of the methyltransferase activity. As previously described, IME4 is homologous to MT-A70 in humans, in Arabidopsis thaliana, to MTA, and recent work has identified a homolog in Drosophila melanogaster, Dmime4 [2,3,4,6]. Mutation of any one of these homologs results in gross developmental defects—in the animal cases, lethality [6]. MUM2 bears homology to the Arabidopsis AtFIP37 at the protein level, which has previously been shown to interact with MTA, the A. thaliana homolog of IME4 (Figure 7). MUM2 also bears homology to Drosophila Fl(2)d and WTAP-1 in humans. Although these latter two genes have been not been tested for interaction with their respective IME4 homologues, both of these genes bear homology to the Arabidopsis AtFIP37 and yeast MUM2. Independent work on the Drosophila and human homologues of MUM2 reveals that, like mutation of IME4 homologues, mutation of these genes results in gross developmental defects in their respective organisms [26,27,28,29,30].

Thus, the necessity of both IME4 and MUM2 for mRNA methyltransferase activity, as well as the apparent essentiality of mRNA methyltransferase activity in higher organisms explains the conservation of these genes in higher organisms.

Slz1 does not appear to be an essential component of the MIS complex. Deletion of SLZ1 still results in methylation activity, albeit reduced compared to wild-type cells. Furthermore,
Figure 6. Ime2 does not accumulate to wild type levels in the absence of IME4.

A) Quantification of IME2-as1-13xmyc transcript levels at 0 hours in meiosis by qPCR in either IME4/IME4 (SAy1193) or ime4Δ/Δ (SAy1192), as labeled.

B) Western analysis of strains in (A) from protein extracted from the same time-point.
Figure 6

(A) Graph showing the relative expression of IME2 in wild type (wt) and ime4Δ/Δ strains. The y-axis represents IME2 relative to ACT1, with values on the x-axis indicating wt and ime4Δ/Δ.

(B) Western blot analysis showing the expression of IME4 and Pgk1 proteins in strains ime2-as1-13xmyc and ime4Δ/Δ.
Figure 7. Comparison of Mum2 homologues.

A) Dendrogram for Mum2 homologues, FL(2)D—*Drosophila melanogaster*, WTAP-1—*Homo sapiens*, AtFIP37—*Arabidopsis thaliana* and yeast Mum2, which serves as an outgroup.

B) Alignment of protein sequences in (A). Blue squares represent partial homology, yellow squares represent partial identity, green squares (also starred) represent conserved identity.
Figure 7

A

- Fl 2 d
- WTAP - 1
- AtFIP37
- MUM2

B

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* * * *

****
deletion of *SLZ1* does not manifest as strong phenotypes as deletion of either *IME4* or *MUM2* in either meiosis or PH development. While it is possible that other meiotic genes may play a functionally redundant role with Slz1—thus, deletion of both genes would be necessary for manifestation of the full methyltransferase defect—it is notable that whereas both Ime4 and Mum2 have well-defined homologues in other organisms, *SLZ1* does not appear to have significant homology to genes any other organisms, supporting a model in which the role that Slz1 plays is dispensable for methyltransferase function in other organisms.

The structural elements of the three components we define here provide some hints as to the mechanism of methyltransferase activity in yeast. As previously described, the conserved catalytic residues on Ime4 are necessary for the methyltransferase activity, suggesting that Ime4 encodes the catalytic module for mRNA methylation. *MUM2* encodes a structurally conserved Smc domain—a chromosome-segregation-associated ATPase domain at its C-terminus, which our data suggest is necessary for interaction with Ime4. Thus, hydrolysis of ATP by Mum2 may provide the energy necessary for the methylation of mRNA by the protein complex. It is notable that deletion of *MUM2* results in a reduction in recombination during meiosis [23,24]. This phenotype has not been observed in deletions of *IME4* or *SLZ1*; thus, outside of the context of the mRNA methylosome, Mum2 may play other roles in DNA recombination.

**An ectopic system for studying RNA methylation**

Expression of all three elements of the MIS complex is sufficient to catalyze the m\(^6\)A-transferase activity in rich medium—a condition under which m\(^6\)A usually does not accumulate.
This result demonstrates the sufficiency of the components of the MIS complex, which we find is usually induced under nutrient starvation conditions in diploid cells. It is notable that \textit{SLZ1} appears to be dispensable for this methyltransferase activity under ectopic conditions, further supporting the notion that Slz1 plays an auxiliary role for mRNA methylation.

The ability to induce methylation of mitotic mRNA provides a powerful tool with which to study this process. Ectopic accumulation of \( m^6 \text{A} \) or mRNA (\textit{i.e.}, outside the context of starvation) is not lethal to cells and does not appear to cause major morphological changes in yeast. Thus, determining the translational burden of these cells (either by \underline{S}table \underline{I}sotope labeling with \underline{A}mino acids in \underline{C}ell culture—SILAC—or by ribosome-footprinting followed by sequencing) will reveal the effect this modification has on mRNA in terms of translation [31,32]. However, central to understanding the translational effects of modified transcripts is identifying which molecules become methylated. Although previous work has isolated putative modified transcripts by immunoprecipitation, this study failed to present controls against un-methylated mRNA to test the fidelity of the antibody, leaving the fidelity of this technique suspect [16]. Developing tools to accurately isolate and identify the individual targets of this methylation remains the central hurdle to understanding this modification to mRNA in both the mitotic and meiotic situations.

\textbf{Translation of mRNA methylation targets depends on Ime4}

In the absence of Ime4, there is a reduction of Ime2. \textit{IME2} is a target of transcript methylation, accumulating \( m^6 \text{A} \) at the 3’ terminus of the transcript. Thus, one explanation for this finding may be that mRNA methylation activates translation of the modified message. In
this model, it is important to note that translation is not absent in methyltransferase-deficient backgrounds: methylation of transcripts appears to enhance a basal level of translation, suggesting that this modification fine-tunes expression of the target gene by modulating the translational capability of individual messages. This model parallels work from Tuck and colleagues in which in vitro translation of methylated dihydrofolate reductase (DHFR) transcript—a transcript that is methylated in vivo—is more efficient than unmethylated transcript; conversely, that inhibiting total RNA methylation (on all nucleotides, at all positions) reduces translation of DHFR in vivo [17,18].

Many questions remain regarding this conclusion. Our study utilized a deletion allele of IME4, resulting in a global loss of mRNA methylation. It is conceivable that mRNA methylation may regulate multiple independent pathways that, in turn, influence translation. Creation of an IME2 allele that abrogates all potential methylation sites will address this question. Furthermore, analyzing the formation of methyl cap and polyadenylation on IME2 transcript in the presence or absence of IME4 will address the question of whether methylation affects mRNA stability and turnover or is a structural component of mRNA that affects the translational capability of a single message.

Our finding, however, suggests that there is fundamental difference in translation between starvation conditions, when mRNA methylation is induced, and rich, vegetative conditions, when there is no detectable level of mRNA methylation. Modulation of transcripts by methylation under starvation conditions is necessary for translation of the efficient
expression of the genes and progression through the starvation-mediated developmental response.
METHODS:

Strains and growth conditions. Strain genotypes are shown in Supplemental Table 1. To induce synchronous meiotic entry, cells were pre-selected on 1% yeast extract, 2% peptone, 2% glycerol, 2% agar for 24 hours at 30°C, grown for 24 hr in 1% yeast extract, 2% peptone, 4% dextrose at 30°C, diluted in BYTA (1% yeast extract, 2% tryptone, 1% potassium acetate, 50 mM potassium phthalate) to OD$_{600}$ = 0.2 and grown for another 16 hr at 30°C, 300 rpm. Cells were then washed once with water and re-suspended in SPO (0.3% potassium acetate) at OD$_{600}$ = 2.0 and incubated at 30°C at 190 rpm. For RTG experiments, cells were removed from SPO at the indicated times, collected by centrifugation, re-suspended in pre-warmed 1% yeast extract, 2% peptone, 2% dextrose and incubated at 30°C at 190 rpm. Pseudohyphal growth was assayed after 6 days of growth on synthetic low-ammonium dextrose (SLAD) medium described in [33] containing 0.5% glucose. The $CUP1$ promoter was induced with 100μM CuSO$_4$ in rich media.

Cell Morphology Quantification. Cells were photographed under 40X magnification and primary bud morphology was quantified using ImageJ (Rasband W., National Institutes of Health, http://rsb.info.nih.gov/ij/index.html).

Co-immunoprecipitation. 50 ml of meiotic culture was harvested 3 hours after meiotic induction in the presence of protease inhibitors (Complete protease inhibitors, Roche). Cells were washed once with 1M Tris-HCl, pH 7.5 and snap-frozen. Frozen pellets were resuspended in lysis buffer (150mM NaCl, 50mM Tris pH7.5, 1% NP40, 10mM PMSF, Complete mini protease inhibitors (Roche) at 2x concentration) and homogenized three times for 5 minutes at 4°C. Debris was pelleted by centrifugation for 10 minutes, and supernatant was incubated with HA-conjugated agarose beads (Pierce) with head-over-tail rotation for three hours. Beads were
washed 5x in lysis buffer and boiled in reducing loading buffer, followed by the standard protocol for Western analysis as described below.

**Quantitative PCR.** Total RNA was obtained by standard phenol:chloroform:isoamyl alcohol extraction. cDNA was generated using random hexamers or strand-specific primers and the Qiagen QuantiTect Reverse Transcription Kit. Transcript abundance was quantified using reagents from Applied Biosystems and the ABI 7500 real-time PCR system. Primer sequences are provided in Supplemental Table 2.

**Other Techniques.** Flow cytometric analysis of DNA content, 4',6-diamidino-2-phenylindole (DAPI) staining for DNA segregation analysis and cell staging by spindle morphology using tubulin indirect immunofluorescence were performed as described in [34]. Northern blots were performed against total RNA as described in [35] with a strand-specific ribo-probe against either *IME4* or *RME2* as generated by the Ambion MaxiScript T7 kit. Western analyses were performed as described in [36], with anti-c-myc (9E11, Covance) or with anti-HA (HA.11, Covance) at a concentration of 1:1000. TLC analysis was carried out as in [2]; mRNA was purified with the Dynabeads mRNA purification system (Invitrogen) and analyzed on cellulose plates (20cm x 20cm) from EMD.
References:


### SUPPLEMENTAL MATERIALS:

#### Supplemental Table 1. Strains and Genotypes. All strains are of the SK1 background.

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Supplemental Table 2. Primer sequences:

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*T7 promoter sequence is underlined
Chapter IV: Conclusions and Perspectives
This thesis has investigated the role of *IME4*, in its capacity as mRNA methyltransferase, in regulating developmental responses to nutrient stress in diploid yeast. This analysis has revealed consequences and mechanism of mRNA methylation: chapter 2 discussed the role mRNA methylation components play in negotiating a decision between meiosis and PH development in diploid yeast. Chapter 3 identified the components of a putative protein complex involved in catalyzing the $N^6$-adenosyl methyltransferase reaction and suggests a role for this modification in regulating the fate of transcripts in nutrient-limiting conditions.

This chapter discusses future work for elucidating the function of transcript methylation in regulating mRNA fate and identifying targets of mRNA methylation. These tools developed towards this end will be useful for gaining an understanding for the role of mRNA methylation in responses to nutrient-starvation in diploid yeast, as well as elucidating the function of this modification in regulating transcript fate and development in higher eukaryotes, specifically metazoans and humans.

**Identifying targets of mRNA methylation**

Isolation of the targets of mRNA methylation remains a central issue in understanding the consequences of this modification. Previous work has used immunoprecipitation to identify targets of methylation, although it is unclear how specific this antibody is towards pulling down only those targets that are methylated [1,2]. Regardless, future work will address two major issues that arise with respect to this technique: first, where the $m^6$A is located on the target molecule? and second, what percentage of total transcripts is methylated?
To answer the first question, that of mapping m\textsuperscript{6}A on individual targets after pull-down: immunoprecipitation of potential methylated transcripts (as Bodi and colleagues have done for the yeast meiotic initiator, *IME2*) will reveal the general region of the transcript that accumulates m\textsuperscript{6}A. It is notable that random fragmentation of methylated transcripts, followed by immunoprecipitation and sequencing of immunoprecipitated fragments will give some sense of where m\textsuperscript{6}A accumulates on transcripts on a genomic scale—similar to techniques identifying methylated CpG islands throughout the genome [3], or identifying transcription factor binding sites by ChIP-seq [4].

The second question as to how one determines the fraction of methylated molecules in a pool of methylated mRNA is easily resolvable at the genomic level, but is a significantly more difficult question at the single-molecule scale. Globally, quantification of immunoprecipitated transcript relative to the input will reveal what fraction of transcripts contains m\textsuperscript{6}A. However, it remains a distinct possibility that the m\textsuperscript{6}A accumulation pattern is not the same among two otherwise identical transcripts. Resolutions of these issues will require mapping which fraction of transcripts is methylated at a given position in the transcript.

Many methods are conceivable for answering this latter question for major sites of m\textsuperscript{6}A accumulation. The presence of m\textsuperscript{6}A inhibits RNA-mediated DNA ligations where the complementary base-pair to the m\textsuperscript{6}A base pair encodes a G [5]. To test if a given site is methylated, two complementary DNA oligos may be synthesized, replacing the putative m\textsuperscript{6}A complement (*i.e.*, T) with a G. Unsuccessful ligations will suggest m\textsuperscript{6}A at the position, while unsuccessful ligation will suggest the presence of unmodified nucleotide at the position; the
successful or unsuccessful ligation maybe quantified by quantitative PCR, or by radio-labeling oligos and quantifying successful or unsuccessful ligation by the migration pattern on polyacrylamide gel. Although proof-of-principle experiments have shown this technique to be effective for highly-conserved sites of m\(^6\)A accumulation (Dai, and colleagues, who developed this technique utilized a modified tRNA from human cells), this approach is unlikely to be effective for minor sites of methylation in which only a small fraction of transcripts are methylated at a given site, and is not easily-adapted for global analysis of m\(^6\)A accumulation (Pan, T--personal communication).

Role of mRNA methylation in transcript fate

Direct effects of m\(^6\)A on translation

In agreement with previous work, I have shown here that m\(^6\)A accumulation affects translation of mRNA. In IME4 deletion cells, there is significantly less IME2 protein accumulation than in wild-type cells. As IME2 is a well-characterized target of mRNA methylation [1], these data raise the possibility that mRNA methylation is an activator of translation in yeast as has been suggested to be the case in other in vitro and mammalian systems [6,7].

Further work will investigate this possibility. Deletion of IME4 may result in multiple defects in the cell that culminate in the loss of translation of IME2 transcript. Future work will re-code the IME2 transcript, mutating the potential methylation sites so that it does not accumulate m\(^6\)A. Assaying protein-accumulation of the un-methylate-able IME2 with the wild-type IME2 will assay the direct effects of mRNA methylation on protein accumulation, although
in this experiment, codon bias may remain a considerable issue to concluding whether m\textsuperscript{6}A affects translation of mRNA. Alternatively, some transcripts may naturally not accumulate m\textsuperscript{6}A, even in wild-type cells—translation of these transcripts should not be affected by the deletion of IME4 if there is a direct effect of m\textsuperscript{6}A on the translation of modified transcripts. These may serve a better control than even the un-methylate-able IME2 experiment described earlier.

It is notable that in vitro transcription of methylated DHFR, followed by in vitro translation resulted in higher protein yields than an un-methylated control [6,7]. These data, though difficult to interpret, suggest a direct effect of m\textsuperscript{6}A on translation of transcripts. Given these data, future work will examine the translation efficiency of transcripts throughout meiosis. Specifically, recent work has developed a method of identifying ribosomal occupation of actively-translated transcripts using a procedure termed “ribosomal footprinting” [8]. Briefly, translation is globally paused in the cell by the addition of cycloheximide. Poly-ribosomal fractions are then isolated, fragmented and digested. Ribosome-protected fragments, or “footprints” are then subjected to sequencing, revealing the occupation of ribosomes throughout the transcriptome at a given time. If m\textsuperscript{6}A accumulation on transcript affects translation, I would expect to see a difference in ribosomal occupation in cells that accumulate m\textsuperscript{6}A in meiosis as compared to cells that do not accumulate m\textsuperscript{6}A. As before, it is notable that these differences in ribosomal footprint may arise from a difference in the timing of meiotic induction or a wide variety of effects that loss of mRNA methylation may inflict on the cell. To account for this, it will be important to normalize ribosomal occupation to the total level of transcript available in the cell. Also, to control for this, I show that m\textsuperscript{6}A accumulation on mRNA is ectopically inducible in media in a strain that encodes inducible alleles of the mRNA
methylosome components. Checking whether the translational changes observed in m$^6$A+ versus m$^6$A- cells in meiosis are sufficient to confer changes on m$^6$A+ versus m$^6$A- in rich media, will speak more directly towards the direct effect of this modification on mRNA fate.

Translational changes in m$^6$A+ versus m$^6$A- cells can be further monitored by SILAC—Stable Isotope Labeling with Amino acids in Cell culture [9]. In this technique, one set of cells (without loss of generality, either m$^6$A+ or m$^6$A- strains) are labeled with heavy isotopes of amino acids while the other set remains un-labeled. After induction of meiosis, total protein is extracted from both strains and subjected to mass spectrometry. Differences in accumulation of individual proteins are discernable by this technique. If m$^6$A affects translation in meiosis, I would expect to see significant changes in translation in m$^6$A+ versus m$^6$A- cells undergoing meiosis. As before, strains encoding the inducible methylosome remain a powerful tool in understanding these changes. If m$^6$A accumulation on mRNA is sufficient for these changes, I would expect to see these changes paralleled in induced versus uninduced cells in ectopic conditions.

**Indirect effects of m$^6$A on mRNA fate**

Methylation may affect various other aspects of mRNA structure and stability. Bodi and colleagues hypothesize that m$^6$A accumulation may affect mRNA binding to Khd1—an RNA-binding protein which has been shown to be necessary for transcript localization to the bud tip in yeast [1,10]. Despite the lack of data suggesting that m$^6$A influences the transcripts with which Khd1 interacts, and although this specific interaction is speculative$^1$, this hypothesis

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$^1$ Bodi and colleagues, in their analysis of IME2 methylation during yeast meiosis, note the presence of two tracts of CNN motifs in the 3’ region of IME2, surrounding three GAC triplets—i.e., m6A accumulation motifs—in the
raises the possibility that m\textsuperscript{6}A may affect mRNA localization. That mRNA localization to subcellular compartments affects gene function and even translation is part of an emerging field of post-transcriptional mRNA operons [12]. Recent developments in visualizing mRNA localization using fluorescent \textit{in situ} hybridization (specifically, the STAR-FISH procedure developed by the Raj, and colleagues) has dramatically increased the resolution of mRNA visualization in a cell without genetic manipulation of the transcript itself, thereby minimalizing perturbations to transcript localization [13]. Observation of localization of methylated or unmethylated transcripts (either by recoding of the transcript itself, or by deletion of \textit{IME4}—although this latter method may result in pleiotropic effects) will resolve whether m\textsuperscript{6}A accumulation influences transcript localization.

m\textsuperscript{6}A accumulation may affect various other aspects of transcript structure and stability. Both polyadenylation and capping of transcripts may be affected by the accumulation of m\textsuperscript{6}A, both of these processes regulate transcript stability, and both encode features that regulate the translational activity of transcripts. Indeed, methylation may affect the intrinsic mRNA half-life, independent of effects conferred by cap and poly(A) tail. Further investigation of how m\textsuperscript{6}A accumulation affects translation of the modified transcript will monitor both of these structural aspects of mRNA.

As previous work has suggested that the Khd1 binding motif is comprised of CNN (\textit{i.e.}, cytidine, followed by two degenerate bases), and that, despite being grown in nutrient-rich medium in haploid cells—conditions under which \textit{IME2} has not been known to be expressed in wild-type situations and under which ectopic expression of \textit{IME2} results in an extreme growth defects, often inviability—Khd1 is somehow associated with meiosis-specific \textit{IME2} transcript (Hasegawa Y, Irie K, Gerber AP (2008) Distinct roles for Khd1p in the localization and expression of bud-localized mRNAs in yeast. RNA 14: 2333-2347.). Disregarding these caveats, Bodi and colleagues hypothesized that the methylation at the GAC triplets between the Khd1 binding motifs somehow influences Khd1 binding to transcripts through an unknown mechanism. It should be noted that deletion of \textit{KHD1} does not result in any major meiotic defect, suggesting that the effect, should there be any, of Khd1 on \textit{IME2} transcript localization is nominal. It should also be noted that \textit{KHD1} transcript is not detectable in diploid cells undergoing meiosis, suggesting it does not play a major role in meiosis.
mRNA methylation in other organisms

mRNA methylation in *Candida albicans*

In this thesis, I have shown that mRNA methylation independently regulates entry into pseudo-hyphal development and meiosis in diploid yeast upon nutrient starvation. However, it is notable, that entry into either pathway is regulated by the early meiosis genes *IME1* and *IME2*. Although *S. cerevisiae* is capable of undergoing both developmental pathways, the human pathogen *C. albicans* is incapable of meiosis but is capable of forming either pseudo-hyphae or true filaments upon either nutrient starvation, exposure to serum, or various other cues. *C. albicans* is incapable of meiotic development, however, has maintained full-length genes that bear strong homology to *IME1*, *IME2*, and *IME4* [14]. These genes may play a role in a parasexual cycle in *C. albicans* in which the genome duplicates, recombination is initiated, but no meiotic divisions occur, my findings raise the intriguing possibility that the early meiotic network may be necessary specifically towards filamentous development in *C. albicans*. Recent work showing that over-expression of the Ime1 interactor, Ume6—also conserved in *C. albicans*—leads to hyper-filamentation suggests that over-expression of these early meiotic genes in *C. albicans* may influence hyphal formation [15,16,17].

mRNA methylation in higher eukaryotes

As previously noted, not only m^6^A-transferases, but the interacting partners necessary for m^6^A accumulation in yeast are conserved in both plant and animal species that also accumulate m^6^A on mRNA. Although mRNA methylation regulates developmental progression in response to nutrient-starvation in diploid yeast, this modification is far more ubiquitous in
other organisms; significant evidence suggests that this modification may play different roles in these organisms.

The role of m$^6$A in mRNA stability is reinforced by work in mouse [18]. Knock-down of the putative protein that interacts with MT-A70 in mouse, WTAP, leads to lethality in mouse embryos and results in G2 cell cycle arrest of cultured mouse cells. This defect is due to the reduced expression of cyclin A2 and B in these cells; over-expression of cyclin A2 mRNA is able to relieve this arrest. Furthermore, similar to m$^6$A accumulation at the 3’ terminus of IME2 mRNA in yeast, WTAP binds to cyclin A2 mRNA at the 3’ UTR. Although this study did not investigate m$^6$A accumulation on transcripts in this system, these data suggest that m$^6$A may play a role in mRNA stability in mammalian systems.$^2$

In contrast, mRNA methylation in the fruitfly Drosophila melanogaster appears to play a role in mRNA processing. The Drosophila homologue of IME4, Dmime4, is restricted to the gonads in adult flies. Recent work has demonstrated that this expression is necessary for development of these tissues in both male and female fruit flies, as well as development of larvae [20]. Although the Drosophila m$^6$A-transferase regulates signaling via the Notch pathway, its putative interactor, Fl(2)d, which bears homology to the yeast Ime4 interactor, Mum2, has been shown to play a role in alternative splicing of multiple genes, including Sex-lethal (Sxl), transformer (Tra) and Ultrabithorax (Ubx), suggesting that m$^6$A may play a role in regulating mRNA splicing [21,22]. This finding is reinforced by work by Carroll and colleagues,$^2$

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$^2$ It should be noted that the meiosis-specific IME2 in yeast functions as a Cdk fused to a cyclin, and bears significant homology to CDK2—which interacts with cyclin A—in human cells (19. Szwarcwort-Cohen M, Kasulin-Boneh Z, Sagee S, Kassir Y (2009) Human Cdk2 is a functional homolog of budding yeast Ime2, the meiosis-specific Cdk-like kinase. Cell Cycle 8: 647-654.). That WTAP interacts with cyclin A2 may reflect the methylation of IME2 transcript in yeast meiosis.
that mapped m$^6$A in the intron of the bovine prolactin mRNA, and suggested that these modifications were necessary for splicing of this intron$^3$ [23]. It may be significant that the targets that have been isolated in yeast do not contain introns.

**Concluding remarks**

mRNA methylation is a necessary part of the mRNA processing machinery in virtually all eukaryotic species. The finding that diploid yeast methylate mRNA upon nutrient starvation has significantly furthered the study of this process. My work has elucidated the functionality of this modification in regulating a decision between meiosis and pseudo-hyphal development. In addition, my work has identified a putative complex that is necessary for this process. My findings, combined with previous work investigating these conserved components in higher eukaryotes, suggest that the role of mRNA methylation is conserved from yeast to metazoans and perhaps even plants. My findings will aid in gaining an understanding of this yet-unexplored modification in yeast in terms of its regulation of mRNA translation and stability.

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$^3$ This work inhibited m6A accumulation by treatment of neplanocin A, a general inhibitor of RNA methylation—this work did not differentiate between whether this effect is due to loss of m6A accumulation of defects in accumulating other mature RNA species.
References


Appendix I: Antisense Transcription Regulates IME4 in *Saccharomyces cerevisiae*

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**IME4** expression is restricted to diploid cells. Haploid cells transcribe a non-protein-coding antisense transcript, *RME2*, through the *IME4* open reading frame. Transcription of this antisense transcript works in cis- to inhibit transcription of the protein-coding message and subsequent expression of the gene. In *MAT a/α* cells, the a1/α2 protein heterodimer encoded by both the *MAT* loci binds downstream of the *IME4* open reading frame, inhibiting production of the regulatory antisense transcript. Strains in which the a1/α2 protein hetero-dimer binding site is mutated to prevent binding of the heterodimer, produce the antisense *RME2* transcript ectopically. This ectopic transcription is sufficient to inhibit induction of protein-coding *IME4* transcript.

We attempted to find activators of the antisense transcript *RME2* in diploid cells. Initial *in silico* analysis of binding sites downstream of *IME4* revealed a strong consensus motif for Reb1 (figure 1A). Reb1 is an essential gene that regulates transcription of ribosomal proteins. Chromatin Immunoprecipitation of epitope-tagged Reb1 revealed that, as predicted, Reb1 bound downstream of the *IME4* locus. We mutated the Reb1 binding site downstream of *IME4* to test if antisense regulation of the locus was relieved in this background. Mutation of the binding site downstream of *IME4* resulted in a loss of Reb1 binding regardless of the presence or absence of the a1/α2 protein heterodimer, and the a1/α2 protein heterodimer bound downstream of *IME4* regardless of the presence or absence of Reb1 (Figure 1B, C).

Northern analysis revealed a new antisense transcript against the *IME4* locus that migrated at a higher molecular weight than *RME2*. Mapping of *RAI1* 3’ and 5’ ends revealed start and stop sites consistent with this higher-molecular-weight species of antisense transcript.
**Figure 1.** \( a_1/\alpha_1 \) and Reb1 bind downstream of *IME4*.

A) Schematic of binding sites downstream of *IME4*. Distances are represented as numbers of nucleotides. Orange line represents the antisense transcript against *IME4, RME2*.

B) Chromatin IP in untagged diploids (SAy355), and strains in which Reb1 is epitope tagged, but in which no sites downstream of *IME4* have been mutated (SAy275), or the \( a_1/\alpha_2 \) binding site has been mutated (*IME4-a_1/\alpha_2—SAy284*), or the Reb1 binding site has been mutated (*IME4-Reb1—SAy287*) or in which both the \( a_1/\alpha_2 \) and Reb1 binding site have been mutated (*IME4-a_1/\alpha_2-Reb1—SAy312*).

C) Chromatin IP in untagged diploids (SAy355), and strains in which the \( a_1/\alpha_2 \) is epitope tagged at \( a_1 \), but in which no sites downstream of *IME4* have been mutated (CHy10), or the \( a_1/\alpha_1 \) binding site has been mutated (*IME4-a_1/\alpha_1—SAy244*), or the Reb1 binding site has been mutated (*IME4-Reb1—SAy247*) or in which both the \( a_1/\alpha_1 \) and Reb1 binding site have been mutated (*IME4-a_1/\alpha_1-Reb1—SAy250*).
Figure 1

A

B

C

A

IME4

a1

α2

binding

Reb1p

binding

12 nt

113 nt

101 nt

70 nt

IME4

Fold Enrichment

untagged wild-type IME4-a1x2 IME4-Reb1 IME4-a1x2-Reb1

Fold Enrichment

untagged wt IME4-a1x2 IME4-Reb1 IME4-a1x2-Reb1

Fold Enrichment

untagged wt IME4-a1x2 IME4-Reb1 IME4-a1x2-Reb1
(figure 2A). We term this transcript Reb1 Associated IME4-antisense 1 (RAI1). Mutation of the Reb1 binding site resulted in transcription of RAI1 only (figure 2B).

Like RME2, RAI1 inhibited transcription of sense IME4. Even under nutrient-starvation conditions, diploid cells that transcribed RAI did not induce IME4 transcript. These cells showed defects in meiosis comparable to cells deleted for IME4. Furthermore, these cells formed hyper-filamentous colonies on nitrogen-starvation medium were adherent to agar—all phenotypes associated with IME4 deletion (figure 3).

Further in silico analysis revealed a putative Phd1 binding site upstream of RAI1. Although deletion of PHD1 partially relieved some of the RAI1-associated phenotypes (i.e., a larger percentage of cells were capable of going through meiosis and cells were less-agar adherent), Northern analysis revealed that it did not inhibit the antisense transcription against the IME4 locus (figure 4).

In an attempt to find other transcription factors that regulate RME2, we replaced the entire transcript with the URA3 open reading frame (figure 5A). Thus, cells that transcribe RME2 (i.e., a or α haploid cells) should be URA+. Although this was the case, we found that these cells were also resistant to 5-FOA\(^1\) (figure 5B, C). Although this would provide us with a method of screening both activators and inhibitors of RME2—i.e., by transforming in a high-copy genomic library and screening for clones that were either more URA+ than background (in the case of RME2 activators) or more URA- than background (i.e., FOA resistant—in the case of RME2 inhibitors), our screen, instead could not differentiate between background and real positives.

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\(^1\) 5-fluoroornotic acid—5-FOA—is a counter-selection for URA3 expression. Cells that do not express URA3 are able to grow on 5-FOA. Cells that express URA3 convert 5-FOA to a toxic intermediate and die.
**Figure 2.** Mutation of the Reb1 binding site results in *RAI1* transcription.

A) Mapping of 5’ and 3’ ends of *RAI1* by RACE (*Rapid Amplification of CDNA Ends*). Number of ends mapped ending on a particular nucleotide is represented on y-axis. *IME4* genetic locus is represented on the x-axis—*IME4* ATG begins at 0.

B) Northern analysis of antisense transcription at the *IME4* locus in haploid cells (*MAT a*, first three columns, *MAT α*, second three columns). Mutation of the a1/α2 binding site (labeled BamHl) does not affect transcription of *RME2*. However, mutation of the Reb1 binding site resulted in transcription of *RAI1* instead of *RME2*. Strains in the Northern follow: (L6437, L8257, SAy60, L6441, L6441, L8258, SAy64, L6445).
Figure 2

A

![Graph showing transcript ends and sequence numbers.](image)

B

![Image of gel with bands labeled RAI1 and RME2.](image)
Figure 3. Reb1 binding site mutants exhibit ime4Δ/Δ phenotypes.

A) Agar adhesion phenotypes of wt (SAy355), MAT a (SAy349), ime4Δ/IME4 (SAy443), ime4Δ/Δ (SAy446), ime4-Reb1/IME4 (SAy431), ime4-Reb1/ime4-Reb1 (SAy434). Plates were grown on YPD for 3 days (left), and were washed to test for agar adhesion (right).

B) Filamentous colony morphology of wt (SAy355), ime4-Reb1/ime4-Reb1 (SAy434) and ime4-Reb1/ime4-Reb1 (SAy434).
Figure 3

A

Wild type
IME4 Reb1 /IME4 Reb1
ime4/ime4

B
Figure 4. \textit{phd1}\textDelta/\Delta\text partially alleviates \textit{ime4-reb1}/\textit{ime4-reb1} defects.

A) Northern analysis for \textit{RAI1} in wt (SAy355), \textit{ime4-Reb1}/\textit{ime4-Reb1} (SAy434), \textit{phd1}\Delta/\Delta (SAy191) and \textit{phd1}\Delta/\Delta \textit{ime4-Reb1}/\textit{ime4-Reb1} (SAy197).

B) Agar adhesion phenotypes of wt (SAy355), \textit{MAT a} (SAy349), \textit{ime4}\textDelta/\Delta (SAy446), \textit{phd1}\Delta/\Delta (SAy191), \textit{phd1}\Delta/\Delta \textit{ime4-Reb1}/\textit{ime4-Reb1} (SAy197) and \textit{phd1}\Delta/\Delta \textit{ime4}\textDelta/\Delta (SAy331). Plates were grown on YPD for 3 days (left), and were washed to test for agar adhesion (right).
Figure 4

A

Probe:
Antisense IME4
ACT1

B
**Figure 5.** A reporter construct for *RME2*

A) Schematic for replacing *RME2*, the antisense transcript against *IME4* with the *URA3* open reading frame.

B) Growth of haploid constructs on complete (left), -Uracil (center) and +5-FOA(right) media. 1) MAT\(\alpha^{+}\) *URA3*, 2) MAT\(\alpha^{+}\) *URA3*, 3) MAT\(\alpha^{+}\) *ura3* (L6437), 4) MAT\(\alpha^{+}\) *ura3* (L6441) 5) MAT\(\alpha^{+}\) *rme2::P\text{rme2}::URA3* (SAy16) and 6) MAT\(\alpha^{+}\) *rme2::P\text{rme2}::URA3* (SAy19). Strains remain constant in all panels.

C) Growth of diploid constructs on complete (left), -Uracil (center) and +5-FOA(right) media. 1) MAT\(\alpha^{+}/\alpha^{+}\) *URA3/URA3*, 2) MAT\(\alpha^{+}/\alpha^{+}\) *URA3/ura3*, 3) MAT\(\alpha^{+}/\alpha^{+}\) *ura3/ura3* (L6445), 4) MAT\(\alpha^{+}/\alpha^{+}\) *ura3/ura3* *RME2/ rme2::P\text{rme2}::URA3* (SAy43), 5) MAT\(\alpha^{+}/\alpha^{+}\) *ura3/ura3* *rme2::P\text{rme2}::URA3/ rme2::P\text{rme2}::URA3* (SAy40). Strains remain constant in all panels.
Figure 5

A

B

C
It remains possible, however, that this screen could be carried out in diploid cells. We found that diploid cells are incapable of expressing \textit{rme2::URA3}, which is silenced by the diploid-specific $\alpha_1/\alpha_2$ protein heterodimer. Thus, mating of \textit{rme2::URA3} haploids with a library encoding high-copy plasmids of the yeast genome would result in diploid cells that may or may not express \textit{URA3}, depending on the plasmid expressed. This screen would result in identification of factors that are capable of overcoming \textit{MAT}-inhibition of \textit{RME2}, and may provide valuable cues as to the inhibition of mating type with meiotic initiation.
Appendix II: Specific Aspects of the Fungal Cell Wall Elicit an Adaptive-Immune-
Like Response in *Drosophila melanogaster*

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Abstract: Although the immune system of the fruit fly Drosophila melanogaster lacks a heretofore-identified lymphoid cellular lineage, we have determined that it has a form of immune memory. Pre-infecting adult D. melanogaster with non-lethal concentrations of Candida albicans protects against subsequent infection by the same pathogen. This Pre-Infective Protection (PIP) is stronger when the pre-infection consists of non-lethal concentrations of pathogen as compared to mock pre-infection or pre-infection with another fungus, Saccharomyces cerevisiae. Furthermore, PIP does not appear to result from hyper-elicitation of antimicrobial peptides. This immunological memory is specific for epitopes that are exposed on the fungal cell wall. Our data on PIP suggest that the immune system of the fruit fly is capable of adaptive-like responses to pathogens despite its lack of a lymphoid-like cellular lineage.
Introduction: Mechanisms of immune response in the fruit fly *Drosophila melanogaster* have important analogues in mammalian immunity. In response to infection by fungi or gram-positive bacteria, Toll, a transmembrane receptor in *D. melanogaster*, initiates a signaling cascade that affects transcription of a class of antimicrobial peptides (AMPs) (Fehlbaum 1994; Lemaitre 1996; Lemaitre 1997). These AMPs are thought to prevent progression of microbial pathogenesis (Fehlbaum 1994; Tzou 2002). Identification of Toll receptor’s function in *D. melanogaster* immunity led to the identification of mammalian counterparts, the Toll Like Receptors (TLRs) (Medzhitov 1997). In mammalian immunity, these TLRs mediate specific immune responses when stimulated by molecular signatures associated with particular classes of pathogens (reviewed in (Akira 2006)).

The ability to study immunological memory using infection of *D melanogaster* with *C. albicans* provides an informative and rapid assay both for the insect and fungal vector. Previous work by Pham, *et al.*, has demonstrated that the fungus *Benthamiana bassiana*, a known vector of *D. melanogaster*, can elicit immunological memory in fruit flies (Pham 2007). The ability to analyze immunological responses to *C. albicans*, a human fungal pathogen, in the model system *D. melanogaster*, offers a number of advantages: the genomes of both organisms have been sequenced, both are easily manipulated genetically and both have an abundance of mutants that can help to dissect the course of infection (Alarco 2004). Given the facile manipulation of both organisms, we tested whether *D. melanogaster* is capable of sustaining immunological memory against *C. albicans*. Here, we report the fruit fly’s ability to maintain memory against the human fungal pathogen. Furthermore, we find that components of the
fungal cell wall are important for eliciting this adaptive-immunity-like response in *D. melanogaster*.
Results & Discussion: A wild-type strain of Drosophila melanogaster (Oregon®) shows increased susceptibility to progressively higher concentrations of C. albicans. Injecting flies with a needle dipped in a low concentration (1e9 cell/mL) of C. albicans affects a mortality that is comparable to mock infections (figure 1A) or infections with a low concentration (1e9 cell/mL) of the non-pathogenic fungus S. cerevisiae (data not shown). Flies infected with the low concentration of C. albicans result in a fungal burden of approximately 16 +/- 10 cfu/fly, which is comparable to the fungal burden of flies infected with a low concentration of S. cerevisiae (35 +/- 14 cfu/fly). As expected, mock infections do not confer a fungal burden. The similarity between the mortalities conferred by the three different treatments suggests that this mortality is due to injury caused by surgery. Increasing the C. albicans fungal burden results in higher mortalities. Infections with a needle dipped in an intermediate concentration of C. albicans (5e9 cell/mL) cause significantly higher mortality, while infections with a needle dipped in a high concentration (1e10 cell/mL) caused a still higher mortality after four days. We quantified the fungal burden as 616 +/- 644 cfu/fly and 5586 +/- 4213 cfu/fly for intermediate and high concentrations, respectively. Notably, lethality in the fly population scales linearly as a function of fungal burden: the fungal burdens we tested with our concentrations of C. albicans reveal a dynamic range in which fly immune systems can negotiate pathogenesis.

C. albicans proliferates in several morphological forms: yeast cells, pseudohyphae and hyphal filaments; upon cell division each form can give rise to one of the others (Calderone 2002). The ability to switch between these forms is necessary for the course of C. albicans virulence in mammals and is controlled by diverse genetic and environmental cues ((Liu 1994; Koehler 1996; Lo 1997; De Bernardis 1998; Calderone 2002; Chamilos 2006) reviewed in (Brown 1999)).
Figure 1. *D. melanogaster* is sensitive to different concentrations of *C. albicans*. (a) Different concentrations of *C. albicans* caused different mortalities in *D. melanogaster*. Needles dipped in different concentrations of *C. albicans* were pricked into the dorsal thorax of seven-day-old males. Flies were collected five hours after treatment and mortality was observed for four days afterwards. (b) *dif*¹/*dif*² flies are more susceptible than wild-type *OreR* flies to the same concentrations of *C. albicans*. Error bars for (a) and (b) represent standard deviations for at least two independent infections of twenty flies each. (c) qPCR quantification of three antimicrobial peptides (*Drosomycin, Metchnikowin, Diptericin*) following either injury or infection with *C. albicans*. AMP profiles were assayed for wild-type and *dif*¹/*dif*² flies. Error bars represent standard deviation of six replicates spanning two separate infections of twenty flies each. (d) *C. albicans* form hyphae in the fruit fly model. Flies were infected with a prototrophic GFP-expressing strain of *C. albicans* SC5314 (SAY1). The thorax was crushed beneath a coverslide and visualized at various timepoints. Four hours after infection, flies were found to have both yeast form (solid arrow) and hyphal (dashed arrow) form cells.
FIGURE 1

A

Number flies surviving (of 20)

B

Number flies surviving (of 20)

C

antimicrobial mRNA levels

D

Image of experimental setup or observation.
C. albicans yeast-form cells develop hyphae after introduction into fruit flies as they do in mammalian cells (Koehler 1996). In order to visualize fungal morphology after infection, we constructed C. albicans strain that expressed GFP from a virulence-neutral locus in the prototrophic background, SC5314. This line, SAY1, has virulence in the fly model as well as a growth curve that is comparable to wild type C. albicans (supplemental figure 1). Morphologically, C. albicans forms the hyphal morphology that is necessary for its virulence in other model systems when infected into the fruit fly dorsal thorax (figure 1D). Both yeast- and hyphal-form cells were found in the abdomen and head (data not shown). These observations, as well as the correlation between fungal load and mortality suggest that differences in mortality affected by different doses of C. albicans in wild-type flies are due to the number of fungal cells that enter into the fruit fly upon infection.

Flies that are deficient in eliciting the antimicrobial peptide DROSOMYCIN are more susceptible to candidiasis than wild-type flies. The low, intermediate and high concentrations of C. albicans consistently cause greater mortality in dif\textsuperscript{1}/dif\textsuperscript{1} flies as compared to wild-type flies (figure 1B). Quantitative PCR of antimicrobial transcript levels reveal that these dif\textsuperscript{1}/dif\textsuperscript{1} flies are deficient in their induction of Drosomycin mRNA as compared to wild-type (figure 1C). These data suggest that Drosomycin is necessary for defending against infections with even our lowest concentrations of C. albicans.

We infected flies with either a mock infection, an infection with a low dose of C. albicans (1e9 cell/mL) or a low dose of S. cerevisiae (1e9 cell/mL). After an immunizing period of two days, we determined the ability of each group of flies to survive infection with a high dose of C. albicans. Flies that received a low dose of C. albicans (1e9 cell/mL) survive a subsequent
infection with a high dose of *C. albicans* (1e10 cell/mL) better than flies that received a mock pre-infection or a pre-infection with *S. cerevisiae* (figure 2A). Furthermore, an immunizing period of seven days results in stronger pre-infective protection (PIP) than that found at an immunizing period of two days (figure 2B). We used a seven-day immunizing period in subsequent assays of PIP, unless otherwise noted.

To uncover the antigenic specificity of PIP, we tested what alterations in the *C. albicans* cell affected PIP. The fungal cell wall, a major component in immune recognition of *C. albicans* in the *D. melanogaster* immune system, consists of an outer layer of mannoproteins covalently bound to an inner layer, the sugar glucan, a polymer of β-1,3- and β-1,6-glucan (Gottar 2006). The outer mannoprotein layer can mask the inner immunogenic molecules in the *C. albicans* cell wall (Wheeler 2006). To determine whether disruption of the outer mannoprotein layer affected PIP, we prepared dead *C. albicans* cells that had a generally intact cell wall and those that had disrupted the outer layer. UV-irradiation of *C. albicans* is lethal but does not disrupt the mannan outer layer, whereas heat inactivation of the fungus disrupts the mannan outer layer and reveals various molecules in the cell wall that elicit mammalian immune responses (Wheeler 2006).

To determine whether fungal cell wall components elicit PIP, we inoculated flies with either UV- or heat-inactivated *C. albicans* (1e10 cell/mL). Flies that were pre-infected with high doses of UV-inactivated *C. albicans* survived infection with *C. albicans* after a seven-day immunizing period to the same extent as flies that received mock pre-infections. Flies that were pre-infected with high doses of heat killed *C. albicans*, however, survived *C. albicans* infections much better than flies that had received mock pre-infections (figure 2C). Notably, the
Figure 2. **pre-infective protection (PIP) in D. melanogaster.** (a) Mortality in pre-infected wild-type *OreR* flies that were infected after a two-day immunization period. (b) Mortality in pre-infected wild-type flies that were infected after a seven-day immunization period. Flies in (a) and (b) were pre-infected with either *C. albicans*, strain SC5314 (*C. albicans*), *S. cerevisiae*, strain Σ1278b (*S. cerevisiae*), or were infected with a sterile needle (mock). (c) Mortality in wild-type *OreR* flies that were pre-infected with either heat-inactivated *C. albicans*, strain SC5314 (HKC. albicans) or UV-irradiated *C. albicans*, strain SC5314 (UVC. albicans). (d) Quantification of AMP mRNA profiles for uninfected (1d), mock (2d), live *C. albicans*, strain SC5314 (3d), heat-inactivated *C. albicans* (4d), and UV-irradiated *C. albicans* (5d) infected wild-type OreR flies, six hours after treatment. (e) Mortality in wild-type *OreR* flies that were pre-infected with UV-irradiated KAH3 strain (UVKAH3) a generous gift from C Abeijon, a kre5/kre5 constructed in the CAI-4 background (Herrero, 2004), or heat-inactivated *S. cerevisiae*, strain S288C (HKFY4/FY5). Error bars for (a), (b), (c) and (e) represent standard deviation for at least four independent infections of twenty flies each. Error bars for (d) represent standard deviations of four replicates spanning two separate infections of twenty flies each.
difference in this PIP is not due to differences in AMP transcript elicitation: the AMP elicitation profile of flies infected with a high dose of heat-inactivated *C. albicans* are comparable to the AMP elicitation profile of flies infected with a high dose of UV-inactivated *C. albicans* (figure 2D).

To test the role of cell wall components involved in PIP further, we pre-infected flies with a UV-inactivated *C. albicans* mutant strain with a disruption in cell wall architecture due to the homozygous deletion for *KRE5*. This strain has exposed fungal cell wall components under our growth conditions (Herrero 2004; Wheeler 2006). Flies that were pre-infected with the UV-inactivated *kre5/kre5* strain survived infections with *C. albicans* as well as flies that were pre-infected with heat-inactivated *C. albicans*. These data indicate that a compromised cell wall induces strong PIP (figure 2E) and are consistent with a model in which pre-infections serve as “vaccinations” against the infecting pathogen.

PIP is specific for the antigenic context of the immune elicitors. The *S. cerevisiae* cell wall has an organization of the mannan and glucan layers similar to that of *C. albicans* (reviewed in (Sohn 2006)). Thus, *S. cerevisiae* might have been expected to elicit PIP in subsequent *C. albicans* infections. However, flies that were pre-infected with heat-inactivated *S. cerevisiae* negotiated *C. albicans* infections as poorly as flies that received a mock pre-infection: heat-inactivated *S. cerevisiae* failed to induce a strong PIP response (figure 2E). Notably, although it has a similar cell wall architecture to *S. cerevisiae*, *C. albicans* cell walls have additional cell wall components that are not present in *S. cerevisiae* (Martínez-Esparza M 2006; Sohn 2006; Schaffer 2007). Our data suggest that the PIP response in the fruit fly immune system
differentiates between these subtle differences in cell wall architecture between the two species.

One explanation for the PIP response is that pre-infection leads to hyper-elicitation of AMPs. Previous work has shown that constitutive expression of a single AMP can increase immune defense against infections (Tzou 2002). Thus, as flies negotiate a pre-infection of a low dose of \textit{C. albicans} by eliciting AMPs through the \textit{Toll} pathway, these AMPs could remain systemically expressed and protect against future infections. This explanation has multiple predictions:

1) PIP should be abolished in flies that are deficient in AMP-elicitation.

2) Extension of the time between the pre-infection and infection beyond the point of systemic AMP expression should decrease PIP.

3) Flies that have increased systemic levels of AMPs should survive better than wild-type flies.

4) PIP should be non-specific for the infecting pathogen: a heterologous pre-infection should be able to elicit AMPs and thus protect against \textit{C. albicans}.

Our data are inconsistent with these predictions of the AMP hyper-elicitation model for PIP. First, \textit{dif}^{1}/\textit{dif}^{1} flies, which are deficient in their ability to induce \textit{Drosomycin}, still manifest PIP: \textit{dif}^{1}/\textit{dif}^{1} flies that are pre-infected with \textit{C. albicans} survive better than mock or \textit{S. cerevisiae} pre-infected flies when challenged with an intermediate dose of \textit{C. albicans} after an immunizing period of two days (figure 3A). As previous data demonstrated that the induction of \textit{Drosomycin} is necessary for appropriate protection against systemic candidiasis, the persistence of PIP in the \textit{dif}^{1}/\textit{dif}^{1} background argues that AMPs are not necessary for this effect.
Figure 3. Anti-microbial Peptide model is inconsistent with PIP. (a) $\text{dif}^+/\text{dif}^+$ flies that were infected after a two-day immunization period. Error bars represent standard deviation for at least two independent infections of twenty flies each. (b) Mortality in $\text{Tl}^{10b}$ flies that were infected with needles dipped in 1e9 cells/mL and 5e9 cells/mL, respectively. Flies in (a) and (b) were pre-infected with either $C.\text{albicans}$, strain SC5314 (C. albicans), $S.\text{cerevisiae}$, strain $\Sigma1278b$ (S. cerevisiae), or were infected with a sterile needle (mock).
FIGURE 3

A

B
Second, as described earlier, an immunizing period of seven days still results in strong PIP, arguably stronger PIP than that found at an immunizing period of two days (figure 2B). Previous studies have demonstrated that AMP elicitation after infection is transient at the mRNA level: the majority of AMP transcript levels return to wild-type levels after four to five days (Lemaitre 1997). If PIP were dependent on AMP hyper-elicitation, the protection that we observe should decrease significantly as a function of time possibly in proportion to the AMP transcript levels.

Third, flies with a constitutively active allele of Toll (Tl\textsuperscript{10b}) do not show increased resistance to \textit{C. albicans}. Previous studies have demonstrated that that \textit{Tl\textsuperscript{10b}+/+} flies have constitutively increased levels of AMP elicitation as compared to wild-type flies (Lemaitre 1996). If constitutively increased concentrations of AMPs protect against infections with \textit{C. albicans}, these flies should survive infections with \textit{C. albicans} better than wild-type flies. We find, however, that infection with different low and intermediate concentrations of \textit{C. albicans} causes a significantly higher level of mortality as compared to wild-type flies (figure 3B).

Fourth, pre-infections with both heat- and UV-inactivated \textit{C. albicans} induce the same AMPs to comparable levels (figure 2D), but only heat-inactivated \textit{C. albicans} is able to induce strong PIP against future infections (figure 2C). Taken together these experimental data provide strong evidence against AMP hyper-elicitation as the basis for the PIP effect.

The molecular basis of PIP may have important homologues in the innate immune system of organisms with a well-defined V(D)J-recombination-based mechanism for adaptive immunity. Elucidating these mechanisms in the easily manipulated innate immune system of \textit{D. melanogaster} with the well-characterized fungal pathogen \textit{C. albicans} may reveal an entire
level of immune recognition and defense that have been previously unexplored in human immune systems.
Materials & Methods:

_Drosophila_ stocks and infections. All fly stocks were maintained on standard cornmeal-molasses fly medium at 25°C. Oregon^R_ flies were a generous gift from Terry Orr-Weaver (Whitehead Institute), while dif^3/dif^4_ and Ti^{10b} flies were a generous gift from K.V. Anderson (Sloan-Kettering Institute) and D. Schneider (Stanford University). 7-day-old adult males were injected with a sterile needle dipped in the respective fungal concentrations as described in (Lemaitre 1996). Five hours after injections, surviving flies were maintained at 30°C and monitored for survival (20 flies per experimental group).

Fungal growth and preparation. Fungal strains were grown at 30°C in yeast peptone dextrose (YPD) medium to a density of 1.5-2.5x10^8 cell/mL (C. albicans) or 0.5-1x10^8 cell/mL (S. cerevisiae) as quantified by hemacytometer. Appropriate numbers of cells were washed once in water then resuspended in 100μL of ddH2O. Strain information is noted in figure legends. Heat-inactivation and UV-irradiation were performed as in (Wheeler 2006).

Strain construction. Plasmid pENO1GFP3 (generous gift from J. Staab and P. Sundstrom of Dartmouth University) was digested with KpnI and ligated with KpnI, PstI digest of PCR product from ENO1 locus and KpnI, PstI-released Nat^r_ cassette from pJK795 (generous gift from J. Koehler, Children’s Hospital, Harvard). The resultant plasmid, SAb5, was digested with EcoRV and transformed into C. albicans strain SC5314. Nourseothricin-resistant colonies were tested by PCR for correct integration into the ENO1 locus. Primer sequences are available upon request.

Quantitative PCR analysis. Real-time PCR analysis was conducted using primers against antimicrobial peptide mRNA sequences. Total RNA was isolated from male flies six hours post-
infection (20 flies per treatment) following TRIzol protocol described in (Bogart 2005). RNA was
DNase-treated according to RNeasy mini-prep protocols from Qiagen and 1 μg of RNA was
reverse transcribed using random hexamer primers according to Applied Biosystems's
AmpliGold Superscript reverse-transcription kit. Quantitative PCR reactions employed Applied
Biosystems's SYBR green 2X MasterMix, and amplification was performed using a 7500 ABI
Real-Time PCR machine. All results were normalized to RP49 levels according to Applied
Biosystems' RT-PCR data analysis software. Individual primer sequences are available upon
request.

**FACS.** Fungal strains were grown to mid-log at 30°C in YPD or YPD+100μg/μL filter-sterilized
CloNat. Cells were then washed in PBS and GFP fluorescence was quantified on a FACScalibur
cytometer (Becton-Dickinson, Palo Alto, California, United States).
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References


Schaffer, T., S Müller, B Flogerzi, B Seibold-Schmid, AM Schoepfer, F Seibold (2007). "Anti-Saccharomyces cerevisiae mannan antibodies (ASCA) of Crohn's patients crossreact with mannan from other yeast strains, and murine ASCA IgM can be experimentally induced with Candida albicans." *Inflammatory Bowel Diseases*.


Supplemental Materials

Supplementary figure 1. SAY1 is a Nourseothricin-selectable GFP-positive strain constructed in the SC5314 background of C. albicans. (a) SAY1, either grown in the presence (red square) or absence (blue triangles) of Nourseothricin (NAT). (b) Histogram representing flow-cytometry assay. Fluorescence (x-axis) is plotted against cell numbers (y-axis) for SC5314 (purple), SAY1 grown in the presence of Nourseothricin (green) and SAY1 grown in the absence of Nourseothricin (magenta). (c) SAY1 fluorescence as compared to SC5314. (d) SAY1 and SC5314 show similar mortalities in the Drosophila model (P<0.1, unpaired Student’s T-test). Error bars represent standard deviation of at least five independent infections of twenty flies each.
SUPPLEMENTARY FIGURE 1

A. Growth Curves SAY1 and SC5314 vs. time

B. Death Curves

C. Bright field and fluorescence images for SC5314 and SAY1

D. Death Curves