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Fabrizio Simonetti

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Structure et dynamique des systèmes vivants

Spécialité de doctorat : Sciences de la vie et de la santé

Par

M. Fabrizio Simonetti

Study of the mechanisms of sexual differentiation
in the fission yeast *S. pombe*

Thèse présentée et soutenue à Paris, le 7 Avril 2017

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*To my mother, my father, my brother
and all my family*

SUMMARY

In the fission yeast *S. pombe*, several meiotic genes are constitutively expressed during the mitotic cell cycle. In order to avoid untimely entry into meiosis, cells have adopted a degradation system that selectively eliminates the corresponding mRNAs. The YTH family RNA-binding protein Mmi1 recognizes specific sequence motifs within these transcripts (UNAAAC), and delivers them to the nuclear exosome for degradation. Upon entry into meiosis, Mmi1 is sequestered in a ribonucleoprotein complex made of the meiotic protein Mei2 and the long non-coding RNA meiRNA, thereby allowing meiotic mRNAs to be exported and translated. During my PhD studies, I focused my work on the role of Mmi1 in the degradation of meiotic transcripts during vegetative growth. Consistent with recent studies, we showed that Mmi1 stably interacts with the mRNA deadenylation complex Ccr4-Not. This interaction is functionally relevant because Ccr4-Not is required for the degradation of meiotic mRNAs. Surprisingly, however, the deadenylation activity of the complex is not involved. Rather, our genetic and biochemical analyses indicate that the E3 ubiquitin ligase subunit Mot2 ubiquitinates a pool of the Mmi1 inhibitor, Mei2, to promote its degradation by the proteasome. This regulatory mechanism ensures the maintenance of Mmi1 in a functional state, leading to the persistent repression of meiotic mRNAs in mitotic cells. Thus, Mmi1 has a dual role: in nuclear mRNA surveillance, by targeting meiotic transcripts for degradation by the exosome, and in protein degradation, by recruiting Ccr4-Not to its own inhibitor Mei2. These results have also revealed a novel role for the ubiquitin ligase activity of the Ccr4-Not subunit Mot2 in the control of sexual differentiation in fission yeast.

Further experiments indicate that the YTH RNA-binding domain of Mmi1, but not the non-coding RNA meiRNA, is required for the degradation of Mei2. Intriguingly, our results support the notion that the YTH domain of Mmi1 mediates the interaction with Mei2. This

Summary

strongly suggests that the YTH domain acts as a bifunctional module, binding not only to meiotic RNAs but also to proteins. We discuss these results within the context of the current literature and we propose a model for the control of sexual differentiation by the Mmi1-Mei2 system.

RÉSUMÉ

Chez la levure fissipare *S. pombe*, certains gènes méiotiques sont exprimés de façon constitutive pendant la croissance végétative. Cependant, pour empêcher le déclenchement prématuré de la méiose, la cellule a mis en place un système de dégradation sélective des ARN messagers correspondant. La protéine de liaison à l'ARN Mmi1, de la famille YTH, reconnaît des répétitions de motifs spécifiques (UNAAAC) au sein des transcrits et dirige ces derniers vers la dégradation par l'exosome nucléaire. Lors de l'entrée en méiose, Mmi1 est séquestré par un complexe ribonucléoprotéique comprenant la protéine de méiose Mei2 et l'ARN noncodant meiRNA, ce qui permet aux ARNm méiotiques d'être exportés et traduits.

Au cours de ma thèse, je me suis intéressé au rôle de Mmi1 dans la dégradation des transcrits méiotiques pendant la croissance végétative. En accord avec des études récentes, nos travaux montrent que Mmi1 interagit étroitement avec le complexe Ccr4-Not de déadénylation des ARNm. Cette interaction est fonctionnelle car Ccr4-Not est requis pour la dégradation des ARNs méiotiques. De façon surprenante, cependant, l'activité de déadénylation n'est pas requise. Nos analyses génétiques et biochimiques suggèrent que la sous-unité E3 ubiquitin ligase Mot2 de Ccr4-Not ubiquitine un pool de l'inhibiteur de Mmi1, la protéine Mei2, pour faciliter sa dégradation par le protéasome. Cette voie de régulation permet de maintenir la fonction de Mmi1 et donc la répression des ARNm méiotiques dans les cellules mitotiques. Ainsi, Mmi1 a une double fonction: cibler les ARNm méiotiques vers la dégradation par l'exosome nucléaire, et recruter Ccr4-Not pour ubiquitiner et dégrader son propre inhibiteur Mei2. Ces résultats mettent également en avant un nouveau rôle pour la sous-unité E3 ligase du complexe Ccr4-Not dans le contrôle de la différenciation sexuelle.

Des expériences supplémentaires indiquent que le domaine YTH de liaison à l'ARN de Mmi1, mais pas l'ARN noncodant meiRNA, est requis pour la dégradation de Mei2. De façon

Summary

importante, nos données révèlent aussi que le domaine YTH de Mmi1 a un rôle clé dans l'interaction avec Mei2. Ceci suggère fortement que le domaine YTH agit comme un module bifonctionnel, permettant la liaison non seulement aux ARNs méiotiques mais aussi aux protéines comme Mei2. Nous discutons ces résultats dans le contexte de la littérature actuelle et proposons un nouveau modèle du contrôle de la différenciation sexuelle par le système Mmi1-Mei2.

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INTRODUCTION

1 General principles of sexual differentiation in yeast

In eukaryotes, sexual differentiation is a process that relies on the conversion of external signals into a stable phenotype, through temporary changes in the expression of genes. The events occurring during this process are critical for the generation of diversity and for the production of a normal offspring. In nearly all eukaryotes, meiosis has a central role in sexual reproduction and is a highly-conserved process, from fungi to plants and animals. Meiosis consists in the specialized division that halves the genetic material, reducing the diploid genome of the progenitor cell to the haploid state and producing genetically different daughter cells. The haploid products, also called gametes, are oocytes and sperm in animals, pollen in plants, and spores in yeasts.

Over the last decades, great advancements in the study of meiosis came from a small number of model organisms, including the fission yeast *Schizosaccharomyces pombe*. The employment of fission yeast as a model system allowed a better understanding of the mechanisms that govern sexual differentiation. *S. pombe* is an ideal organism to study the meiotic process for several reasons. For instance, almost all yeast cells in a population, when starved for nutrients, enter meiosis in a synchronous manner. This allows temporal analyses using cytological, biochemical and molecular assays. Yeast meiotic mutants are easy to isolate, and it is possible to rapidly clone and disrupt the corresponding genes.

The use of fission yeast as tool allowed elucidating to a great extent how cells enter and proceed through meiosis.

In fission yeast the initiation of meiosis relies on a decrease of available nutrients in the environment. This event induces yeast haploid cells of opposite mating types to mate and conjugate, forming a zygote that enters the meiotic cell cycle. One round of DNA replication and two meiotic divisions give rise to asci, which contain four haploid spores. Each of these

spores can eventually re-enter the mitotic cell cycle in the presence of nutrients (**Fig. 1**). By analogy, yeast haploid cells behave similarly to gametes in higher organisms, such as oocytes, sperm or pollen.

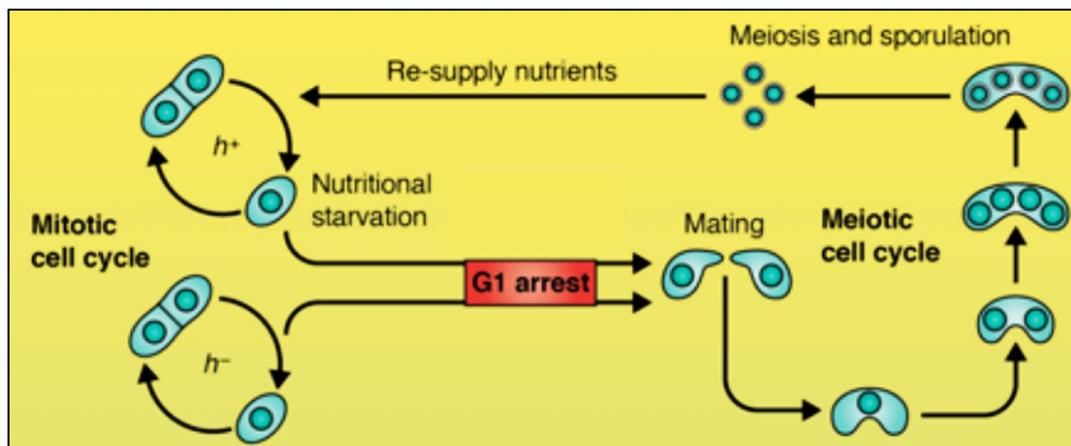


Figure 1. Scheme of the life cycle of the fission yeast *S. pombe*.

Upon nutritional starvation, haploid cells arrest mitotic growth and initiate the mating process. Cells of the M and P mating types conjugate to form a zygote. The diploid zygote then undergoes the meiotic process, generating four ascospores. If nutrients are supplied in the medium the spore can germinate giving rise to a haploid cell. Figure adapted from Otsubo and Yamamoto [1].

Particularly, in *S. pombe*, the cell mating type (h^+ or h^-) is determined by the sequence at the *mat1* locus, which can be either P (*mat1-P* for h^+ cells) or M (*mat1-M* for h^- cells) [2]. h^+ cells synthesize the mating pheromone P-factor as well as the M-factor receptor, whereas h^- cells produce the M-factor and the P-factor receptor. P and M mating pheromones are small peptides that bind to their cognate receptor anchored in the plasma membrane of cells with opposite mating type, thereby promoting mating and conjugation [3, 4]. Upon mating, cells arrest transiently in G1 and undergo one round of DNA replication known as pre-meiotic S phase, in which the DNA content of each cell is doubled. The nuclei of the two progenitor cells then fuse, in a process called karyogamy, and undergo meiotic prophase, which features a so-called ‘horse tail’ stage, because of the peculiar shape of the nucleus. During this stage the

continuous movement of the nucleus, mediated by the spindle body (SPB), favors chromosome pairing, which is essential for the following event of chromosome segregation.

Cells then perform two consecutive nuclear divisions, which are known as meiosis I and meiosis II. During meiosis I, called reductional division, replicated homologous chromosomes align at the equator of the cell and undergo high-frequency meiotic recombination. Then, the first nuclear division occurs and homologous chromosomes segregate, producing two daughter cells with halved genetic material [5-7]. Meiosis II instead is described as an equational division; it resembles the normal mitotic division where sister chromatids are separated. This division produces four genetically different cells, each carrying a haploid content of DNA, which are then packed in order to form mature spores (**Fig. 2**).

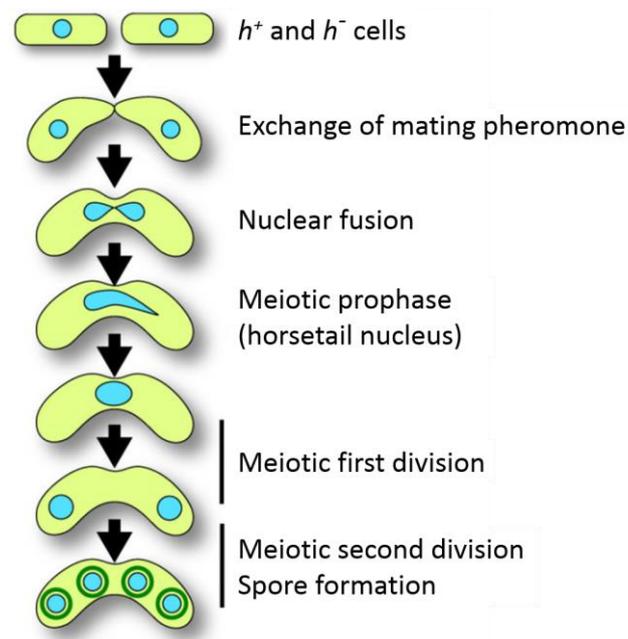


Figure 2. The meiotic process in fission yeast.

Upon nutritional starvation, h^+ and h^- haploid cells arrest in G1 and secrete the respective mating pheromone. Upon exchanging the pheromones, cells elongate towards each other and fuse (conjugation process), followed by the fusion of the haploid nuclei. The diploid nucleus elongates and assumes a horsetail shape, then moves from one end of the cell to the other (meiotic prophase). After the horsetail nucleus ceases to move, it becomes round again and proceeds through the first and second meiotic divisions to form four haploid nuclei. Figure adapted from Asakawa [8].

The meiotic process is characterized by a complex sequence of events that irreversibly alter cell morphology and growth capacities in response to specific developmental and/or environmental cues. In order to prevent the initiation of sexual differentiation in conditions suitable for vegetative growth, mitotic cells have evolved several regulatory mechanisms that remain largely uncharacterized. Indeed, the molecular details underpinning the decision of a cell to switch from mitosis to meiosis are far from being fully understood.

During my PhD studies, I focused my work on the molecular mechanisms involved in the transition from mitosis to meiosis in fission yeast. In the next sections, I will describe the several layers of the regulation of meiosis in *S. pombe*, ranging from gene transcription to protein modification. First, I will give an overview on how environmental cues trigger and establish the meiotic gene expression program, highlighting the main molecular determinants at play. In a second part, I will introduce the factors involved in the control of meiosis initiation and their associated function. Given the results obtained from my work, particular attention will be devoted to the post-transcriptional mechanisms involved in sexual differentiation.

2 The meiotic program: from external signals to transcriptional regulation

Specific environmental and/or developmental cues trigger the entry into meiosis, including nutrient starvation and the synthesis of mating pheromones. These external signals are integrated by the cells, which modify and adapt their gene expression profiles to drive sexual differentiation. The expression of hundreds of meiosis-specific genes is indeed induced in a coordinated manner, thanks to several transcription factors that are themselves activated sequentially. This temporal regulation is essential for the correct execution of the different steps of the meiotic program.

In this section, I briefly summarize the current knowledge concerning the mechanisms that link the sensing of environmental signals to the activation of a complex transcriptional cascade, which ultimately leads to cell differentiation in fission yeast.

2.1 Signaling cascades

Fission yeast cells respond to environmental stimuli (e.g. nutrients, stresses, mating pheromones) and activate specific signaling pathways to initiate the meiotic program. Four transduction cascades, involved in the control of sexual differentiation, have been characterized in fission yeast. Their activation relies on different input signals, including carbon and nitrogen sources, mating pheromones and stress stimuli. These signaling pathways, which I describe succinctly below, all converge to the activation of a master regulator of meiosis, the transcription factor Ste11.

2.1.1 The cAMP pathway

In an environment rich in carbon, which is favorable to vegetative growth, intracellular ATP is converted into cAMP, a key messenger molecule that represses sexual differentiation [9]. High concentrations of cAMP results in the inhibition of the Cgs1 protein and thereby relieve the downregulation of the protein kinase PKA, which in turn phosphorylates and inactivates the transcription factor Rst2 (**Fig. 3**) [10, 11]. As a consequence, expression of the *ste11+* gene is not induced, thereby maintaining cells in the mitotic cell cycle.

Instead, upon depletion of the carbon source in the environment, intracellular cAMP levels rapidly decrease and prevent the activation of PKA. This allows Rst2 to escape inhibition and to induce expression of *ste11+*, which promotes sexual differentiation (**Fig. 3**) [12].

2.1.2 The TORC1 pathway

Nitrogen starvation is a major determinant of sexual differentiation in fission yeast. The signaling pathway that responds to nitrogen availability involves a TOR (Target Of Rapamycin) family protein kinase (**Fig. 3**). Conserved in all eukaryotes, TOR kinases are key regulators of cell growth that modulate gene transcription, protein synthesis and degradation to adapt to environmental changes (reviewed in Loewith and Hall [13]).

S. pombe encodes two TOR homologues, *tor1* and *tor2*, that incorporate in distinct complexes, TORC2 and TORC1, respectively (reviewed in Loewith and Hall [13]). Remarkably, mutations of *tor2+*, but not *tor1+*, mimic nitrogen starvation by initiating sexual development regardless of the nutritional conditions [14, 15]. This is accompanied by an increased expression of *ste11+* and its target genes, indicating that Tor2 normally functions to repress sexual differentiation [15, 16]. Interestingly, Tor2 forms a complex with Ste11, although the biological relevance of this association remains unclear. Whether Tor2 directly phosphorylates to inhibit Ste11, prevents its nuclear accumulation and/or regulate its

transcriptional regulators requires further investigation. More recently, Tor2 was shown to phosphorylate the RNA-binding protein Mei2, another key meiosis inducer, to stimulate its degradation by the proteasome and therefore prevent untimely entry into meiosis ([17]; see next section). Thus, Tor2 is a central effector that functions to maintain robust vegetative growth and inhibit sexual differentiation. Depletion of nitrogen causes the inactivation of Tor2, thereby allowing initiation of the meiotic program.

2.1.3 The mating pheromone-responsive pathway

Upon meiotic conditions, fission yeast cells of either P or M mating type, produce and secrete the mating pheromones (P-factor and M-factor) that will bind to their cognate receptor on the cell membrane [3, 4]. This event allows cells of the opposing mating type to conjugate and it triggers a MAPK (Mitogen Activating Protein Kinase) signaling cascade that leads to the phosphorylation and activation of Ste11 by the Spk1 kinase (**Fig. 3**) [18-20]. Importantly, Ste11 itself is required for the induction of mating-type specific genes, including the mating pheromones and their receptors [21]. This reinforces the commitment of cells in sexual differentiation and ensures that the process is irreversible.

2.1.4 The stress-responsive pathway

Other stimuli, such as heat shock and oxidative stress, activate a fourth signaling pathway that controls *ste11+* expression [22, 23]. The final effector of this pathway is the MAPK Sty1, which accumulates in the nucleus and phosphorylates several targets, including the CREB family transcription factors Atf1 and Pcr1 [24]. These latter are both required for *ste11+* expression (**Fig. 3**) [23, 25, 26], although it is unclear whether they directly control its transcription. Interestingly, Sty1 is also required for the activation of Lsk1, a protein kinase

that phosphorylates the Ser2 within the Carboxy Terminal Domain (CTD) of the Rpb1 subunit of RNAPII, a prerequisite for the correct expression of *ste11+* [27, 28].

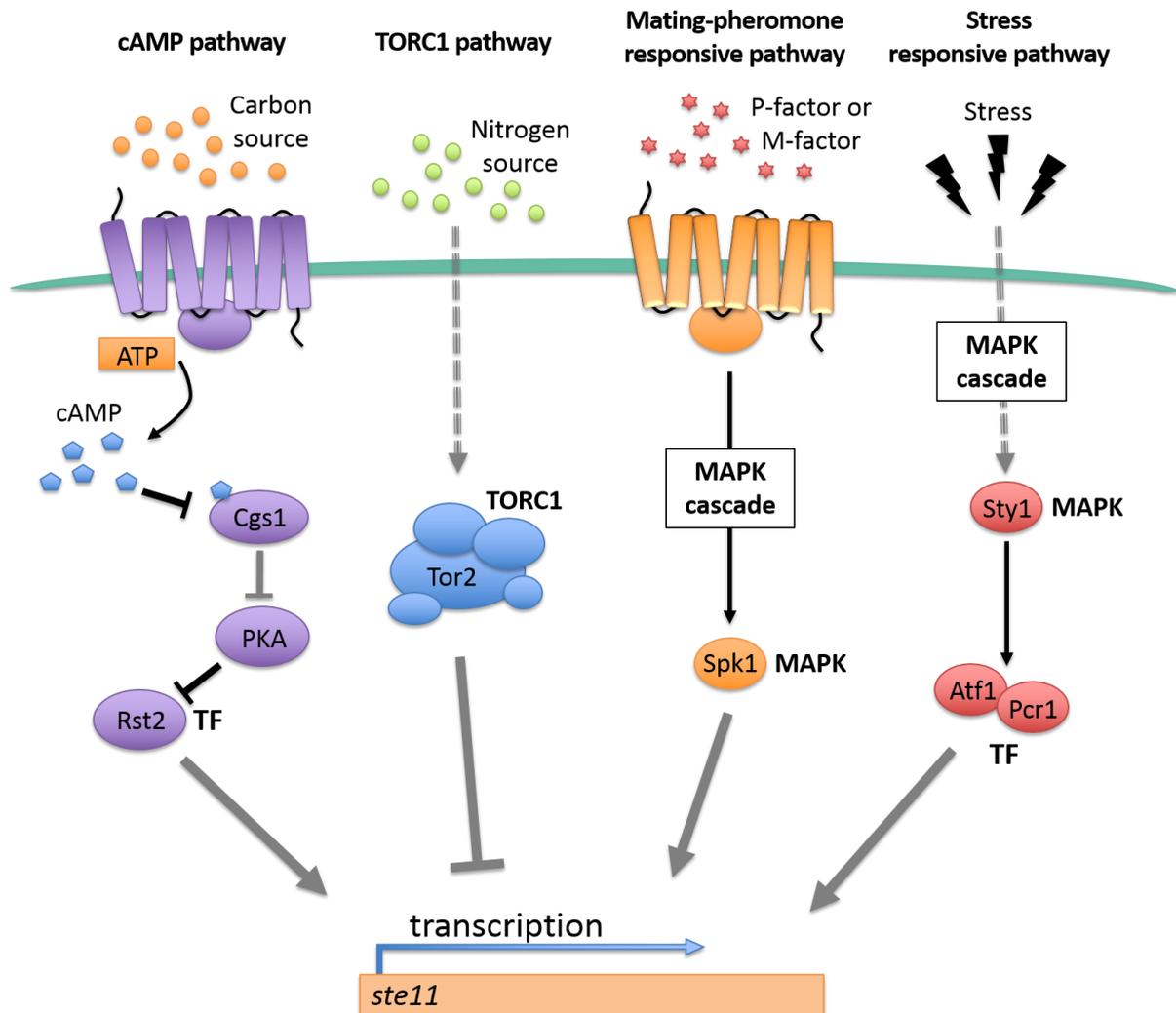


Figure 3. Scheme of the signaling pathways which control meiosis onset in *S. pombe*.

In fission yeast, four pathways mediate the transduction of external signals to the activation of *ste11+* expression. (MAPK: MAP kinase; TF: Transcription factor). Figure adapted from Yamamoto [29].

2.2 The transcription factor Ste11

2.2.1 General properties

ste11+, which belongs to a group of genes whose mutations provoke meiotic deficiency and complete sterility, encodes a major transcription factor required for sexual differentiation [21, 30]. Supporting this notion, ectopic induction of *ste11+* triggers uncontrolled mating and sporulation, irrespective of nutritional conditions [21].

Ste11 is 486 amino acids in length and contains a HMG (high mobility group) domain at its N-terminus that facilitates protein-DNA interactions [31, 32]. It is responsible for inducing the expression of genes required for mating and meiosis, which feature one or several 10-base motifs in their 5'-upstream region (TTCTTTGTTY), called the TR boxes. The selective binding of Ste11 to this cis-regulatory motif is essential for its proper function [21, 33]. Intriguingly, the *ste11+* gene itself harbors a TR box [34], which likely serves to reinforce its own expression and cell fate decision.

Ste11 controls the expression of approximately 80 genes, the majority of which is induced in cells of both mating types [33]. These include the transcription factor Rep1, involved in pre-meiotic S phase [35], Dhc1, a dynein protein necessary for nuclear movement during meiotic prophase [36] and Tht1, required for karyogamy [37]. Other targets encode positive (Mei2, meiRNA) and negative (Pat1) regulators of sexual differentiation, suggesting the existence of complex feedback mechanisms [33] (see next section). Ste11 also promotes the expression of mating type specific genes through its association with either Mat1-Pc or Mat1-Mc, which encode cell type specific transcription factors that assist Ste11 in the binding to suboptimal TR boxes [38].

2.2.2 Regulatory mechanisms of *ste11+* expression

2.2.2.1 Transcriptional control

Given the fundamental importance of Ste11 in sexual development, its expression must be tightly regulated to avoid ectopic induction of meiotic genes during vegetative growth and, conversely, to induce the meiotic program upon nutritional starvation. Interestingly, the *ste11+* gene displays an unusually long 5' UTR of more than 2 kb that comprises several binding sites for various transcription regulators. One of these is the zinc-finger protein Rst2, which recognizes a stress response (STRE) element (i.e. 5'-CCCCTC-3' motif) within the promoter of *ste11+* [34]. Rst2 relays the nutritional status to the expression of *ste11+* via the cAMP pathway [34, 39]. Ste11 itself promotes the transcription of its own gene by binding to a TR box in its promoter [34]. The GATA protein Gaf1 instead represses *ste11+* expression by binding a canonical GATA motif 5'-CTATCT-3' in the promoter, although the precise mechanism at play remains unknown [40].

Interestingly, the conserved coactivator SAGA (Spt-Ada-Gcn5-acetyltransferase) complex also regulates the expression of *ste11+* through the opposing roles of its Gcn5 and Spt8 subunits [41]. It was suggested that the complex, which is recruited by Rst2, fine-tunes the levels of Ste11 in response to cellular signals, although the mechanistic basis of this regulation is still unclear.

As previously mentioned, phosphorylation on Ser2 (S2P) of RNAPII CTD responds to cellular signaling and it is important for proper *ste11+* expression [27, 28]. Generally, the phosphorylation pattern of the RNAPII CTD has a key role in recruiting different factors involved in transcriptional regulation [42]. Accordingly, it has been recently shown that the presence of S2P, which depends on the activity of the Lsk1 kinase, activates *ste11+* expression by hampering the recruitment of the histone methyltransferase Set1, which in turn recruits

histone deacetylases (HDACs) that negatively regulate transcription of *ste11+* [43]. Moreover, it has been shown that the transcriptional activation of *ste11+* also depends on the deubiquitynase Ubp8, which regulates the ubiquitination status of histone H2B to limit the recruitment of Set1 to the *ste11+* promoter [44]. Thus, phosphorylation of S2 on the RNAPII CTD and removal of ubiquitin from H2B act in parallel to impair the Set1-dependent recruitment of HDACs and in turn promote transcriptional induction of the *ste11+* gene.

2.2.2.2 Post-transcriptional control

Beside its transcriptional regulation, *ste11+* is also regulated at the protein level. Upon nutritional starvation, the mating-pheromone pathway activates a signaling cascade and the MAPK Spk1 binds and phosphorylates Ste11 on Thr³⁰⁵ and Thr³¹⁷, triggering its activation [18, 19]. In mitotic cells, the Pat1 protein kinase represses the sexual differentiation program. Indeed, Pat1 phosphorylates directly Ste11 on Thr¹⁷³ and Ser²¹⁸, inhibiting it in two ways: (1) allowing binding of the 14-3-3 protein Rad24 to Ste11, hampering its nuclear accumulation and therefore its role in transcription activation [45], and (2) promoting the ubiquitination and subsequent degradation of the Ste11 protein [46].

Moreover, the Tor pathway, also affects Ste11 at the protein level. Indeed, Tor2 is able to associate with Ste11 and the latter accumulates in the nucleus when the Tor2-containing TORC1 complex is inactivated [14, 15]. It has been speculated that Tor2 might directly phosphorylate Ste11, affecting its nucleocytoplasmic shuttling, although direct evidence is currently lacking [16].

2.3 The meiotic transcriptional program

Following the activation of Ste11, the meiotic program is initiated and leads to profound modifications in gene expression profiles. Hundreds of genes are indeed activated in successive transcriptional waves that correlate with the different phases of meiosis: starvation or pheromone-induced genes, pre-meiotic S-phase and recombination (early meiosis), nuclear divisions (middle meiosis) and spore formation (late meiosis) [47, 48]. Each group of genes is associated with specific promoter motifs and contains defined transcription factors responsible for the stepwise activation of the meiotic program (**Fig. 4**) [47, 48]. Below are summarized the main regulators of meiotic transcription waves:

- Ste11 regulates the genes involved in the response to nutritional changes (e.g. Rep1) (see above). It activates the pheromone communication system and the entry into meiosis. Ste11 also promotes its own expression, therefore reinforcing the commitment of the cell in sexual differentiation.
- The zinc finger transcription factor Rep1 induces genes required for early meiosis, including those involved in premeiotic DNA synthesis and recombination [35, 49, 50]. However, about half of early genes do not depend on Rep1 for proper induction [48], suggesting that additional transcription factors may also contribute to their activation.
- The forkhead transcription factor Mei4 induces the transcription of middle genes and is essential to complete meiosis I [51]. Mei4 binds to the FLEX motif present in the promoter of its target genes via its forkhead domain and stimulates their expression. Interestingly, the *mei4+* gene also contains a FLEX sequence in its promoter, allowing autoregulation through a positive feedback loop [52].

- The bZIP transcription factors Atf21 and Atf31 mediate the transcription of more than half of the late meiosis and sporulation genes in a sequence-dependent manner [23, 47, 48, 53] and are essential to complete the meiotic program. Though, Atf21 and Atf31 do not regulate all late genes and additional factors also contribute to their expression, including the zinc finger protein Rsv2 [48].

The succession of the transcriptional waves ensures that the main biological events occurring during meiosis are tightly coordinated over time. To reinforce the progression through the meiotic cell cycle [48], transcription factors not only activate the expression of transcription regulators involved in the next wave but also switch off the genes from the previous wave. For example, Mei4 induces middle genes and represses some Rep1-dependent early genes. Likewise, Rep1 negatively impacts expression of genes involved in response to nutrient starvation and pheromone signaling (**Fig. 4**). However, the mechanisms at play are likely indirect and additional work is needed to better understand the intricate coordination of the meiotic transcriptional program.

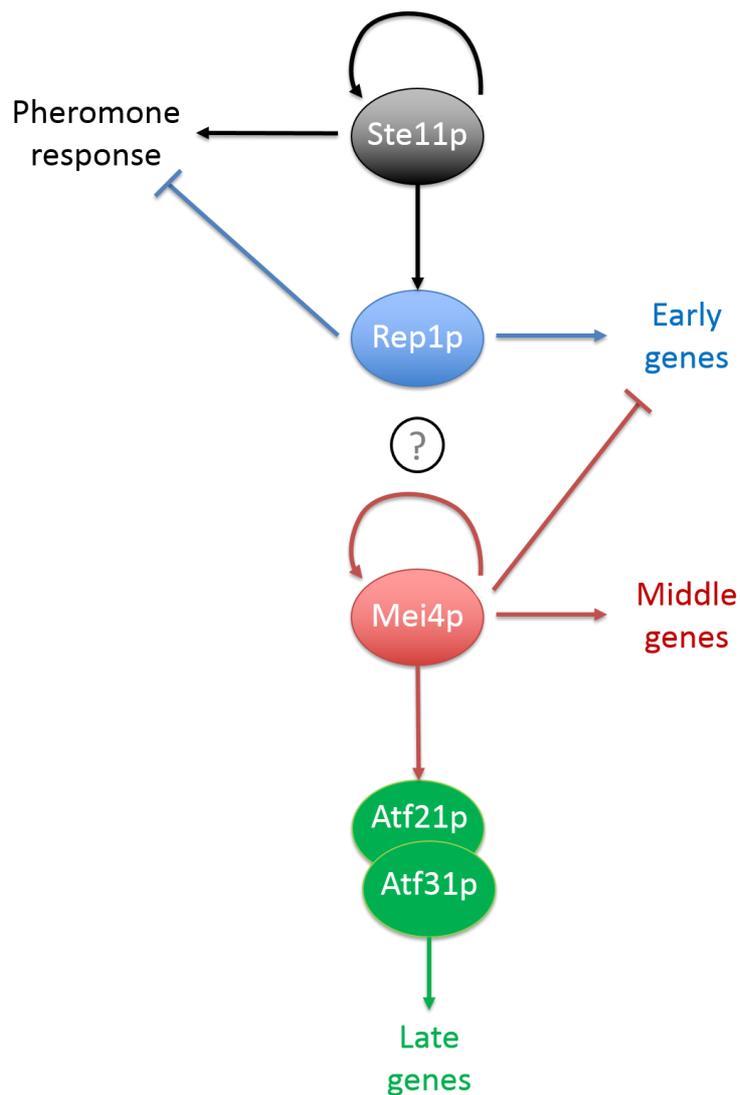


Figure 4. Transcriptional regulatory network regulating meiosis and sporulation.

The colors correspond to different phases of sexual differentiation. Arrows indicate induction and cross bars indicate repression. Mei4 stimulates its own production through a positive feedback loop, but no specific factor was shown to directly control its transcription. Rather, expression of *mei4+* seems to be primarily regulated at the post-transcriptional level [54] (see next section). Figure adapted from Mata [48].

3 Regulatory mechanisms of sexual differentiation

Given the profound changes in gene expression, the meiotic process could be extremely deleterious for the cell if executed in the inappropriate cell cycle context. Cells have therefore evolved regulatory mechanisms that go beyond the transcriptional control of meiotic genes, to avoid untimely entry into meiosis. These regulatory pathways act at the post-transcriptional and post-translational levels to ensure a precise and proper switch from the mitotic to the meiotic cell cycle. Here I will describe the molecular pathways implicated in the mitosis-meiosis decision in fission yeast.

3.1 The Pat1-Mei2 system: a central regulator of meiosis

3.1.1 The Pat1 kinase inhibits entry into meiosis

Three decades ago, the *pat1+* gene was isolated from a mutant undergoing meiosis even in the presence of a nitrogen source and in a haploid state [55, 56]. These observations led to propose that *pat1* is a factor preventing sexual differentiation in nutrient-rich conditions.

The essential *pat1+* gene encodes for a protein kinase for which two substrates have been identified so far. The first one is Ste11 (see previous section), a key HMG domain transcription factor involved in the expression of genes needed for mating and conjugation [35, 57, 58]. The second one is the RNA-binding protein Mei2, a major meiosis inducer [46, 59] (see below).

In nutrient-rich conditions, Pat1 phosphorylates Ste11 at two sites (Thr173 and Ser218) [60], allowing its association with the 14-3-3 family protein Rad24 which in turn inhibits Ste11 activity by preventing its nuclear accumulation [45]. These phosphorylation events have also been suggested to reinforce the ubiquitin-dependent degradation of Ste11, although direct evidence for this is currently missing [46]. Pat1 also phosphorylates Mei2 on two amino acid

residues, Ser438 and Thr527, both *in vitro* and *in vivo* [61]. Similarly to Ste11, phosphorylated forms of Mei2 are bound by Rad24, which inhibits its function [46]. Further analyses showed that phosphorylation of Mei2 is a prerequisite for its rapid turnover by the ubiquitin and proteasome-dependent pathway involving the E2 ubiquitin-conjugating enzyme Ubc2 and possibly the E3 ubiquitin ligase Ubr1 [46]. Remarkably, expression of a non-phosphorylatable form of Mei2 (i.e. the Mei2-SATA mutant protein where serine and threonine residues targeted by Pat1 are substituted by alanines) triggers ectopic entry into meiosis, similarly to the inactivation of Pat1 [29, 61]. Based on these observations, it has been proposed that phosphorylation of Ste11 and Mei2 by Pat1 during the mitotic cell cycle is essential to ensure robust vegetative growth and avoid untimely entry into meiosis.

Upon nutritional starvation, the *ste11+* gene is transcriptionally activated via the downregulation of the cAMP-dependent kinase (PKA) [9, 21, 62]. In turn, Ste11 induces several genes involved in mating and meiosis, including mating type genes, genes encoding mating pheromones and their receptors, and *mei2+* [21]. The binding of mating pheromones to their receptors results in the activation of a MAPK cascade that promotes the induction of mating genes required for the mating process and the formation of a zygote. In the zygote, h⁺-specific Mat1-Pi and h⁻-specific Mat1-Mc cooperate to induce the expression of the *mei3+* gene, whose product inhibits Pat1 [60, 63]. The *mei3+* gene indeed encodes a peptide that functions as pseudosubstrate for Pat1, inhibiting its kinase activity [64]. Consistent with a major role for Mei3 in the regulation of sexual differentiation, ectopic expression of *mei3+* is sufficient to trigger the switch from the mitotic to the meiotic cell cycle [65]. The association of Mei3 results in the complete inactivation of Pat1, thereby allowing Ste11 and Mei2 to escape inhibitory phosphorylation and to initiate the meiotic program [61] (**Fig. 5**).

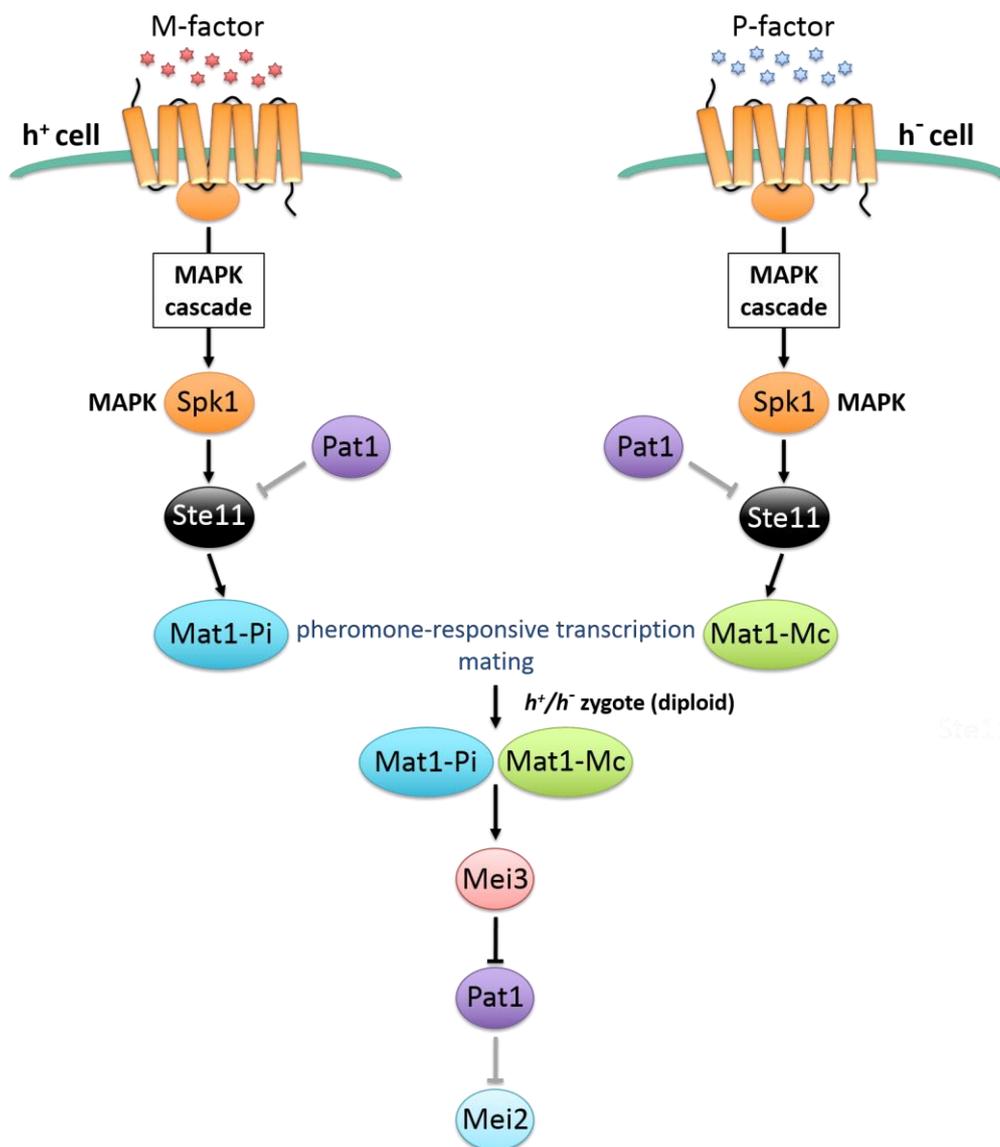


Figure 5. The pheromone responsive pathway regulates the initiation of meiosis by inactivating the Pat1 kinase.

Binding of the P and M pheromones to their relative receptors triggers the activation of the MAPK cascade, which further activates *ste11+*. Ste11 then stimulates the transcription of pheromone-induced genes, which is a prerequisite for the initiation of mating and meiosis. The subsequent formation of a zygote provides the production of cell-type specific Mat1-Pi and Mat1-Mc within a single cell. Both these factors cooperate to stimulate transcription of *mei3+*. Mei3 inhibits the kinase Pat1, which cannot promote the inhibitory phosphorylation of Mei2. Figure adapted from Harigaya and Yamamoto [66].

3.1.2 The RNA-binding protein Mei2: a key meiosis inducer

3.1.2.1 General properties

The *mei2+* gene was originally identified in a screen for fission yeast mutants that failed to enter meiosis and/or to sporulate [67]. Genetic characterization then determined that *mei2+* is required for efficient pre-meiotic DNA synthesis and the first meiotic division (meiosis I) [30, 59, 68-70]. Transcription of *mei2+* is induced by nutrient depletion and is directly controlled by the transcription factor Ste11, which binds to its promoter region in a sequence-dependent manner (e.g. TR boxes) [21, 70]. Later on, the *mei2+* gene was found to encode an RNA-binding protein harboring three RNA recognition motifs (RRM), two positioned in the N-terminal half and one in the C-terminal half (**Fig. 6**) [59]. Importantly, the third RRM was shown to be critical for entry into meiosis, highlighting a physiological role for Mei2 RNA-binding capacity in promoting sexual differentiation.



Figure 6. Schematic illustration of Mei2 protein.

Highlighted are the three RNA-recognition motifs (RRMs) and numbers indicate amino acid residues.

3.1.2.2 Evolutionary conservation

Analyses of amino acid sequences revealed the existence of Mei2-like proteins that belong to an ancient family in eukaryotic organisms. The vast majority of the Mei2-like proteins are found in plants - whereas none are present in mammals - and they are also key regulators of differentiation and meiosis [71, 72].

Almost all these proteins are characterized by three identifiable RNA recognition motifs (RRMs). The third C-terminal RRM (RRM3) is the most highly conserved region, with 25%

identity in plants and fungi [71]. The strong conservation within the RRM3 suggests that all Mei2-like proteins might use a common molecular mechanism. Supporting this notion, expression of the *Arabidopsis* Mei2-like protein AML1 allows *S. pombe* cells that are defective for meiosis to trigger sexual differentiation [73].

3.1.2.3 A key Mei2 cofactor: the long non-coding RNA meiRNA

The *sme2+* gene (suppressor of *mei2+*) was isolated as a multicopy suppressor of a thermosensitive mutant of *mei2+* that does not undergo meiotic divisions [59]. Former analyses showed that *sme2+* encodes a non-coding RNA (named meiRNA) that is essential for meiosis I [59, 74]. The RNA molecule is produced as two isoforms of different length, meiRNA-S, of about 0.5 kb, and meiRNA-L, 1.0 kb longer, that are both polyadenylated [59, 74, 75]. meiRNA directly interacts with Mei2 both *in vivo* and *in vitro*, preferentially via its 5' region [59, 76], and was shown to promote the transport of Mei2 from the cytoplasm to the nucleus, where the latter exerts its function [74]. Because deletion of *sme2+* prevents meiosis I but does not affect pre-meiotic DNA synthesis, it was proposed that meiRNA acts as a key cofactor for Mei2 at a specific stage in sexual differentiation [59].

The *sme2+* gene also harbors multiple hexanucleotide motifs in its 3' region that define the so-called Determinant of Selective Removal (DSR). I will just mention here that these sequences are critical for its degradation and function during vegetative growth (see next section).

Recently, meiRNA was found to accumulate at its site of transcription, which is a critical event to promote efficient pairing of homologous chromosomes during meiosis I [77]. It was proposed that meiRNA, retained at the *sme2+* gene, favors the recognition between homologous chromosomes, although this seems to occur independently of Mei2. Future work will be required to understand the mechanistic details of this fascinating phenotype.

3.1.2.4 Localization

During vegetative growth, Mei2 is lowly expressed and resides essentially in the cytoplasm (**Fig. 7A**). Upon nutritional starvation, Mei2 accumulates and a fraction of the protein becomes apparent in the nucleus, forming a dot-like structure [61, 74]. The Mei2 dot, which correlates with the ability to perform the first meiotic division, is observed in meiotic prophase and persists until the end of the first division, segregating into the two daughter cells (**Fig. 7B-C-D**) [74].

Further studies showed that meiRNA is a key constituent of the Mei2 dot, which overlaps to the *sme2+* locus on chromosome II [59, 74, 78]. It was proposed that meiRNA traps Mei2 as a dot in the nucleus, thereby preventing its export to the cytoplasm [79]. Consistent with this notion, *sme2* Δ cells are not able to form the dot, which prevents Mei2 nuclear accumulation and function.

The exact nature and composition of the Mei2-meRNA dot are far from being fully understood but it is clear that it plays a key role in the initiation of the meiotic program.

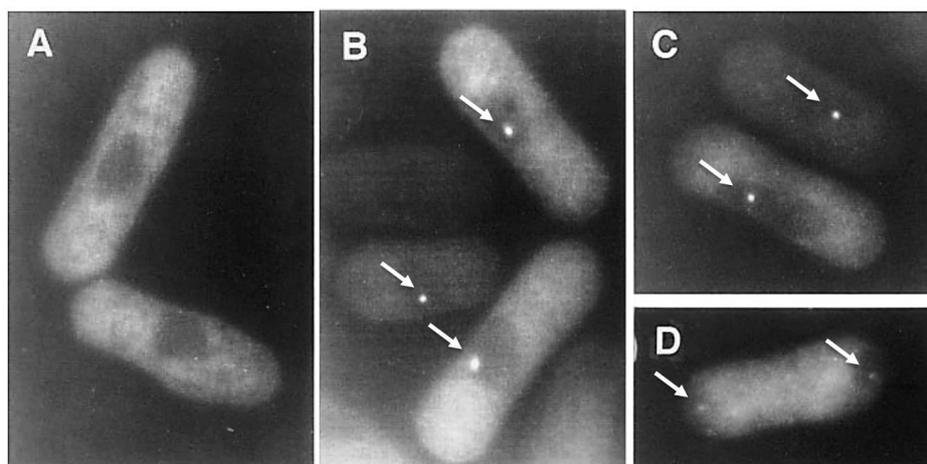


Figure 7. Subcellular localization of the Mei2 protein during the mitotic and meiotic cell-cycle.

Fluorescence of GFP-tagged Mei2 in fission yeast cells, which were grown vegetatively in rich medium (**A**) or depleted of nitrogen for 3,5 hs (**B**), 4 hs (**C**) and 6 hs (**D**). Specifically, cells in (**A**) are in mitotic growth, cells in (**B**) and (**C**) are undergoing meiotic prophase and cells in (**D**) are going through the first meiotic division. White arrows: Mei2 dot. Figure adapted from Yamashita [74].

3.1.2.5 Functions

Mei2 is crucial to initiate sexual differentiation in fission yeast and it has been associated with several functions, although their mechanistic details are not fully understood (**Fig. 8**).

First, Mei2 promotes pre-meiotic DNA synthesis [59]. Interestingly, the RNA-binding capacity of Mei2, but not meiRNA, is required for this function. This led to the suggestion that the binding of Mei2 to another RNA species may account for this phenotype. However, there is currently no evidence for the existence of such a putative RNP complex.

Mei2 also contributes to telomere clustering, which is a prerequisite for the alignment of homologous chromosomes during the horse-tail stage of meiotic prophase [18, 80]. However, and similarly to pre-meiotic DNA synthesis, whether this phenotype underlies a direct function for Mei2 is still unclear but it is tempting to speculate that it is also linked to the formation of an RNP complex with a specific RNA.

Finally, a key function for Mei2 in promoting meiosis I consists in sequestering, in cooperation with meiRNA, a protein called Mmi1 (meiotic mRNA interception 1), which targets meiotic transcripts produced during vegetative growth for degradation. This mechanism is known as selective elimination of meiotic mRNAs, which is described in depth in the next section. I will just mention here that upon nutritional starvation Mei2 and meiRNA trap Mmi1 in the dot-like structure, thereby inhibiting degradation of meiotic transcripts and favoring initiation of meiosis [54, 74].

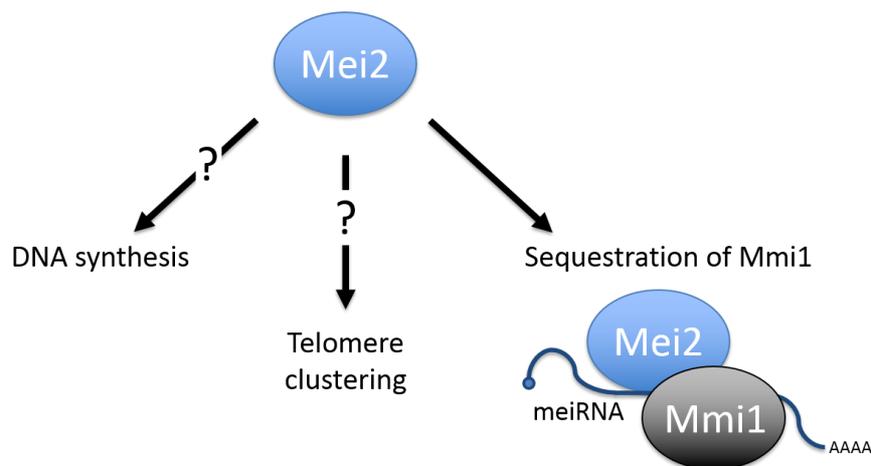


Figure 8. Scheme of the known functions of Mei2.

To date, the role of Mei2 in both pre-meiotic DNA synthesis and telomere clustering is unclear; the most characterized function is the sequestration and inactivation of Mmi1 in an RNP complex containing Mei2 and the long non-coding RNA meiRNA.

3.1.2.6 Regulation of Mei2 expression and activity

As previously mentioned, the abundance and activity of Mei2 are regulated by the Pat1 kinase. Phosphorylated forms of Mei2 are indeed susceptible to ubiquitination and proteasome-dependent degradation [46], and are also bound by the 14-3-3 protein Rad24, which prevents the association of Mei2 with meiRNA [81]. These events prevent ectopic activation of Mei2 and therefore allow cells to sustain vegetative growth. Of note, the role of Pat1 in the inhibition of the transcription factor Ste11 also contributes indirectly to lower *mei2*⁺ expression levels.

Recent work showed that Mei2 is also subjected to phosphorylation by the Tor2 kinase, which, similarly to Pat1, accelerates its degradation through the ubiquitin-proteasome pathway in mitotic cells [17]. Specifically, Tor2 phosphorylates nine residues within Mei2, which are distinct from the ones targeted by Pat1 [17]. Therefore, two essential kinases independently regulate the steady state levels and activity of Mei2.

The current model posits that, upon nutritional starvation, the expression of Tor2 is downregulated, thereby allowing the accumulation of partially dephosphorylated Mei2. This is

accompanied by cell cycle arrest in G1 and mating, which in turn stimulates the transcription of *ste11+* and increases the levels of dephosphorylated Mei2 [28]. The subsequent inactivation of Pat1 by Mei3 in zygotes contributes to the full activation of Mei2, leading to the initiation of meiosis (Fig. 9).

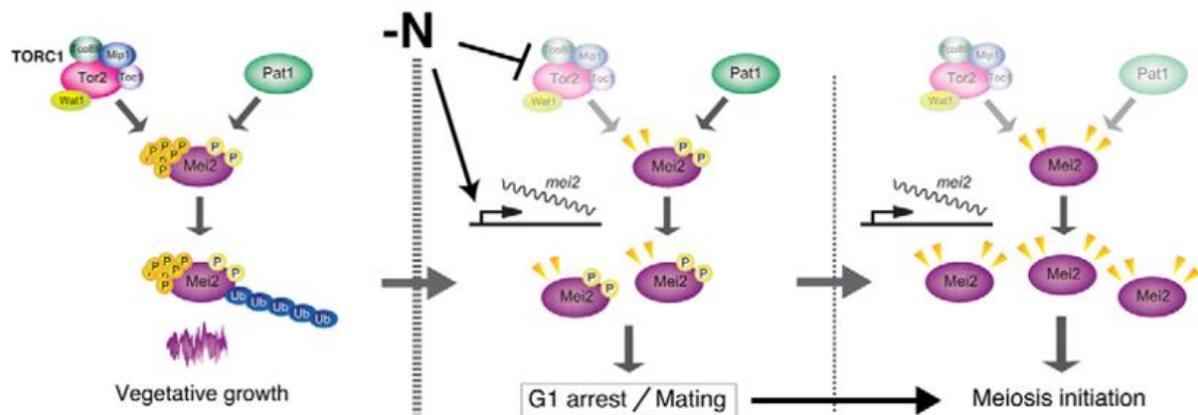


Figure 9. Schematic illustration of the cascade of events leading to Mei2 activation and meiosis initiation.

A hypothetical model displaying the regulation of Mei2 in cells switching from mitotic growth to mating and meiosis. It is thought that, upon nitrogen starvation (-N), Mei2 is still phosphorylated by Pat1, but the inactivation of TORC1 reduces the Mei2 phosphorylation rate, allowing the mating process to occur. Whereas, it has been shown that inactivation of only Pat1 induces cells to enter meiosis without mating. It is likely that the stepwise inactivation of TORC1 and then Pat1 is crucial for the efficient progression of sexual differentiation. P: phosphorylation; Ub: ubiquitination. Figure adapted from Otsubo [17].

3.2 The selective elimination of meiotic transcripts during vegetative growth

The selective elimination of meiotic mRNAs is a post-transcriptional regulatory pathway that triggers degradation of cognate transcripts in a sequence-dependent manner. Several factors recognize and remodel meiotic RNAs prior to target them for decay during the mitotic cell cycle. In this section, I summarize the knowledge on the *cis* elements and the *trans*-acting factors that play a key role in the regulation of this process that is essential for robust vegetative growth and sexual differentiation.

3.2.1 *cis*-regulatory sequences control meiotic mRNA levels

Upon entry into meiosis, hundreds of genes are induced or up-regulated thanks to the activity of specific transcription factors [47] (see above). However, in addition to transcriptional regulation, another regulatory process distinguishes the mitotic and meiotic cell cycles in fission yeast. Indeed, the expression of several meiotic transcripts is suppressed at the post-transcriptional level during vegetative growth. This mechanism, known as the selective elimination of meiotic mRNAs, was proposed to prevent untimely expression of the meiotic program [54].

Originally, the Yamamoto lab made the surprising observation that some meiosis-specific transcripts (e.g. *mei4+*, *spo5+*, *ssm4+*, *mcp5+*) do not accumulate in mitotic cells even when artificially expressed from a constitutive promoter [54]. This suggested the existence of *cis*-acting regulatory sequences that prevent the accumulation of these mRNAs in vegetative cells. A screen for mutations that restore transcript levels allowed the identification of a region responsible for mRNA elimination: the Determinant of Selective Removal (DSR) [54].

Subsequent computational analysis showed that the DSR regions do not share extensive homology between meiotic genes but contain several repeats of the hexanucleotide motif U(U/C/G)AAAC (**Fig. 10**). Genetic experiments demonstrated that the UUAAAC and UCAAAC sequences constitute the core motifs that exhibit functional DSR activity on their own [75]. Insertion of multiple tandem repeats of UUAAAC at the 3' end of a *gfp+* reporter gene expressed from the *adh1+* promoter indeed precludes the accumulation of these transcripts in mitotic cells [75].

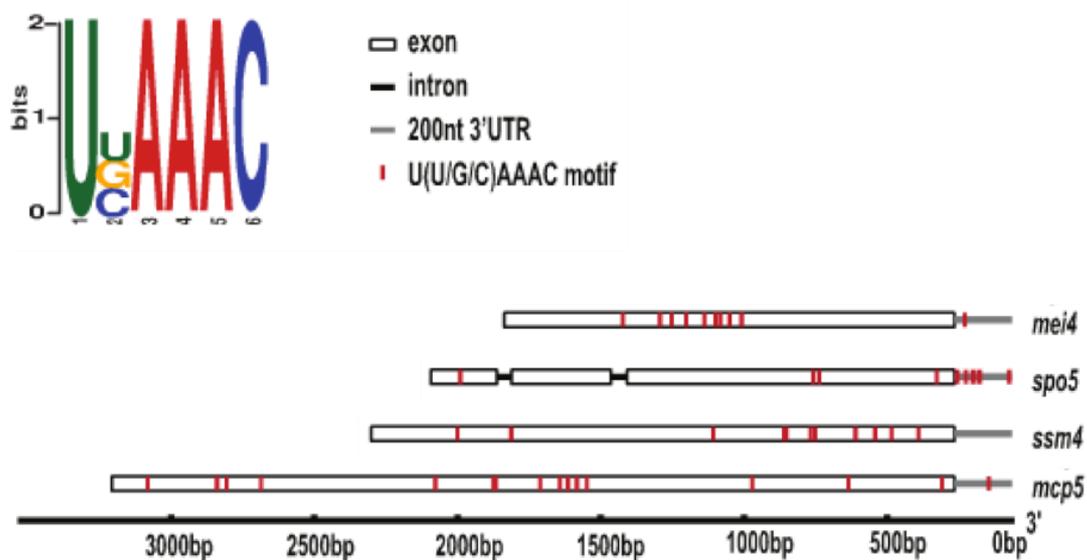


Figure 10. Sequence and distribution of the hexanucleotide motif U(U/G/C)AAAC, in constitutively expressed meiotic genes.

Figure adapted from Chen [82].

Thus, a subset of meiotic transcripts that are constitutively expressed encode in *cis* the elements that dictate their removal during the mitotic cell cycle, thereby preventing inappropriate expression of meiosis-specific transcripts that might be deleterious for robust vegetative growth.

3.2.2 Factors involved in the selective elimination of meiotic mRNAs

Since the original discovery of the post-transcriptional regulation of meiosis-specific transcripts, a number of factors have been shown to participate in this process. In this section, I discuss the proteins involved in the recognition of meiotic transcripts as well as the effector complexes that control and promote their elimination in mitotic cells.

3.2.2.1 The factors that recognize meiotic transcripts

3.2.2.1.1 *The YTH-family RNA-binding protein Mmi1*

Identification

A genetic screen was first designed to identify factors required for selective elimination of DSR-containing meiotic transcripts [54]. Yeast wild type cells expressing a chimeric transcript containing the *ura4+* gene fused to the DSR region of *mei4+* that prevents its own accumulation, were mutagenized and selected for growth in the absence of uracil, i.e. in conditions in which the reporter transcript is derepressed. Four of the isolated clones carried a mutation in a gene encoding an RNA-binding protein of the YTH-family [83], named Mmi1 for meiotic mRNA interception factor 1 [54]. The fact that deletion of *mmi1+* causes severe growth and viability defects due to the ectopic expression of several meiotic genes highlights the physiological relevance of the Mmi1/DSR system in mitotic cells.

Evolutionary conservation and structural properties

The YTH-family RNA-binding proteins are evolutionarily conserved throughout the eukaryotic kingdom. Originally identified by comparing protein sequences with the human splicing factor YT521-B [83], the YTH domain (YT521-B Homology) defines a specific class of proteins with RNA-binding capacity that contains more than 170 members in various species

(Fig. 11). The fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* each encode one member of this family, Mmi1 and Mrb1 (also known as Pho92) respectively, while mammals contain five YTH proteins, including the YTH Domain Family (YTHDF) proteins 1, 2 and 3, and the YTH Domain Containing (YTHDC) proteins 1 and 2.

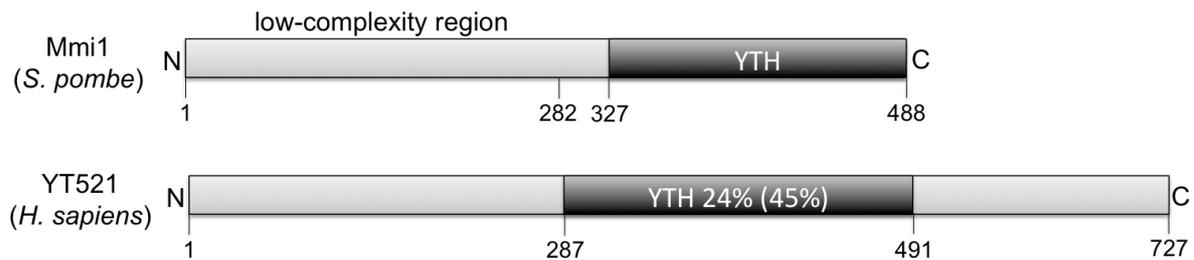


Figure 11. Schematic representation of the YTH-family RNA-binding proteins Mmi1 in *S. pombe* and YT521 in humans.

Numbers indicate amino acid residues. The black boxes correspond to the YTH domains in both proteins. Note that the human domain displays 24% identity and 45% similarity to Mmi1. Figure adapted from Harigaya [54] and Stowell [84].

The precise mode by which YTH proteins bind to RNA was discovered only very recently. A study of the human YTHDF2 protein showed that specific RNA modifications, and more precisely N⁶-methyladenosine (m⁶A) residues, confer selectivity for RNA binding both *in vitro* and *in vivo* [85-87]. Crystal structures of the YTH domains of YTHDF2 and YTHDC1 bound to a methylated RNA revealed the existence of a conserved hydrophobic binding pocket that can specifically accommodate m⁶A [87-89]. Thus, proteins of the YTH family directly “read” RNA methylation patterns, which in turn influence the fate of transcripts, thereby providing an additional layer in the control of gene expression. Landmark studies in metazoans demonstrated that YTH proteins regulate many RNA-related processes, including splicing, degradation, translation and transcriptional silencing [86, 90-92].

Similarly to their metazoan counterparts, the budding and fission yeast YTH proteins Mrb1 and Mmi1 also display an aromatic cage that is a potential m⁶A-binding pocket [92, 93]

(**Fig. 12**). Unexpectedly, however, structural and biochemical analyses demonstrated that m^6A decreases the affinity of Mmi1 for RNA [92]. Contrary to Mrb1 and mammalian YTH proteins, the arrangement of residues in the Mmi1 aromatic cage sterically hinders the recognition of m^6A (**Fig. 12**). Consistent with this notion, mutations of the residues delineating the cage do not impact Mmi1 RNA-binding activity [92]. Another striking difference is the presence of negatively charged residues surrounding the m^6A -binding pocket, which creates a repulsive environment for RNA binding.

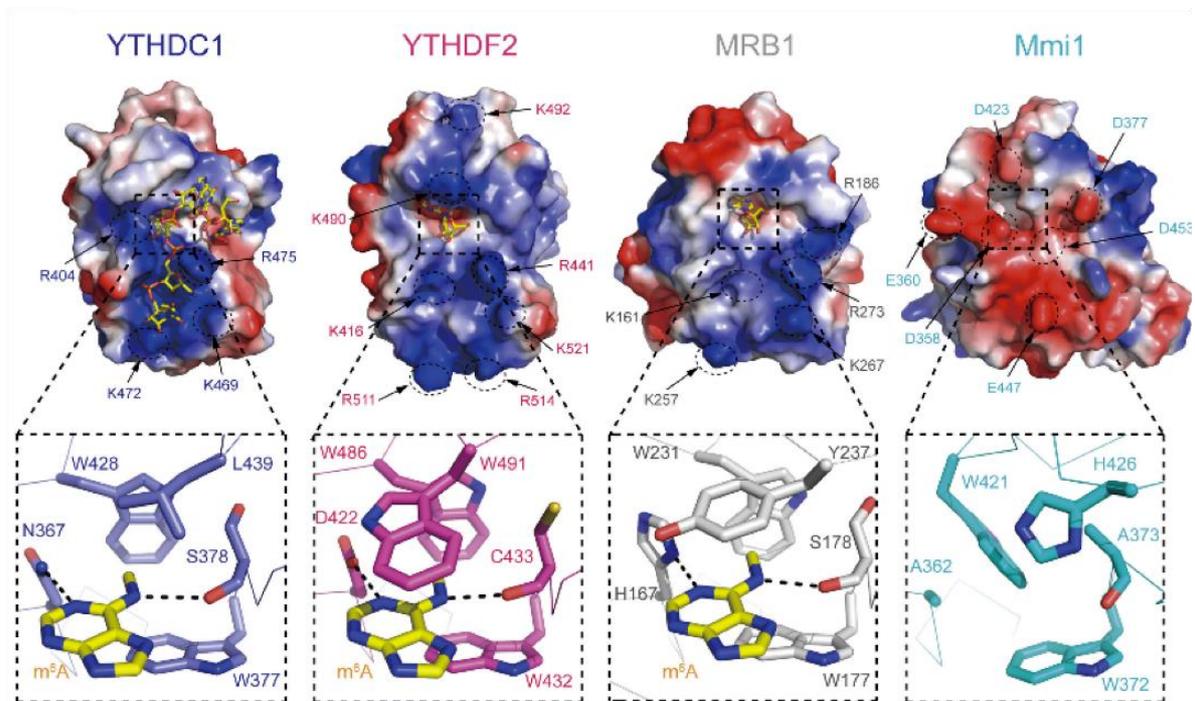


Figure 12. Comparison of the aromatic cages within YTH domains.

Above is the electrostatic potential of the surface, showing the positively charged residues (blue) in the m^6A RNA-binding interfaces of YTHDC1, YTHDF2 and Mrb1 and the negatively charged residues (red) near the aromatic cage of Mmi1. Below is the enlarged view of the respective aromatic cages. Figure adapted from Wang [92].

Mmi1 uses instead a long groove located at the opposite side of the m^6A -binding pocket to associate with RNA, thereby defining a unique binding mode for YTH domains (**Fig. 13**) [92]. This atypical mode of RNA binding is consistent with the absence, in fission yeast, of

enzymes homologous to METTL3 and METTL14 that catalyze methylation of adenosines in metazoans (reviewed in Roundtree [94] and Zhao [95]). This suggests that m⁶A modification has been lost in *S. pombe*, although it encodes a putative RNA methyltransferase, *ime4+*, the homologue of which is responsible for adenosine methylation in budding yeast.

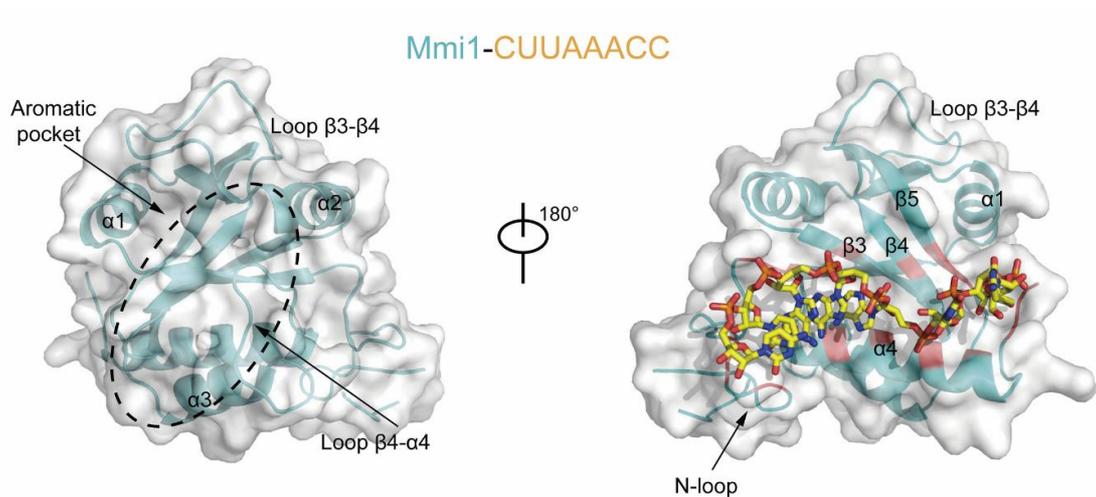


Figure 13. Structural overview of the Mmi1 protein in complex with a DSR-containing CUUAAAC RNA.

The Mmi1 YTH domain is colored in cyan and the RNA-binding regions are in red. The structural elements and aromatic pocket implicated in RNA-binding are highlighted. Figure adapted from Wang [92].

Beyond their RNA-binding domains, YTH proteins do not share significant homology. It has been proposed that the N-terminal regions are involved in protein-protein interactions. Consistent with this notion, Mmi1 contains a serine rich low-complexity region at the N-terminus, which has been shown to mediate interactions with protein partners [84].

Targets

Former micro- and tiling-array analyses allowed the identification of a set of meiotic genes whose expression is increased upon mutation of *mmi1+* during vegetative growth [82, 96]. A common feature in these RNA targets is the presence of multiple DSR motifs (i.e. repeats of the UNAAAC hexanucleotide sequence), generally clustered in the 3' half of the genes. However, and because some Mmi1 targets include transcripts encoding meiotic transcription factors (e.g. *rep1+*, *mei4+*), it has been difficult to discriminate between direct and indirect effects on the expression of putative targets.

Recent genome-wide identification of direct Mmi1 RNA targets by CRAC (UV Crosslinking and analysis of cDNA by high-throughput sequencing) revealed a large repertoire of transcripts produced by all three RNA polymerases (PolI, II, III), including hundreds of protein-coding and ncRNAs transcribed by PolII, many PolIII transcripts and a PolI-dependent ribosomal RNA precursor [97]. Interestingly, many transcripts associated to Mmi1 display fewer UNAAAC motifs, indicating flexibility in target recognition [97-99]. However, whether this can impact Mmi1 function is not understood.

One peculiar target of Mmi1 is the lncRNA *meiRNA*, encoded by the *sme2+* locus. This non-coding RNA contains a DSR region with 13 copies of the core motif, especially in the 3' part of the transcript to which Mmi1 binds [75]. Its expression is increased in meiotic cells [59], like DSR-containing meiotic transcripts, and *sme2Δ* cells are not able to inhibit Mmi1 via the Mei2 dot [54]. It has been proposed that *meiRNA* serves as a decoy to lure Mmi1, favoring its inhibition upon meiotic conditions (see below).

Functions

As described above, Mmi1 recognizes, via its YTH domain, a *cis*-acting region within meiotic transcripts (i.e. DSR motifs) [54, 75], which is essential to prevent their ectopic expression in mitotic cells [54].

I will simply mention here that one of the key functions of Mmi1 is the activation of RNA degradation. Over the last few years, many studies have pointed a key role for the nuclear exosome in the degradation of Mmi1 targets during vegetative growth (see below). The current model posits that Mmi1 is co-transcriptionally loaded onto DSR-containing mRNAs and recruits several factors that contribute to their rapid decay (**Fig. 14**).

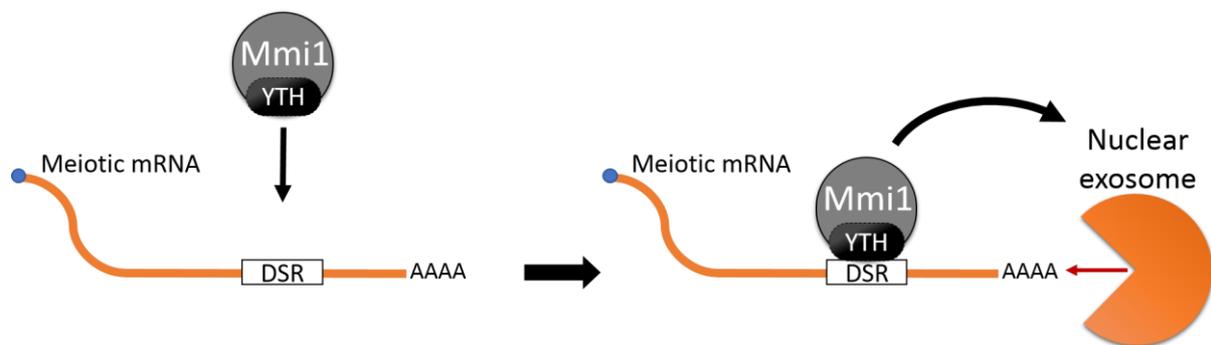


Figure 14. Mmi1 promotes meiotic mRNA degradation by the nuclear exosome.

Mmi1 recognizes the DSR motif within meiotic mRNAs via its YTH domain (**left**) and then cooperates with other factors to promote RNA degradation by the exosome (**right**).

Previous work showed that Mmi1 also regulates the splicing of several intron-containing meiotic mRNAs [82, 100]. Mutants of *mmi1*⁺ lead to the accumulation of spliced *rec8*⁺ and *crs1*⁺ transcripts, suggesting that Mmi1 may be involved in intron retention. The use of artificial constructs indicated that Mmi1 also affects intron splicing without impacting transcript stability [82]. Recently, Kilchert and colleagues showed that mRNAs containing introns with an Mmi1 binding site are rapidly targeted for degradation in conditions of inefficient splicing [97]. Conversely, fast splicing events prevent the recruitment of Mmi1 and degradation factors, thereby allowing accumulation of mRNAs [97].

Another function of Mmi1 lies in the control of transcription termination. It has been shown that deletion of *mmi1*⁺ triggers the accumulation of RNAPII downstream of canonical termination signals [99]. The presence of the DSR region is essential for this function.

However, the mechanism by which Mmi1 may affect transcription termination is not fully understood. One possibility is that Mmi1 co-transcriptionally loads onto meiotic transcripts and promotes the recruitment of additional factors that promote the dismantling of elongation complexes.

Localization and regulation

In mitotic cells, Mmi1 localizes to one or several scattered nuclear foci (**Fig. 15A**) [54, 76]. These nuclear bodies have been proposed to reflect RNA processing/decay centers, whereby Mmi1 and several of its protein partners assemble onto newly synthesized meiotic transcripts to mediate their elimination [76]. Microscopy experiments indicated that the dots do not overlap with the transcription sites of canonical Mmi1 target genes, such as *mei4+*, although a transient association of these bodies with meiotic gene loci cannot be formally excluded [101]. One of the dots, however, was shown to precisely map to the *sme2+* locus, which encodes the DSR-containing lncRNA *meiRNA* that has been suggested to function as a decoy to lure Mmi1 [76]. Removal of the Mmi1-binding sites within *meiRNA* (i.e. DSR motifs) abolishes formation of the dot, suggesting that a pool of Mmi1 is associated with *meiRNA* at the *sme2+* locus in vegetative cells [76].

Upon nutritional starvation, the multiple Mmi1 foci converge into a single spot that persists until meiotic prophase I before dispersing after metaphase I [54]. RNA-FISH and immunofluorescence experiments revealed that this unique Mmi1 dot, observed upon meiosis onset, overlaps with the Mei2 dot (**Fig. 15B**) [76]. Crucially, deletion of either *mei2+* or *sme2+* prevents the convergence of Mmi1 dots (**Fig. 15C**) and results in major sporulation defects, highlighting the biological relevance of the Mei2 dot in sexual differentiation [54]. It has been suggested that, upon meiosis, Mei2 anchors Mmi1 in a dot-like structure, to inhibit its function in DSR-dependent meiotic mRNA degradation (**Fig. 15D**).

However, and despite recent progresses, the exact composition and function of the Mmi1-containing nuclear foci remain elusive.

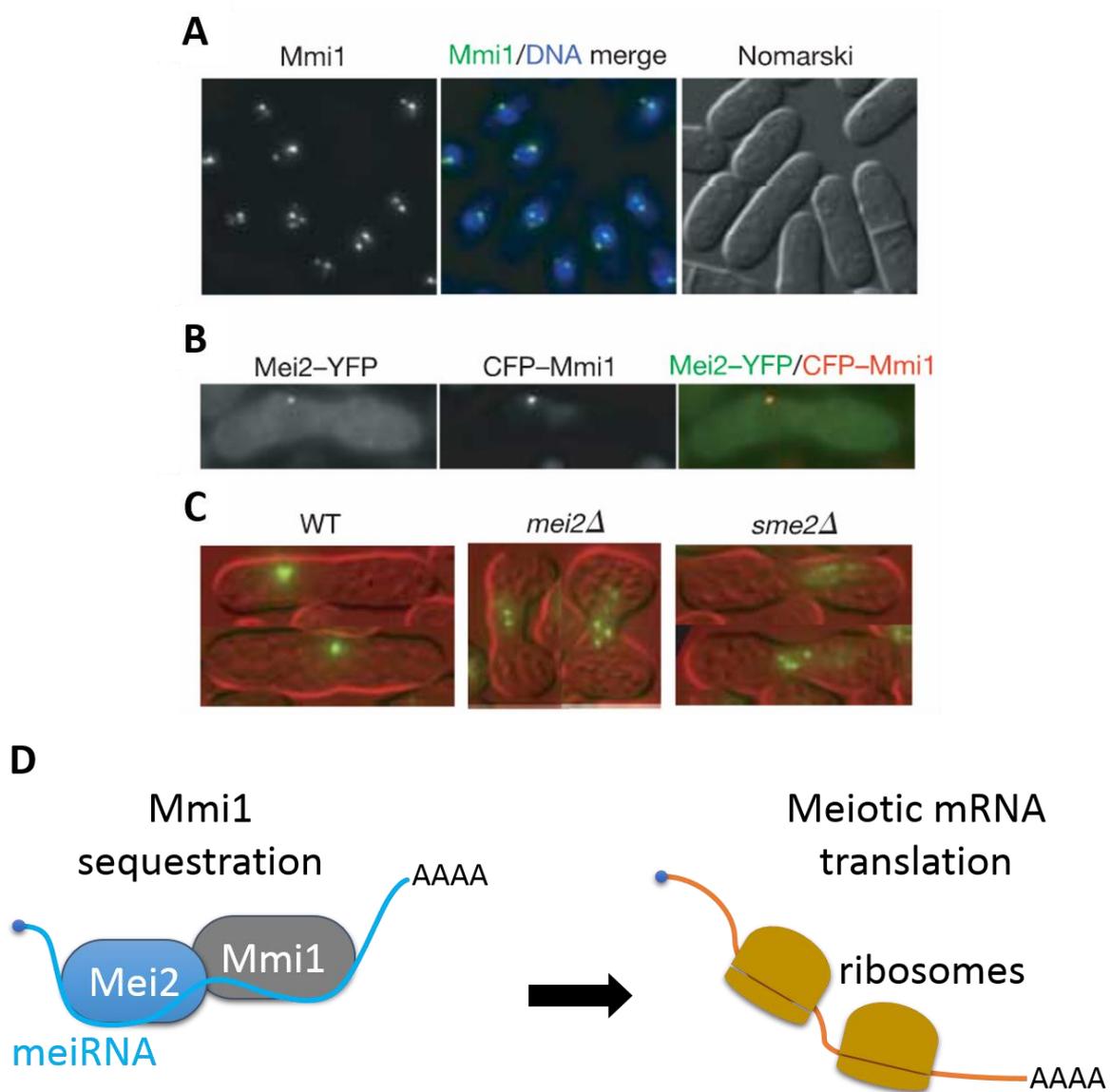


Figure 15. The Mei2 dot sequesters Mmi1 allowing meiotic mRNAs to be translated

(A) Mitotically growing cells expressing GFP-tagged Mmi1 are shown; nuclei are counterstained with Hoechst 33342. (B) Cells during meiotic prophase, expressing CFP-Mmi1 and Mei2-YFP. (C) Meiotic cells, expressing GFP-Mmi1, show scattered foci when defective for *mei2*⁺ or *sme2*⁺. Figures in A-B-C adapted from Harigaya [54]. (D) Model showing the sequestration of Mmi1 by Mei2 and the non-coding meiRNA (left), which allows meiotic mRNAs to be free from degradation and therefore translated (right).

3.2.2.1.2 3'-end processing and polyadenylation factors

The Mmi1-dependent meiotic mRNA elimination process was initially found to require components of the 3'-end processing and polyadenylation machinery. Yeast two-hybrid, biochemical and genetic analyses uncovered several potential Mmi1 protein partners, including Rna15, a subunit of the cleavage factor CF1A involved in mRNA processing, the canonical mRNA poly(A) polymerase Pla1, the poly(A) binding protein Pab2 and the Rrp6 subunit of the nuclear exosome [54, 102]. Functional analyses revealed that these factors cooperate with Mmi1 to target DSR-containing meiotic mRNA for degradation [100, 102, 103]. Mechanistically, it was proposed that Mmi1 first binds to the transcript and, by virtue of its affinity for Rna15 and Pla1, promotes its hyperadenylation. The resulting poly(A) tail is subsequently bound by Pab2, which in turn recruits the exosome for rapid decay.

Recently, the 5' to 3' exoribonuclease Dhp1, the homologue of Rat1/XRN2, was also shown to prevent meiotic mRNAs expression in vegetative growth, possibly through a mechanism that couples premature transcription termination to RNA decay [104]. However, the requirement for the catalytic activity of Dhp1 is still unclear and it has been suggested that the protein may serve as a scaffold for the recruitment of RNA elimination factors, including Mmi1 and MTREC (see below) [104].

Interestingly, all of the above-mentioned factors form patchy subnuclear structures that overlap with Mmi1 foci in mitotic cells. This further suggests that the polyadenylation control of meiotic mRNAs is an important molecular facet of their elimination.

3.2.2.1.3 *The MTREC complex*

A major effector in the selective elimination of meiotic mRNAs that is tightly associated to Mmi1 is the multisubunit MTREC complex. Core components of this complex include the zinc finger-containing protein Red1 (RNA elimination defective 1) and the RNA helicase Mtl1 [101, 105].

Red1 was initially found as a factor localizing to nuclear foci in mitotic cells [106]. Functional analysis revealed that Red1 encodes a 712 amino acids protein harboring a zinc-finger domain (often found in DNA/RNA-binding protein) that is required for robust vegetative growth and efficient mating/sporulation. Red1 was shown to associate with Mmi1 in mitotic cells and to colocalize with Pla1, Pab2 and the exosome in Mmi1 nuclear foci. Expression profiling of *red1Δ* cells revealed that many meiotic genes are up-regulated in vegetative cells which strongly overlap Mmi1 RNA targets. Importantly, point mutants in the zinc finger of Red1 also display defects in meiotic mRNAs degradation, strongly suggesting that RNA binding is important for function [106].

Subsequent affinity purifications provided for the first time a global picture of the composition of MTREC. These include, together with Red1, the essential Mtr4-like RNA helicase Mtl1, the Pro/Ser rich factor Iss10/Pir1, the zinc finger-containing protein Red5, the RRM-containing protein Rmn1, and Ars2, the homologue of which is known to promote the recruitment of the exosome and the cap-binding subunits Cbc1/Cbc2 in humans [101, 105, 107, 108]. Immunofluorescence experiments indicated that most MTREC subunits localize to nuclear foci, overlapping those formed by Mmi1 [101, 105]. Deletion or mutation of the MTREC subunits generally lead to a strong accumulation of meiotic transcripts, although some components appear to have a weaker role [101, 105, 108]. It was suggested that submodules of MTREC target distinct sets of RNA substrates to the nuclear exosome, including unspliced pre-mRNA and cryptic unstable transcripts (CUTs) [105, 108]. Indeed, specific MTREC

subunits associate independently with the splicing factors Ctr1 and Nrl1, and the nuclear exosome (e.g. Rrp6).

The precise mechanism by which MTREC promotes meiotic mRNA degradation remains elusive. It has been proposed that MTREC may physically bridge RNA-bound Mmi1 to the nuclear exosome. Supporting this, *Iss10*, previously found in a genetic screen designed to identify factors involved in the Mmi1/DSR system [109], is essential for the interaction between Mmi1 and Red1. However, *iss10Δ* cells show only a moderate increase in meiotic mRNAs steady state levels [101, 109], suggesting that the Mmi1-Red1 association is not critical for meiotic mRNA degradation. It was proposed that MTREC also directly binds to transcripts, which is supported by the presence of several RNA-binding motifs in its subunits.

A general model has emerged in which MTREC submodules target specific classes of RNA substrates (e.g. DSR-containing meiotic mRNAs, unspliced pre-mRNA, CUTs, etc.) and deliver them to a larger machinery that comprises MTREC itself, the poly(A) polymerase Pla1 and the nuclear exosome (**Fig. 16**) [108]. The reported physical interactions between Red1, Mtl1, Pla1 and Rrp6 strongly support this idea. Polyadenylation by Pla1 is believed to provide single-stranded A tails, thereby facilitating exonucleolytic attack by Rrp6. Whether the catalytic activity of Mtl1 is required to unwind putative RNA secondary structures to further assist the exosome remains to be established. This would be reminiscent of the function of the Mtr4 subunit of the TRAMP complex in budding yeast, which facilitates exosome-dependent degradation by remodeling RNA substrates. Interestingly, in human cells, there is only one Mtr4-like protein that associates with two distinct complexes, one of which is similar to the TRAMP complex of budding and fission yeast, and a second - known as CBCN complex - similar to the fission yeast MTREC [110, 111].

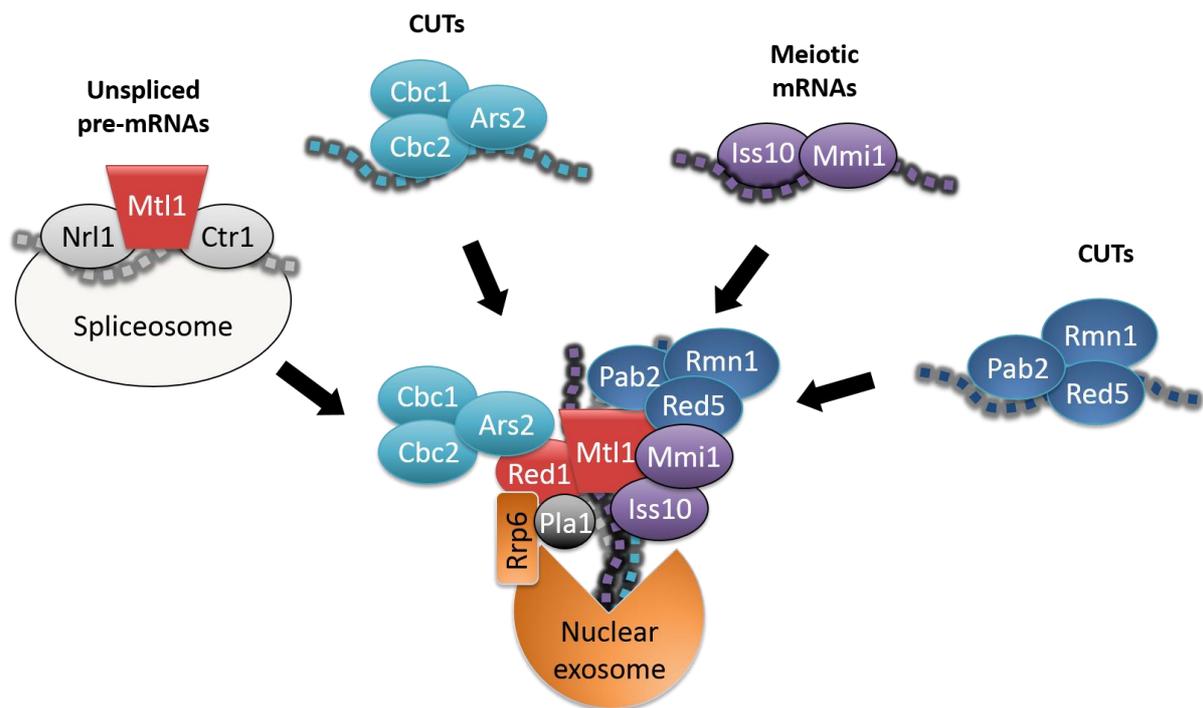


Figure 16. Current model displaying the role of different submodules of the MTREC complex in RNA surveillance.

Different classes of unspliced pre-mRNA, CUTs or meiotic mRNAs are targeted by protein submodules, which in turn deliver them to the MTREC complex. The canonical poly(A) polymerase is responsible for the polyadenylation of these transcripts and MTREC can associate to the nuclear exosome via Red1-Rrp6 interaction. It appears that the helicase activity of Mtl1 is required to feed the targeted RNA into the exosome channel. Figure adapted from Zhou [108].

MTREC has also been involved in the positive regulation of meiotic gene expression during meiosis [112], but the molecular basis for this phenotype is not completely clear. It was proposed that MTREC facilitates the maturation of meiosis transcripts through the recruitment of RNA processing factors, including the splicing and 3'-end processing factors.

3.2.2.1.4 *Erh1*, enhancer of rudimentary homologue

Erh1 was initially discovered in the same genetic screen that led to the identification of *Iss10* [109]. Like *Mmi1* and MTREC, *Erh1* promotes degradation of DSR-containing meiotic mRNAs. A recent study by the Grewal lab showed that *Erh1* (enhancer of rudimentary 1)

associates stoichiometrically with Mmi1, forming a defined Erh1-Mmi1 complex (EMC) [113]. EMC was proposed to engage the MTREC and Ccr4-Not effector complexes (see below) to promote meiotic mRNA suppression and silencing at the rDNA locus, respectively. In light of the findings that mammalian ERH1 forms homodimers in solution [114, 115], it has been hypothesized that Erh1 might contribute to Mmi1 dimerization, perhaps to facilitate its binding to RNA substrates [113]. Future work is needed to understand its precise relationship with Mmi1 and its spectrum of action regarding RNA-related processes.

3.2.2.2 The factors that contribute to meiotic mRNA elimination

3.2.2.2.1 The nuclear exosome

The RNA exosome is a large multi-subunit complex, conserved from archaea to higher eukaryotes, that is found in both the nucleus and the cytoplasm. It is the major 3' to 5' RNA degradation machinery in the cell and it plays key roles in RNA degradation, processing and surveillance [116-118]. I will focus here on the properties and the functions of the nuclear form of the complex, which has a critical role in meiotic mRNA suppression.

Composition and structure

The core exosome is composed of nine subunits that organize in a barrel-shaped structure from yeast to humans (reviewed in Chlebowski [119], Januszyk [120] and Kilchert [121]). The RNase PH-like proteins Rrp41, Rrp42, Rrp43, Rrp45, Rrp46 and Mtr3, which contain both the S1 and KH RNA-binding domains, assemble into a ring complex. Three additional subunits, including Rrp4, Rrp40 and Csl4, form a cap structure on the top of the ring to constitute the core complex that is catalytically inactive. The core associates with two active RNases: Dis3 (or Rrp44), a processive 3' to 5' exo- and endoribonuclease that locates at the bottom of the ring [122, 123], and Rrp6, a nucleus-specific subunit associated with the cap and endowed with a distributive 3' to 5' exonuclease activity (**Fig. 17**).

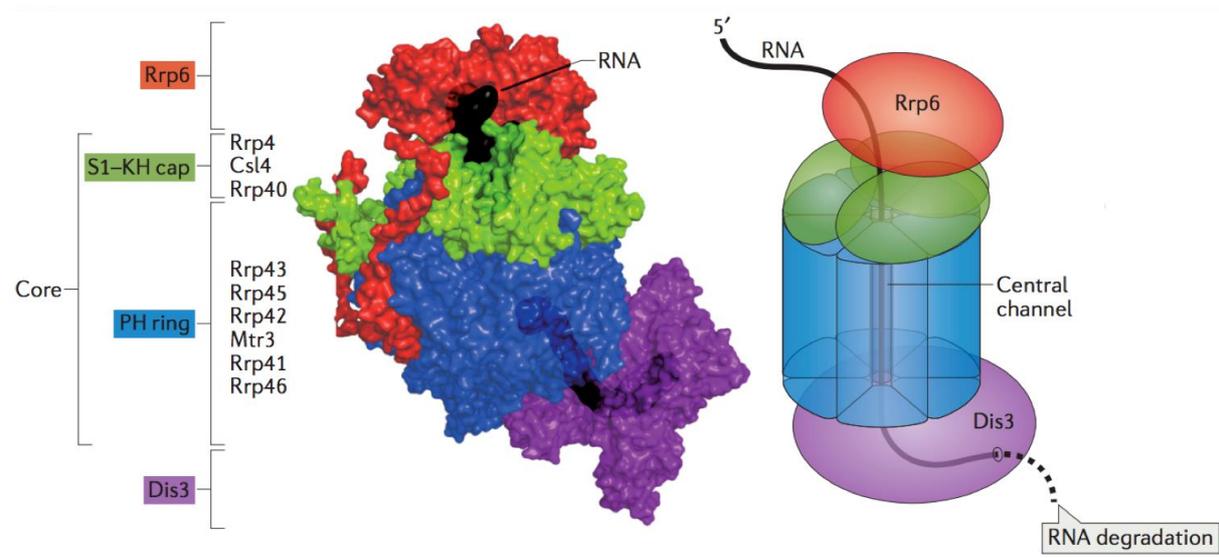


Figure 17. Structure of the exosome complex.

On the left a model of the exosome is shown, Rrp6 is displayed in red, the cap complex in green, PH ring complex in blue, Dis3 in purple and RNA in black. On the right a scheme of the structure of the exosome, showing an RNA molecule entering 3' to 5' inside the central channel, passing through the cap and PH ring until the Dis3 catalytic site, in which it is degraded. Figure adapted from Kilchert [121].

The vast majority of RNA substrates that are targeted to the exosome enter the barrel-like structure via a pore located at the center of the cap and thread through the central channel in a 3' to 5' direction. Single-stranded RNA molecules move inwards the ring until they reach the catalytic site of Dis3, which faces the central channel [124, 125]. However, in some cases, the RNAs do not enter the barrel-like structure of the exosome and are directly targeted to either Dis3 or Rrp6, whose endonuclease and exonuclease domains respectively are exposed to solvents. The global structure of the exosome therefore suggests the existence of different routes for decay, which might underlie different regulatory mechanisms.

Regulators of the nuclear exosome

The core exosome, as an isolated complex, is catalytically inactive. Its association with specific accessory co-factors is essential to mediate RNA processing/degradation [124, 126, 127].

Intriguingly, all the exosome regulating complexes include a conserved RNA helicase and the RNA-unwinding activity appears to be crucial for the regulation of the exosome activity. It has been proposed that helicases would facilitate the delivery of the RNA substrate inside the central channel of the exosome [128-130].

A well-characterized exosome cofactor is the polyadenylation TRAMP complex, which contains the DEAD-box helicase Mtr4 and assists the exosome in a number of RNA quality control and 3'-end processing events [131-133]. In fission yeast, the TRAMP complex targets rRNA precursors and heterochromatic transcripts for degradation by the exosome [134-136]. Another co-factor, the MTREC complex (see above), is essential for the degradation of spurious transcripts, unspliced pre-mRNAs and meiotic mRNAs [108]. Interestingly, and as mentioned above, MTREC also contains a helicase subunit, called Mtl1 (Mtr4-like 1), which is highly similar to the Mtr4 subunit of TRAMP and favors the channeling of RNA molecules within the exosome.

Factors that target RNA substrates to the nuclear exosome

The exosome and its associated helicases display low specificity with respect to RNA substrates. This is acquired thanks to dedicated co-factors, which recognize and deliver different subsets of transcripts for correct processing or efficient decay.

The first examples of exosome-specificity factors have been the proteins that bind AU-rich elements (AREs), located in the 3' untranslated regions of unstable transcripts [137]. In humans, ARE-binding proteins associate with the RHAU helicase, which assists the exosome and stimulate ARE-dependent RNA degradation [138].

In *S. cerevisiae*, the trimeric NNS complex, which contains the RNA-binding proteins Nrd1 and Nab3 as well as the helicase Sen1, recognizes short sequence motifs within transcripts and targets them for degradation by the exosome. Mechanistically, NNS interacts

with phosphorylated Serine 5 of RNAPII CTD in the early stage of transcription and couples transcription termination to RNA decay by the exosome through a direct interaction with the TRAMP complex (**Fig. 18A**) [139-142].

In *S. pombe*, the RNA-binding protein Mmi1 is also recruited co-transcriptionally thanks to the presence of DSR motifs within targeted transcripts [54, 96]. Mmi1 associates with the MTREC helicase Mtl1, which is believed to feed the exosome during vegetative growth (**Fig. 18B**) [54, 97-99].

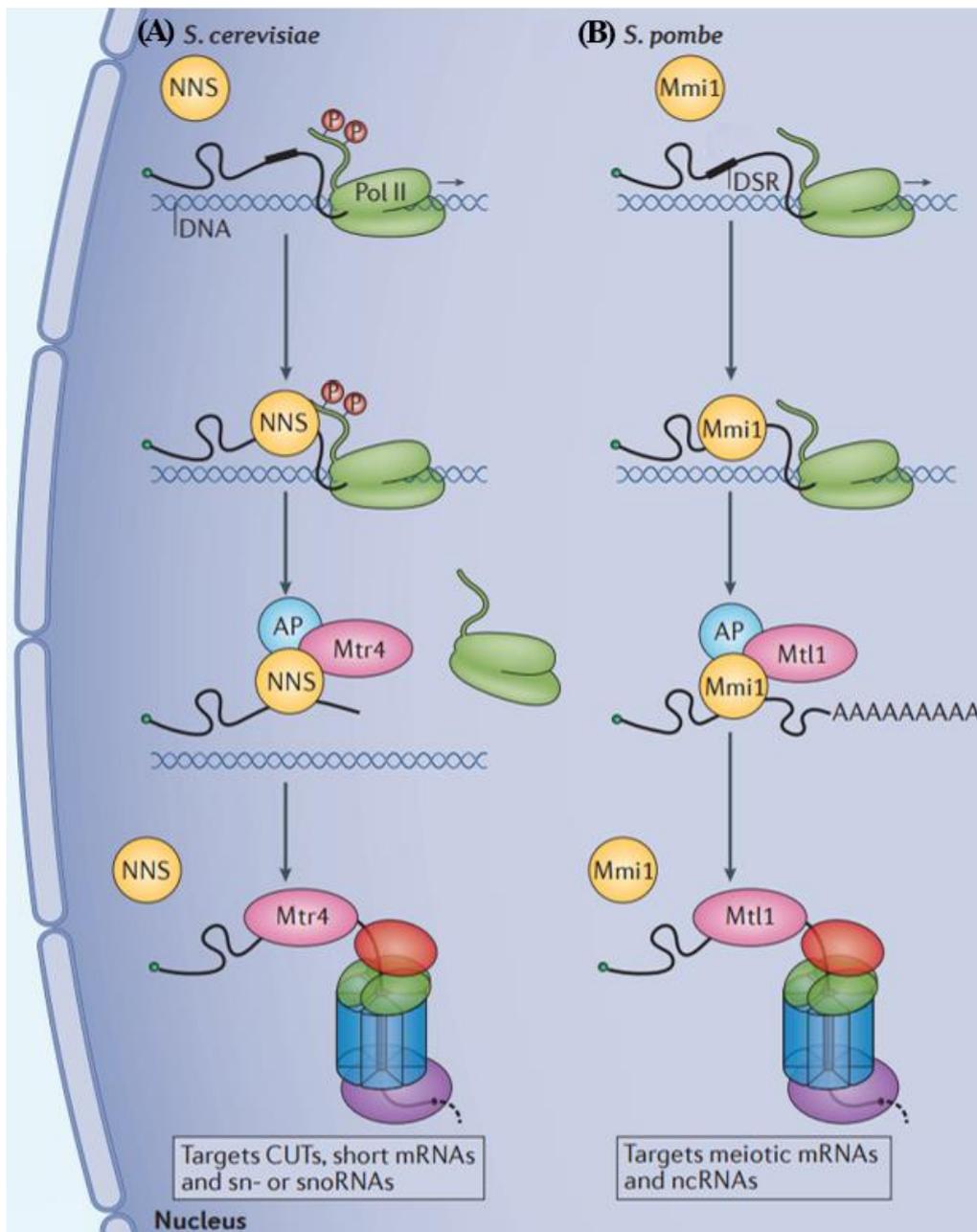


Figure 18. Exosome-targeting mechanisms display conserved features among species.

(A) In *S. cerevisiae*, the NNS complex recruits the Mtr4 helicase, which favors RNA unwinding and RNA degradation by the exosome. (B) In fission yeast, the YTH protein Mmi1 cooperates with the Mtl1 (Mtr4-like protein 1) helicase, which assists the exosome in RNA degradation. Figure adapted from Kilchert [121].

Functions of the exosome

3'-end processing

Originally, the RNA exosome was shown to promote nuclear pre-rRNA processing in yeast, and subsequently its function extended to the processing of small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) precursors [143, 144]. The exosome trims extended 3'-ends of primary transcripts down to their mature length [116]. It has been proposed that specific RNA secondary structures and/or bound proteins block the complete degradation of these species, allowing the production of functional transcripts [116, 145].

mRNA quality control

The exosome has a crucial role in the degradation of improperly processed mRNAs, such as those produced in splicing or export mutants [121]. However, limited evidence has been provided on how these aberrant RNAs are targeted by the exosome and thus distinguished from normal processing substrates. It is thought that these RNA species are targeted for degradation because they lack protective features, such as a proper poly(A) tail, secondary structures or RNA-binding proteins [116, 132, 146, 147].

Elimination of non-coding transcripts

One major function of the exosome is to degrade RNAs species that arise spuriously in the genome due to the leaky control on transcription. Several studies have shown eukaryotic genomes are indeed pervasively transcribed, leading to the production of a myriad of non-coding transcripts that are sense or antisense to known ORFs or arise from intergenic regions [148]. To avoid interference with the expression of protein-coding genes, these transcripts are rapidly targeted for degradation soon after their synthesis. Other noncoding substrates include transcripts produced from heterochromatin regions, such as the pericentromeric repeats, subtelomeres and the silent rDNA locus [134, 135, 149]. Thus, the exosome acts as a genome

surveillance factor, preventing the accumulation of undesired transcripts that are potentially toxic for cell growth.

Regulation of meiotic mRNA degradation in *S. pombe*

The exosome is the final effector of the selective elimination of Mmi1-bound meiotic mRNAs during vegetative growth. Previous studies showed that both Rrp6 and Dis3 subunits are required for proper degradation of meiotic transcripts [82, 102]. Interestingly, it was recently shown that the catalytic activity of Rrp6 has only a partial role. This led to the hypothesis that Rrp6 might mostly play a structural role, favoring the channeling of RNA substrates to Dis3 [124, 150, 151].

Interestingly, moreover, the TRAMP complex is dispensable for meiotic mRNAs degradation [102]. Rather, the process involves the canonical mRNA poly(A) polymerase Pla1 as well as 3'-end processing factors (see above). Thus, the rules that dictate the degradation of meiotic transcripts are distinct from those involved in ncRNA decay.

3.2.2.2.2 The Ccr4-Not complex

The Ccr4-Not complex is a highly conserved macromolecular and multifunctional machinery that is involved in the control of gene expression at multiple levels [152]. Over the last two decades, numerous studies, in organisms ranging from budding yeast to humans, indicated that the complex regulates chromatin modification, transcription, mRNA export and quality control, RNA deadenylation and turn-over, translational repression and protein degradation [153, 154]. Through its many functions, the Ccr4-Not complex, which localizes throughout the cell, is a master regulator that integrates different signals to fine-tune gene expression and ensures robust cell proliferation.

Discovery of the complex

The Ccr4-Not complex has been first identified and studied in budding yeast [155-159]. The majority of its components were found through genetic selections aimed at identifying factors regulating transcription [155-157, 159]. This led to the discovery of Ccr4 (Carbon Catabolite repressor), which is required for the expression of glucose-repressible enzymes [155, 158]. Ccr4 was first found to be part of a multi-subunit complex that includes Caf1 (Ccr4-associated factor 1) and Dbf2, a cell cycle-regulated protein kinase [160, 161]. Mutations in *ccr4+*, *caf1+* and *dbf2+* showed similar phenotypes, suggesting that the Ccr4 complex functions to ensure the correct expression of many genes [160, 161]. Affinity purification and mass spectrometry analyses next revealed that the Ccr4 complex contains additional factors, the so-called Not proteins (negative on TATA-less) [162]. These factors, which include Not1, Not2, Not3 and Not4, were originally identified in a screen for genes that repress the expression of TATA-less promoters [157]. These findings led to the proposal that the Ccr4-Not complex defines a unique molecular entity that affects gene transcription both positively and negatively. Since then, considerable amount of work has been performed to precisely determine the composition, biochemical activities and mechanisms of action of the complex. Below, I summarize this knowledge to provide a general picture of the properties and functions of the Ccr4-Not complex, with a particular emphasis on yeast biology.

Composition

The Ccr4-Not complex consists of a core containing about ten polypeptides depending on the organism studied [163]. The complex organizes around its large scaffolding subunit Not1 to which associate different modules and components. In budding and fission yeasts, at least six components anchor directly or indirectly to Not1. These include the RNA deadenylases Ccr4 and Caf1/Pop2, the E3 ubiquitin ligase Not4/Mot2 as well as the Not2, Not3 and Caf40/Rcd1 subunits without catalytic activity *per se* (**Table 1**). The *S. cerevisiae* complex

contains two additional subunits, namely Caf130 and Not5, the latter being a protein homologous to Not3 that is believed to originate from a gene duplication event [164, 165]. In *S. pombe*, Mmi1 has been proposed to be an integral component of Ccr4-Not [166], although it is becoming clear that it acts as an adaptor to recruit the complex to specific targets (see above). In metazoans, two paralogues of both Ccr4 (CNOT6C, CNOT6L) and Caf1 (CNOT7, CNOT8) can individually incorporate in the complex in addition to the CNOT10, and CNOT11 subunits (**Table 1**) [167-169].

<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>Drosophila</i>	<i>H. sapiens</i>	Function
Not1	Not1	Not1	CNOT1	Scaffold
Not2	Not2	Rga	CNOT2	NOT box
Not3, Not5	Not3	Not3	CNOT3	NOT box
Not4	Mot2	Not4	CNOT4	RING E3
Ccr4	Ccr4	twin	CCR4a/CNOT6C and CCR4b/CNOT6L	Nuclease
Pop2	Pop2	Pop2	Caf1a/CNOT7 and Caf1b/CNOT8	Nuclease
Caf40	Rcd1	Rcd1	CNOT9	Protein Interactions
	Mmi1			RNA binding
			CNOT11	RNA binding
Caf130				
			CNOT10	

Table 1. Ccr4-Not subunits in fission yeast (*S. pombe*), budding yeast (*S. cerevisiae*), *Drosophila* and human.

Structure and organization

The structure of the whole Ccr4-Not complex is not known in details. However, several crystal structures of domains of Not1 in association with its partners have been described in yeast, *Drosophila* and humans [169-171]. Electron microscopy experiments of the budding and fission yeast Ccr4-Not complexes [166, 172] also provided three-dimensional maps defining L-shaped particles with two arms of similar length (**Fig. 19**). Immunolocalization of the different Ccr4-Not subunits combined with 3D reconstruction and docking of available structures of components and/or domains allowed to propose a general architecture of the complex in fission yeast [166].

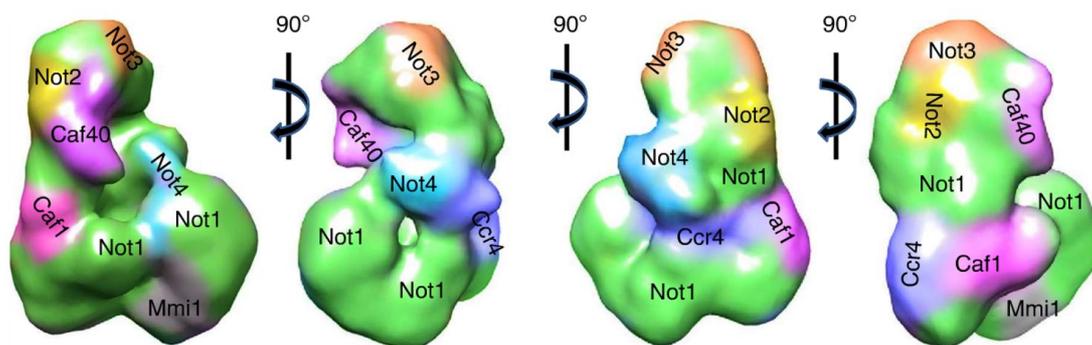


Figure 19. Structural organization of the Ccr4-Not subunits.

Pseudo-atomic model of the Ccr4-Not complex. The location of highly disordered regions is indicated by the color codes. Figure adapted from Ukleja [166].

As mentioned earlier, the complex assembles around its L-shaped scaffolding subunit Not1, a large protein of about 240 kDa. In yeast, the N-terminal portion, described as the shorter arm, contains HEAT-repeats that are involved in protein-protein interactions. The HEAT-repeats of Not1 also superpose with a MIF4G (middle domain of eukaryotic initiator factor 4G) domain [173], which is responsible for the interaction with the RNA deadenylase Caf1 that in turn binds the LRR domain of Ccr4, thereby forming the nuclease module of the complex [168, 170, 174] (**Fig. 20**).

The C-terminal portion of Not1 is dedicated to the interaction with Not2 and Not3 (or Not5 in budding yeast), which constitute the Not module [175, 176] (**Fig. 20**). Not2 and Not3 contain extended regions that wrap around Not1, the Not boxes [175]. Similarly, a largely extended region within the C-terminal of Not4 wraps around a C-terminal HEAT-repeat of Not1 [171]. Interestingly, the C-terminal region of Not4 is only partly conserved, rationalizing its weaker Not1-binding properties in metazoans. Finally, Caf40 was shown to occupy a region of Not1 located between the nuclease and Not modules [166, 169]. Therefore, Not1 can incorporate the deadenylation, ubiquitination and No2-Not3/5 modules concomitantly to form the Ccr4-Not complex.

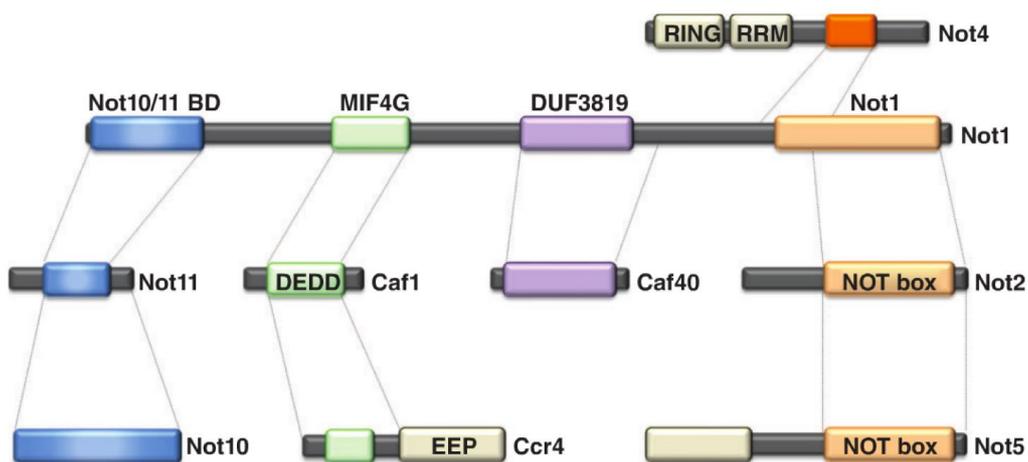


Figure 20. Schematic representation of the human Ccr4-Not subunits

The linear scheme of the Not1 subunit displays the docking sites for the other core subunits, which are conserved among eukaryotes. Shown are the catalytic domains of the Ccr4 and Caf1 deadenylases (EEP and DEDD), the ubiquitin ligase RING domain and the RNA recognition motif (RRM) of Not4, and the homology domain shared by several Not proteins (NOT box). Figure adapted from Collart [163].

Biochemical activities of the complex

The Ccr4-Not complex is endowed with two major catalytic activities: Ccr4 and Caf1-dependent RNA deadenylation and Not4/Mot2-mediated protein ubiquitination.

Caf1 belongs to the family of DEDD nucleases with 3' to 5' exoribonucleolytic activity [177]. It is characterized by the presence of the conserved amino acids Asp-Glu-Asp-Asp within the active site [177]. Its activity depends on divalent ions (Zn^{2+} , Mn^{2+}) that modulate its deadenylation kinetics [178, 179]. Ccr4 is a member of the exonuclease-endonuclease-phosphatase (EEP) family of proteins that possesses 3' to 5' exoribonuclease activity [177]. Interestingly, in budding yeast, Ccr4 is the main active subunit of the complex [180-182]. This is in contrast with other species, including fission yeast and metazoans, in which Caf1 has a predominant role in RNA deadenylation [179, 183, 184]. The presence of a noncanonical SEDQ motif in the budding yeast enzyme is the likely reason why the protein has a weak catalytic activity [178, 185]. Rather, it is believed to play a structural role by anchoring Ccr4 to Not1 [173].

The Not4/Mot2 subunit of Ccr4-Not is an E3 ubiquitin ligase of the RING family that functions to modulate protein function and/or degradation [186]. It is the final enzyme of a stepwise process, which consists in the addition of one or multiple ubiquitin molecules to a protein substrate to regulate its function and/or degradation. Briefly, an E1 enzyme first activates free ubiquitin in an ATP-dependent manner and transfers it to an E2 conjugating-enzyme. Then, the E3 ligase, associated with the substrate and the E2 enzyme, covalently attaches ubiquitin to the target protein [187, 188]. Reiteration of the process can lead to the formation of a polyubiquitin chain that marks the protein for degradation by the proteasome [188, 189] (**Fig. 21**).

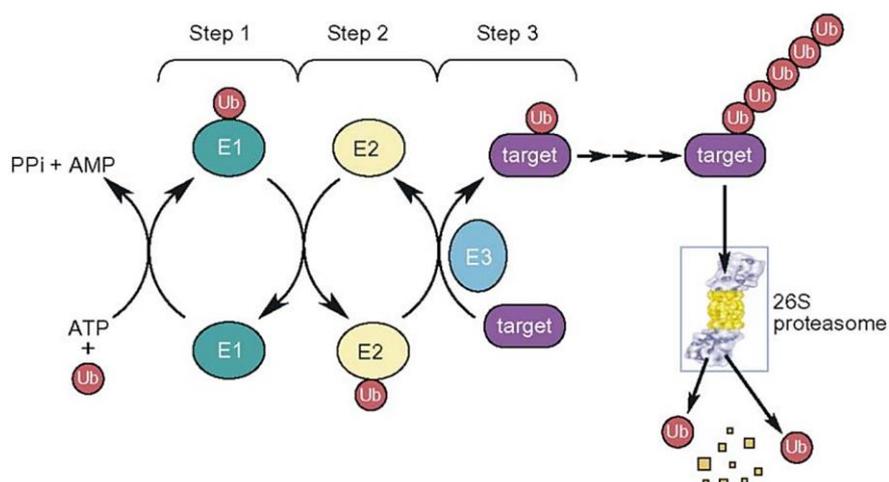


Figure 21. Ubiquitination of target proteins and degradation by the proteasome.

The activating enzyme E1 binds ubiquitin and transfer it to the ubiquitin conjugating E2 enzyme; then the E3 ubiquitin ligase transfer the ubiquitin to the protein substrate. After multiple rounds a chain of polyubiquitin is attached to the target protein which is then recognized and degraded by the proteasome. Figure from Adams [187].

In yeast, the RING finger domain of Not4/Mot2 that locates in the N-terminus is responsible for the interaction with the E2 enzymes Ubc4 and Ubc5 (**Fig. 22**) [171, 190], and this association is critical for efficient ubiquitination. Structural analyses in both budding yeast and human allowed determining how key cysteine residues within the RING domain coordinate zinc ions and precisely contact the E2 enzymes [171, 186]. Mechanistically, RING-containing proteins associate simultaneously with both the E2 enzyme and its substrate and directly transfer ubiquitin from the former to the target protein [191-193]. In contrast, E3 ligases of the HECT family form covalent intermediates with ubiquitin, before transfer to the substrate [191, 192, 194].

Not4/Mot2 also contains an RNA recognition motif (RRM) [167], followed by a second zinc finger of the C3H1 type (**Fig. 22**) [195], but the respective functions are currently unknown. Moreover, a putative coiled coil region has been found between the RRM and the RING domain (**Fig. 22**) [195].



Figure 22. Scheme of the yeast Not4 protein.

The different domains are shown: RING (RING finger domain), CC (coiled coil), RRM (RNA recognition motif), C3H1 (zinc finger domain) and the Not1 interaction domain. Figure adapted from Collart [195].

Functions

Transcription

As mentioned above, the Ccr4-Not complex has been initially described as a global regulator that affects transcription both positively and negatively. Early studies showed that Ccr4-Not is enriched at gene promoters, regulates the recruitment of the transcription factor TFIID, and genetically interacts with several transcription elongation factors [196-200]. The complex has also been functionally linked to the coactivator SAGA complex [201] and genome-wide expression profiling revealed that the Not proteins affect SAGA-controlled genes [202]. Direct evidence for a role of Ccr4-Not in transcription came from recent studies in yeast showing that the complex is recruited to genes in a transcription-dependent manner, interacts with and stimulates the activity of RNAPII both *in vivo* and *in vitro* [203-205]. This was suggested to occur through an association between Not5 and Rpb4 [206]. Recent work also linked the RNA deadenylase Ccr4 to the regulation of ribosomal RNA synthesis by RNA polymerase I in response to nutrient signaling [207].

Despite substantial evidence linking Ccr4-Not to transcription regulation, the precise mechanisms by which the complex influences gene expression is far from being fully understood.

mRNA turnover and targeted deadenylation

The Ccr4-Not complex is the major deadenylase in yeast that is required for global mRNA turn over in the cytoplasm [208]. This function is conserved in flies, mouse and human [180, 185, 209, 210].

The deadenylation process is a biphasic and rate-limiting reaction [211-213], which initiates by mRNA poly(A) tail shortening before complete removal and subsequent RNA degradation. Previous studies have indicated that the pathway is the result of a sequential action of the deadenylation enzymes Pan2-Pan3, which act during the first phase, and the Ccr4-Not complex, which is responsible for the second phase [154, 210]. The removal of poly(A) tails hampers the binding of the poly(A) binding protein Pab1, which prevents further translation [211]. This in turn triggers mRNA decapping by the Dcp1/Dcp2 enzymes and 5' to 3' degradation by the Xrn1 exoribonuclease. Alternatively, RNA decay can occur in a 3' to 5' orientation thanks to the activity of the exosome [211] (**Fig. 23**). In mammals, it has been shown that proteins of the BTG/TOB family physically bridge the poly(A)-binding protein PABP to the Caf1 deadenylase [211], thereby providing a mechanistic basis for the role of Ccr4-Not in general mRNA turnover. Nonetheless, how the yeast complex is recruited to RNA-bound Pab1 remains unclear.

Beyond its role in the degradation of the bulk of cellular mRNAs, the Ccr4-Not complex also targets specific transcripts for deadenylation. This is achieved thanks to physical interactions with sequence-specific RNA-binding proteins, which provides a mean for the post-transcriptional regulation of mRNAs. Several examples in yeast, flies and humans have shown how Ccr4-Not can be recruited. RNA-binding proteins of the PUF family recognize sequences within the 3' UTR of targeted transcripts and associate with the Caf1 deadenylase to mediate RNA decay [214, 215]. Another example is provided by the human zinc-finger containing protein tristetraprolin (TTP), which binds to short A/U rich elements present in many mRNAs

and interacts with the scaffolding subunit CNOT1 to promote Caf1-dependent deadenylation and degradation [216]. The *Drosophila* RNA-binding protein Smaug has also affinity for sequences in the 3' UTR of specific mRNAs and for Ccr4-Not, although it is unclear which subunit is bound [217]. Finally, recent studies in fission yeast revealed that the RNA-binding protein Mmi1 recruits Ccr4-Not to meiotic mRNAs, although evidence for deadenylation and degradation *in vivo* is currently lacking [84, 113, 166, 218]. In metazoans, the miRNA machinery also provides an additional way to recruit Ccr4-Not: the Argonaute protein Ago1 favors base-pairing between miRNA and targeted transcripts and indirectly recruit Ccr4-Not for targeted deadenylation and decay [219-221].

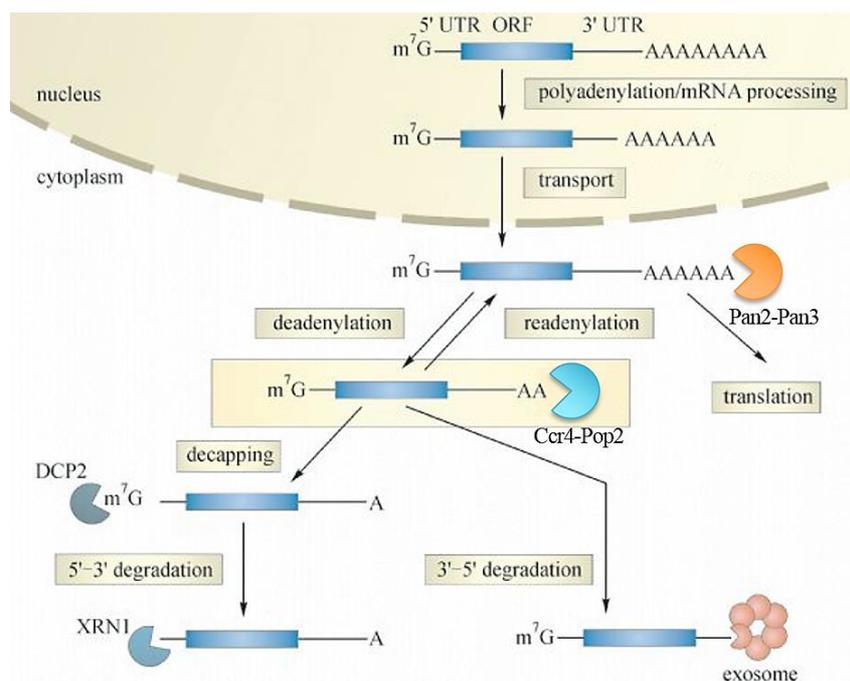


Figure 23. Model for the stepwise mRNA deadenylation pathway.

The first step of the deadenylation process comprises the shortening of the poly(A) tail by the Pan2-Pan3 complex. After this event the mRNA could still be readenylated and eventually translated. The second deadenylation step involves the Ccr4/Pop2 subunits of the Ccr4-Not complex and they further shorten the poly(A) tail, triggering the degradation of the mRNA via two general pathways. One involves the decapping enzyme complex, Dcp1-Dcp2, which hydrolyzes the 5' cap, stimulating the 5' to 3' digestion by Xrn1. In the other pathway, the deadenylation process exposes the body of the transcript for 3' to 5' degradation by the exosome. Figure adapted from Bartlam and Yamamoto [211].

mRNA synthesis and degradation rate

Recent studies have shown that the degradation of mRNAs in the cytoplasm is coordinated with the transcription process in the nucleus [222, 223]. The decrease in mRNA decay rates observed upon deletion of the deadenylase subunits of the Ccr4-Not complex indeed correlates with a decrease in mRNA synthesis, likely to buffer increased steady state levels of transcripts [224, 225]. It has been proposed that the Rpb4 and Rpb7 subunits of the RNA polymerase II form a module that follows the newly synthesized mRNA and, by virtue of its association with Ccr4-Not, impacts translation and degradation in the cytoplasm [204, 226, 227]. However, the precise mechanisms linking Ccr4-Not to transcription and RNA decay remain elusive and further investigation is required.

RNA export and quality control

The Ccr4-Not complex is also involved in the metabolism of mRNAs in the nucleus. Previous studies in budding yeast showed that Ccr4-Not physically and functionally interacts with RNA export factors as well as components of the nuclear pore complex [228]. Moreover, improperly processed or packaged mRNAs are targeted by the deadenylases Ccr4 and Pop2 for degradation [229], implicating Ccr4-Not as a nuclear mRNA surveillance factor. Several small noncoding RNAs (sn and snoRNAs) also accumulate as polyadenylated species in the absence of Ccr4 or Pop2 [230], suggesting that Ccr4-Not may cooperate with the nuclear exosome to promote RNA processing/decay, although evidence for a catalytic function awaits further investigation.

Translation

Previous studies showed that artificial tethering of the Ccr4-Not complex to a reporter mRNA represses its translation independently of deadenylation [231, 232]. Similar effects have been observed with mRNAs targeted by the microRNA machinery in metazoans (reviewed in

Inada and Makino [233]). Nevertheless, the specific mechanism of action is still unknown. It has been proposed that the MIF4G (middle domain of eukaryotic initiator factor 4G) domain of Not1, otherwise found in other proteins implicated in translation regulation [234-236], associates with and stimulates the activity of the helicase DDX6 (Dhh1 in yeast) (reviewed in Collart [163]). It has been postulated that Ccr4-Not might help the recruitment of mRNAs to P-bodies, cytoplasmic structures involved in mRNA storage in which Dhh1 and/or other factors would inhibit their translation (reviewed in Miller and Reese [153]).

Moreover, the E3 ubiquitin ligase subunit Not4 plays a role in co-translational protein quality control [237, 238]. In budding yeast, indeed, *not4* Δ cells accumulate polyubiquitinated proteins independently of the deadenylation module. It has been proposed that Not4 ubiquitinates nascent polypeptides and targets them for degradation by the proteasome [239]. However, recent evidence challenged this idea, showing that Not4 does not promote the turnover of arrested polypeptides, but is involved in global translational repression of ribosome stalling mRNAs, most likely in collaboration with decapping factors [240].

Recent findings also showed that the Not5 subunit is important for the presence of some mRNAs in polysomes [206]. Moreover, Not5 favors the co-transcriptional association of Not1 with ribosomal protein mRNAs, which is critical for efficient translation [241]. Thus, Ccr4-Not coordinates transcription and translation to produce more ribosomes and thus increase the efficiency of translation globally [241].

Protein ubiquitination

The presence of the E3 ubiquitin ligase subunit Not4/Mot2 provides a link between the Ccr4-Not complex and the ubiquitin-proteasome pathway [186, 190]. Previous studies in budding yeast showed that Mot2 targets several factors for ubiquitination to regulate various cellular processes. Notably, Mot2 promotes polyubiquitination and subsequent proteasome-dependent degradation of Jhd2, a histone demethylase specific to H3K4, both in yeast and humans [242]. Mot2 also affects the turnover of the transcription activator Yap1 [243] and cyclin C, which is required for the transcriptional repression of stress response genes [244]. Likewise, the human homologue CNOT4 controls the levels of the PAF1 subunit of the evolutionary conserved PAF transcription complex [245]. These functions of Mot2 provide an additional layer in the regulation of gene expression and it has been suggested that some of them may account for the requirement of Ccr4-Not in transcription. Moreover, Not4 mediates the destabilization of Cdc17, the catalytic subunit of polymerase α , indicating that Ccr4-Not also controls the efficiency of DNA replication [246]. Finally, our results indicate that the fission yeast Not4/Mot2 contributes to the ubiquitination and subsequent degradation of a positive regulator of meiosis, the Mmi1 inhibitor Mei2, thereby ensuring efficient meiotic mRNAs degradation during vegetative growth (see Results section for more details).

Not4 not only triggers polyubiquitination-dependent protein degradation but also mediates regulatory ubiquitination, which alters the function of substrates. For example, Mot2 ubiquitinates the Egd1 and Egd2 subunits of the conserved ribosome-associated complex (called NAC in mammals for Nascent polypeptide-associated complex) [247], which is essential for the correct folding of newly synthesized proteins. Because ubiquitination of Egd proteins is important for their association with both the ribosome and the proteasome [248], it has been suggested that Not4 may coordinate protein synthesis and degradation. In addition, Not4 targets the ribosomal protein Rps7A for ubiquitination, which negatively affects the

interaction of Ccr4-Not with ribosomes [222]. It was therefore proposed that the E3 ligase activity of Not4 controls the association of the Ccr4-Not complex with polysomes [222].

Proteasome assembly

The Ccr4-Not complex was also reported to impact the assembly of the proteasome itself [249]. In *not4Δ* cells, polyubiquitinated proteins accumulate and the structural integrity of the proteasome is altered. It is believed that Not4 controls the interaction between the proteasome and the chaperone protein Ecm29, involved in proteasome assembly and stability [195]. However, the mechanism by which the complex regulates the assembly of the proteasome is not fully understood. Indeed, deletion of the RNA deadenylase Caf1 leads to similar defects and mutations that disrupt the interaction of Not4 with its partner E2 enzymes instead preserve proteasome integrity and function [237, 249].

3.2.2.2.3 The RNA interference machinery

Previous works showed that the RNAi machinery, implicated in the maintenance of genome integrity through the degradation of noncoding transcripts emanating from heterochromatin regions [250, 251], also contributes to the degradation of meiotic mRNAs targeted by Mmi1 [96, 252]. Indeed, deletion of the Argonaute protein Ago1 or of the Dicer family endoribonuclease Dcr1 triggers the accumulation of DSR-containing mRNAs [96, 252]. Moreover, Mmi1 recruits the Ago1-containing RITS complex to its target genes [96]. However, the effect of RNAi factors is considerably weaker than the ones observed in the absence of Mmi1 or the exosome, indicating that the RNAi machinery is not a major player in meiotic mRNA suppression. Nonetheless, removal of Ago1 or Dcr1 partially suppresses the sporulation defects associated with the deletion of the *sme2+* locus, which implicates a physiological role for RNAi in sexual differentiation.

3.2.3 A chromatin-based regulation of meiotic mRNA suppression?

Several studies have reported the presence of repressive chromatin marks (e.g. dimethylated histone H3 lysine 9 or H3K9me2) at a subset of meiotic genes during vegetative growth [96, 113, 252-254]. Interestingly, H3K9me2 marks disappear when cells are starved for nitrogen, suggesting that the expression of meiotic mRNAs might be under the control of an epigenetic mechanism [254]. In addition, the MTREC subunit Red1 was found to associate with the sole H3K9 methyltransferase in *S. pombe*, Clr4 [254]. This led to the proposal that Mmi1 and MTREC may co-transcriptionally recruit the heterochromatin machinery to further repress meiotic mRNAs expression at the chromatin level in mitotic cells. However, the biological relevance of such heterochromatin marks remains unclear. Indeed, deletion of Clr4, which disrupts H3K9me2 at meiotic genes, does not lead to an accumulation of meiotic transcripts [252, 254], our unpublished data). It was proposed that H3K9me2 by Clr4 regulates the balance between silencing and anti-silencing activities, through the recruitment of factors with opposite function (i.e. RITS and Epe1 respectively) [252]. The effects of these downstream actors are however rather limited with respect to the expression of meiotic genes [96, 252]. Similarly, the requirement for the Ccr4-Not complex, including the E3 ligase subunit Mot2, in the deposition of H3K9me2 did not correlate with an accumulation of meiotic transcripts in the conditions examined [218]. Instead, the post-transcriptional regulation exerted by Mmi1, MTREC and the nuclear exosome, and the post-translational control of Mei2 levels by Ccr4-Not (see “results” section) have predominant roles in meiotic mRNA suppression. Importantly, moreover, only a subset of Mmi1 target genes are covered by silent chromatin, indicating that H3K9me2 is not part of a general pathway that controls the expression of developmental genes. It is possible that H3K9me2 deposition might be the

Introduction

consequence of an active RNA degradation mechanism rather than a regulatory process *per se*. Alternatively, other chromatin signatures may act redundantly with H3K9me2 to maintain low expression levels of meiotic gene.

RESULTS

1 Summary and contribution

In the fission yeast *S. pombe*, an RNA degradation system selectively eliminates meiosis-specific transcripts produced during the mitotic cell cycle, thereby inhibiting sexual differentiation. The YTH-family RNA-binding protein Mmi1 (meiotic mRNA interceptor 1) mediates this regulatory process by recognizing *cis*-acting elements within meiotic mRNAs, called DSR (Determinant of Selective Removal), which allow their targeting to the nuclear exosome for degradation. Mmi1 associates and cooperates with a multi-subunit complex called MTREC, which has been proposed to physically bridge RNA-bound Mmi1 to the nuclear exosome.

Upon nutritional starvation, Mmi1 is sequestered in an RNP complex, which include the RNA-binding protein Mei2, essential for the first meiotic division, and the DSR containing, lncRNA meiRNA, encoded by the *sme2+* gene. The formation of this RNP complex has a key role in sexual differentiation through the inactivation of Mmi1, which ensures the correct expression of meiotic mRNAs.

We have shown that Mmi1 associates with the evolutionarily conserved Ccr4-Not deadenylation complex to promote meiotic mRNAs suppression in mitotic cells. Intriguingly, the function of Ccr4-Not does not depend on its deadenylation activity but requires its E3 ubiquitin ligase subunit Mot2. Specifically, Mot2 targets a pool of the Mmi1 inhibitor Mei2 for ubiquitination and subsequent degradation by the proteasome, therefore maintaining Mmi1 in a functional state. Importantly, Mot2 is unlikely to mediate constitutive degradation of Mei2, which depends for its largest share on Ubr1, another E3 ubiquitin ligase. Rather, Mot2 targets a fraction of Mei2, which is critical for the persistent repression of meiotic mRNAs in vegetative cells.

Our work unveils a novel regulatory circuit involved in the control of meiosis onset whereby a protein (i.e. Mmi1) controls the levels of its own inhibitor (i.e. Mei2). It also reports a novel role for the ubiquitin ligase activity borne by the Mot2 subunit of the Ccr4-Not complex in the control of sexual differentiation in *S. pombe*. The results obtained from this work are included in a scientific paper that has been submitted for publication (see below).

Specifically, my contribution relates to the analysis of the interaction between Mmi1 and the Ccr4-Not complex and to the identification and characterization of Mot2 as a key player in meiotic mRNA suppression, through the downregulation of Mei2. I confirmed and dissected the interaction between Mmi1 and the Ccr4-Not complex found by mass spectrometry. I analyzed the levels of meiotic transcripts using gene-specific (e.g. RT-qPCR) and genome-wide approaches (RNA-seq) in various mutant strains and also contributed to the biochemical analysis of Mei2 protein by the E3 ligases Mot2 and Ubr1. Finally, I performed additional experiments to further support our conclusions.

2 Article

Ubiquitination-dependent control of sexual differentiation in fission yeast

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ABSTRACT

In fission yeast, meiosis-specific transcripts are selectively eliminated during vegetative growth by the combined action of the YTH-family RNA-binding protein Mmi1 and the nuclear exosome. Upon nutritional starvation, the master regulator of meiosis Mei2 inactivates Mmi1, thereby allowing expression of the meiotic program. Here, we show that the evolutionarily conserved Ccr4-Not deadenylation complex promotes suppression of meiotic transcripts expression in mitotic cells. Surprisingly, suppression is not linked to the deadenylase activity of Ccr4-Not but depends on its E3 ubiquitin ligase subunit Not4/Mot2 that ubiquitinates Mei2 to restrict its levels during vegetative growth. We demonstrate that Mmi1 recruits the Ccr4-Not complex to limit the accumulation of its own inhibitor Mei2, thereby locking the system in a stable state that ensures the repression of the meiotic program by Mmi1. This study discloses unprecedented functions for Mmi1 and the conserved Ccr4-Not complex.

INTRODUCTION

The cell cycle switch from mitosis to meiosis is associated with profound changes in gene expression. In yeast, initiation of meiosis occurs upon nutrient starvation and depends on well-characterized signaling pathways¹. Several hundred genes are induced thanks to specific transcription factors, which define the key steps of the meiotic program². Previous work in *S. pombe* has revealed the existence of an additional mechanism that controls the onset of meiosis. An RNA degradation system selectively eliminates meiosis-specific transcripts produced during the mitotic cell cycle, thereby inhibiting sexual differentiation³. Essential to this regulatory process is Mmi1 (meiotic mRNA interception factor 1), a member of the conserved YTH family of RNA-binding proteins that localizes exclusively to the nucleus. Mmi1 recognizes a *cis*-acting region within targeted mRNAs, called DSR (Determinant of Selective Removal), which confers sensitivity to nuclear exosome-mediated degradation. DSR regions are enriched in repeats of the hexanucleotide motif UNAAAC to which Mmi1 binds to via its C-terminal YTH domain^{4,5}. Mmi1 also associates to several coding and non-coding RNAs with fewer UNAAAC motifs, indicating flexibility in target recognition⁶⁻⁸.

Several factors identified both by genetic screens and biochemical analyses, cooperate with Mmi1 to promote meiotic mRNA suppression during vegetative growth. Previous studies demonstrated a role for components of the 3'-end processing machinery as well as the canonical poly(A) polymerase Pla1 and the poly(A) binding protein Pab2^{9,10}. Mechanistically, it was first proposed that Mmi1 promotes hyperadenylation of targeted transcripts and binding of Pab2 onto mRNAs poly(A) tails, which in turn recruits the nuclear exosome subunit Rps6 for degradation. Mmi1 was next found to associate and cooperate with a multi-subunit complex called MTREC or NuRS to mediate meiotic mRNAs suppression¹¹⁻¹⁵. Core components of this complex, hereafter referred to as MTREC, include the zinc finger-containing protein Red1 and

the Mtr4-like RNA helicase Mtl1¹³⁻¹⁵. MTREC is crucial for meiotic mRNA degradation and has been proposed to physically bridge RNA-bound Mmi1 to the nuclear exosome. Recent studies also reported an interaction between Mmi1 and the Ccr4-Not complex¹⁶⁻¹⁹, the major cytoplasmic mRNA deadenylation machinery conserved from yeast to humans²⁰. However, although Mmi1 recruits Ccr4-Not to its RNA targets *in vivo*¹⁶ and stimulates its deadenylation activity *in vitro*¹⁹, the complex is not required for the turnover and the translation of DSR-containing meiotic mRNAs^{16,18}.

A subset of Mmi1-regulated meiotic genes is covered by repressive chromatin marks (i.e. dimethylated histone H3 lysine 9 or H3K9me2) during vegetative growth, which disappear upon entry into meiosis²¹⁻²³. Mmi1 recruits the H3K9 methyltransferase Clr4 via the MTREC subunit Red1 and directs components of the RNAi machinery to these loci²¹⁻²³. However, the contribution of heterochromatin and RNAi factors to the silencing of DSR-containing meiotic mRNAs is moderate²¹⁻²³. Rather, the post-transcriptional degradation pathway mediated by Mmi1, MTREC and the exosome is predominant.

Upon nutritional starvation, Mmi1 is sequestered in an RNP complex, which allows translation of meiotic mRNAs and progression of the cell through meiosis³. This inhibitory complex includes the nucleocytoplasmic shuttling RNA-binding protein Mei2, required for pre-meiotic DNA synthesis and the first meiotic division, and the DSR-containing, lncRNA meiRNA, encoded by the *sme2+* gene²⁴⁻²⁶. Sequestration of Mmi1 by the Mei2-meiRNA complex occurs at the *sme2+* locus^{26,27} and failure to assemble this structure prevents entry into meiosis, highlighting its biological relevance for sexual differentiation³.

Mitotic cells exploit transcriptional and post-translational mechanisms to control Mei2 abundance and activity. *mei2+* expression depends on the meiosis-specific transcription factor Ste11²⁸ and its activity and stability are regulated by phosphorylation via two protein kinases, Pat1 (also known as Ran1) and Tor2, both of which are essential for vegetative growth and

inhibition of sexual differentiation²⁹⁻³¹. Phosphorylated forms of Mei2 are inhibited by 14-3-3 family proteins³² and are targeted for degradation by the proteasome in an ubiquitination-dependent manner^{29,31}.

Despite recent progresses, a full understanding of how Mmi1 cooperates with its cofactors during vegetative growth to prevent initiation of the meiotic program is still elusive. Using affinity purification and co-immunoprecipitation assays, we show that Mmi1, but not MTREC, stably associates *in vivo* with the evolutionarily conserved Ccr4-Not complex. This interaction is functionally relevant because integrity of the Ccr4-Not complex is required for meiotic mRNA suppression during vegetative growth. Surprisingly, we show that the RNA deadenylases Ccr4 and Caf1/Pop2 are dispensable, while the E3 ubiquitin ligase subunit Not4/Mot2 is essential for controlling the levels of meiotic transcripts. Importantly, biochemical and genetic analyses indicate that this function is mechanistically linked to the ubiquitination and proteasome-dependent degradation of a pool of the Mmi1 inhibitor Mei2. Our data demonstrate that Mmi1 fine-tunes the levels of its own inhibitor by inducing its degradation. They also unveil an important role of the Ccr4-Not complex in providing an additional level of control to the repression of the meiotic program in vegetative cells.

RESULTS

Mmi1 associates independently with the MTREC and Ccr4-Not complexes *in vivo*

To obtain a comprehensive view of Mmi1 protein partners during vegetative growth, we affinity-purified a C-terminally TAP-tagged version of the protein and analysed interacting proteins by mass spectrometry (Fig. 1a,b; Supplementary Fig. 1a). We identified several factors that co-purify with Mmi1, even when extracts were treated with RNases (Fig. 1b, Supplementary Table 1). As previously reported^{12-15,18}, we found components of the MTREC complex, the Mmi1 cofactor Erh1 as well as splicing factors. Strikingly, the most abundant interacting factors were the subunits of the Ccr4-Not complex (Fig. 1b), which was also reported independently while this work was in progress¹⁶⁻¹⁹. Other associated proteins include subunits of the proteasome, transcription and chromatin remodeling factors as well as components of signaling pathways (Supplementary Table 1). We further focused on the functional implications of the interaction between Mmi1 and Ccr4-Not.

The Ccr4-Not complex assembles around its scaffolding component Not1 and contains three catalytic subunits, the Ccr4 and Caf1/Pop2 deadenylases and the E3 ubiquitin ligase Not4/Mot2¹⁷. We validated the interactions revealed by the MS analysis using co-immunoprecipitation experiments. Mmi1 efficiently pulled down Not1, Pop2 and Mot2 in an RNA-independent manner (Fig. 1c,d,e). These interactions were preserved in the absence of Red1, which is required for MTREC integrity, possibly supporting the existence of alternative Mmi1-containing complexes. Consistent with this notion, Mtl1 and Red1 did not interact with Not1 and Pop2 in our assays (Fig. 1f and Supplementary Fig. 1b). Together, our data demonstrate that Mmi1 associates independently with MTREC and Ccr4-Not during vegetative growth.

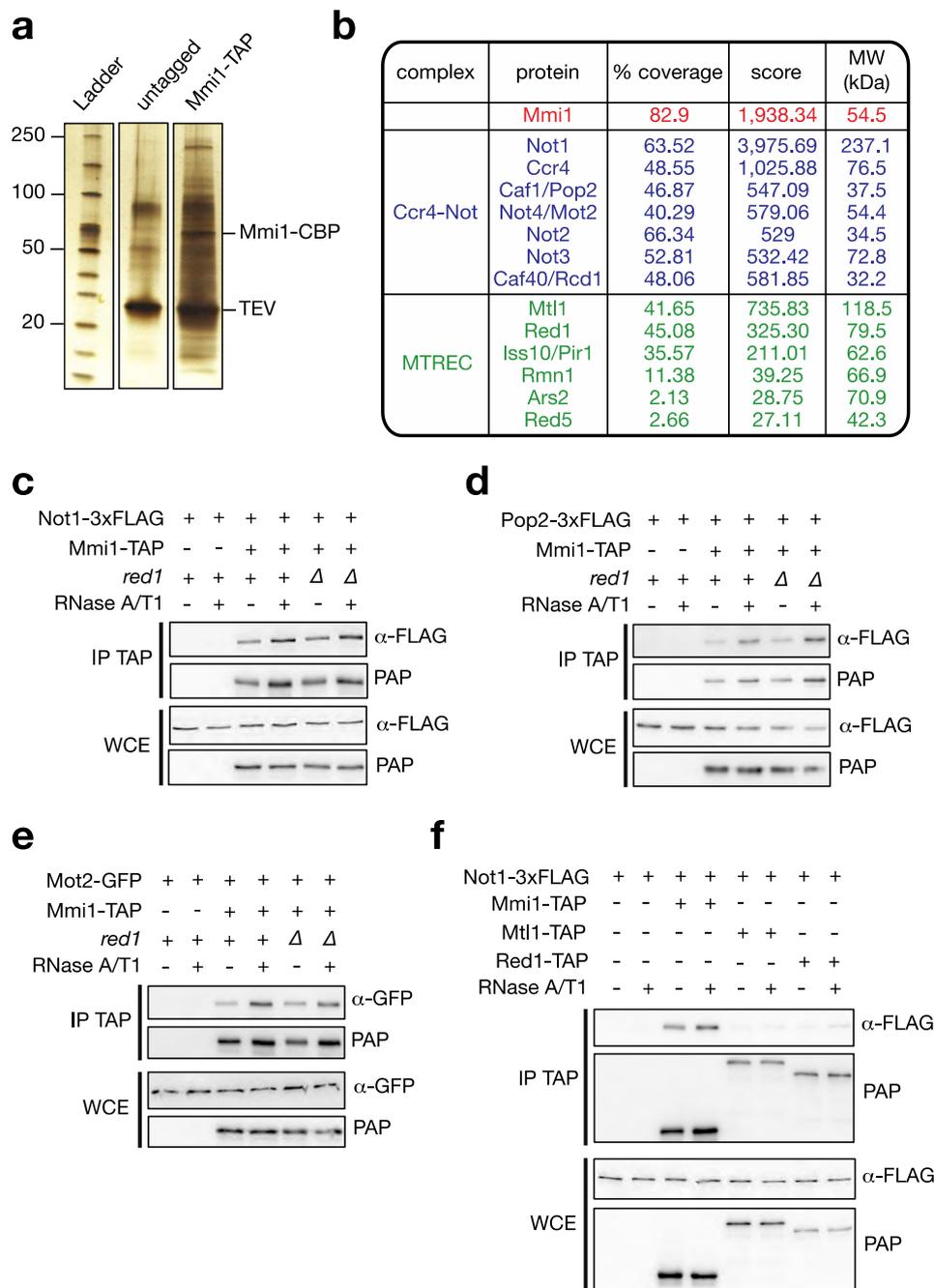


Figure 1. Mmi1 associates independently with the MTREC and Ccr4-Not complexes *in vivo*.

(a) Silver-stained SDS polyacrylamide gel showing proteins co-eluting with TAP-tagged Mmi1 in minimal medium (EMM0.5X) after one-step affinity purification. Extracts were treated with RNaseA/T1 before immunoprecipitation and TEV cleavage. As a control, extracts from cells expressing untagged protein were used. The positions of the bait protein (Mmi1-CBP) and TEV are indicated. (b) Results of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of Mmi1-TAP-associated proteins. The percentage of sequence coverage, scores (i.e. significance of the identified peptides represented as the $-\log_{10}$ of the Posterior Error Probability provided by the Percolator algorithm) and molecular weights are indicated.

(c-e) Western blots showing that Not1-3xFLAG (c), Pop2-3xFLAG (d) and Mot2-GFP (e) co-immunoprecipitate with Mmi1-TAP in minimal medium (EMM0.5X) in an RNA- and Red1-independent manner. (WCE) Whole Cell Extract; (IP) Immunoprecipitation. (f) Western blots showing that Not1-3xFLAG co-immunoprecipitates with Mmi1-TAP in minimal medium (EMM0.5X), but not with Mtl1-TAP and Red1-TAP. (WCE) Whole Cell Extract; (IP) Immunoprecipitation.

The E3 ubiquitin ligase Mot2 of the Ccr4-Not complex is required for meiotic mRNAs suppression

The Ccr4-Not complex localizes throughout the cell and is involved in essentially all steps in gene expression, including chromatin modification, transcription, nuclear and cytoplasmic mRNA degradation, translational repression, protein degradation and quality control^{33,34}. To assess whether Ccr4-Not plays a role in the suppression of meiotic mRNAs by Mmi1 during vegetative growth, we first examined steady state levels of meiotic transcripts by RT-qPCR in strains deleted for its non-essential subunits (i.e. all but the scaffolding component Not1). Neither the RNA deadenylases Ccr4 and Pop2 nor the E3 ubiquitin ligase Mot2 were required for meiotic mRNAs degradation when cells were grown in rich medium, in contrast to the nuclear exosome subunit Rrp6 (Fig. 2a). However, a significant role for the complex became apparent when cells were cultured in minimal medium. Indeed, several selected meiotic mRNAs, including *mei4+*, *ssm4+* and *mcp5+* were upregulated specifically in the *mot2Δ* mutant (Fig. 2b), in some cases to levels comparable to those observed in *rrp6Δ* cells (e.g. *mcp5+*). Interestingly, the lack of meiotic RNAs stabilization in *pop2Δ* and *ccr4Δ* mutants indicates that the deadenylation activity of the complex is not required for RNA degradation, consistent with recent work^{16,18}. Rather, the specific requirement for the Mot2 subunit strongly suggests the unprecedented possibility that meiotic mRNA suppression involves protein ubiquitination.

To establish whether the requirement for Mot2 in meiotic mRNA suppression is general, we analyzed the transcriptomes of wild type, *mot2Δ* and *rrp6Δ* strains grown in

minimal medium by RNA-seq. Statistical analyses revealed increased expression for 622 and 393 transcripts in the *mot2* Δ and *rrp6* Δ mutants respectively, when compared to wild type cells (Fig. 2c, Supplementary Table 2). A highly significant overlap of 100 transcripts indicates that Rrp6 and Mot2 function to repress expression of a common set of targets (Fig. 2d). Importantly, a large fraction of these RNAs contain DSR elements and are known targets of Mmi1, MTREC and the nuclear exosome during vegetative growth^{13,22}. Gene ontology analysis indicated that upregulated transcripts in *mot2* Δ cells fall into three distinct functional categories (Supplementary Table 2). The most significant group included genes involved in meiosis or that show an increase in expression upon meiotic induction. The second group includes genes required for mating and conjugation, and the third class contains genes encoding protein kinases (Supplementary Table 2). However, these two latter categories were not over-represented in the *rrp6* Δ mutant, suggesting that the genes they belong to are not regulated in an Mmi1-dependent manner. Whether this relates to other functions of Mot2 and/or indirect effects remains to be determined. Together, our results reveal a global requirement for Mot2 in suppressing meiotic transcripts, among which Mmi1 targets are significantly enriched. These data further indicate a prominent role for the Ccr4/Not complex in inhibition of sexual differentiation.

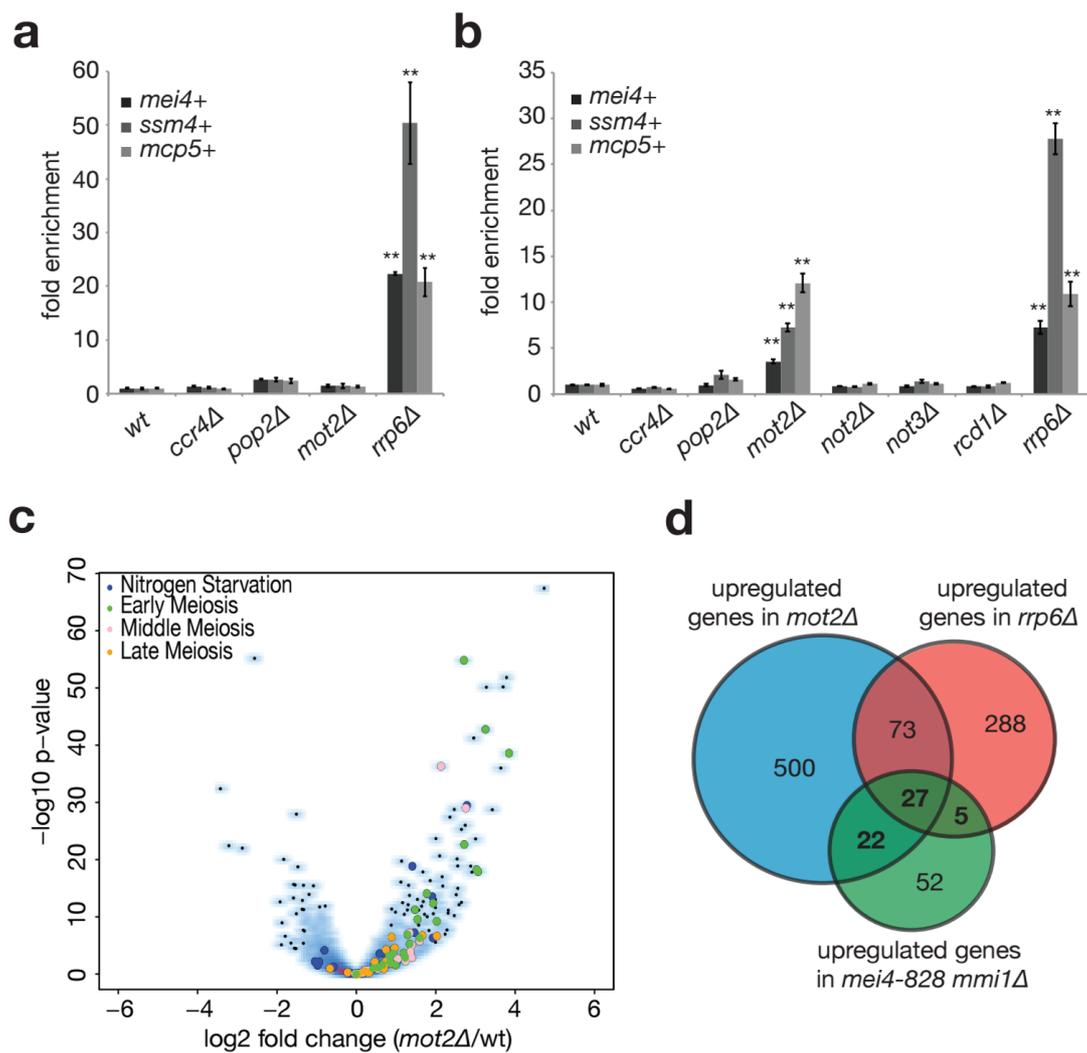


Figure 2. Mot2 is required for meiotic mRNAs suppression in nutrient-limiting growth conditions.

(a) RT-qPCR analysis of the *mei4+*, *ssm4+* and *mcp5+* meiotic transcripts in cells grown in rich medium (YE) and deleted for Ccr4, Pop2, Mot2 or Rrp6. Signals were normalized to *act1+* mRNA levels and expressed relative to the wild type strain. Error bars represent the standard deviation from at least three independent experiments. Stars denote statistical significance relative to wild type cells (see Supplemental Information). (b) RT-qPCR analysis of the *mei4+*, *ssm4+* and *mcp5+* meiotic transcripts in cells grown in minimal medium (EMM0.5X) and deleted for all non-essential Ccr4-Not subunits (i.e. all but Not1) or Rrp6 as a control. Signals were normalized to *act1+* mRNA levels and expressed relative to the wild type strain. Error bars represent the standard deviation from at least three independent experiments. Stars denote statistical significance relative to wild type cells (see Supplemental Information). (c) Comparison of the *mot2Δ* (duplicate) and wild type (triplicate) transcriptomes in minimal medium (EMM0.5X). Volcano plot shows the fold change (log₂) on the x axis and the *P*-value distribution (-log₁₀ *P* value) on the y axis for the transcripts identified in RNA-seq analysis. Each dot represents one transcript and the color code refers to the different functional categories of Mmi1 targets, as described in ²². (d) Venn diagram showing the overlap of transcripts stabilized in *mot2Δ* and *rrp6Δ* strains and compared to Mmi1 targets²².

Ccr4-Not promotes meiotic mRNA suppression by limiting the accumulation of the Mmi1 inhibitor Mei2

The E3 ubiquitin ligase Not4/Mot2 has various functions in protein metabolism. Previous work showed that Mot2 controls protein turnover through ubiquitination and proteasome-dependent degradation to regulate chromatin modification, DNA replication, transcription as well as translation³⁵⁻⁴¹. Other studies revealed roles in the functional integrity of the proteasome⁴² and in translational quality control⁴³⁻⁴⁵. Mot2 also mediates non-destabilizing protein ubiquitination⁴⁶, highlighting its functional versatility.

The requirement of Mot2 for meiotic mRNAs suppression might reflect the need for degrading a protein inhibitor of nuclear RNA decay during vegetative growth. One possible candidate is the RNA-binding protein Mei2, which functions to inhibit the activity of Mmi1³. We therefore hypothesized that Mmi1 might recruit the Ccr4-Not complex to Mei2 to promote its ubiquitination by Mot2 and subsequent proteolysis.

To test this hypothesis, we first determined Mei2 protein levels in wild type and *mot2Δ* cells grown in different conditions. In the presence of Mot2, Mei2 was not detected in rich medium and only to low levels in minimal medium. As expected from its role in meiosis onset, its levels increased in sporulation medium (Fig. 3a). Importantly, deletion of *mot2+* resulted in a strong accumulation of Mei2 in all growth conditions, with levels in minimal medium that were even higher than those observed in the wild type strain grown in sporulation medium (Fig. 3a, Supplementary Fig. 2a, compare lanes 6 to lanes 8). Co-immunoprecipitation assays and MS analyses of Mmi1 purified from the *mot2Δ* mutant grown in minimal medium indicated that Mei2 levels were also increased in the Mmi1-Ccr4-Not complex (Supplementary Fig. 2b, Supplementary Table 1). Higher Mei2 levels in *mot2Δ* cells might result from an effect on the production (or stability) of *mei2+* mRNAs. Indeed, we found increased *mei2+* transcript levels in the *mot2Δ* mutant (Supplementary Fig. 2c), possibly suggesting a negative control exerted

by Mot2 on the promoter of the *mei2+* gene. To assess whether Ccr4-Not regulates Mei2 levels in a transcription-independent manner, we replaced the endogenous *mei2+* promoter with the *nmt1+* promoter, which is active in minimal medium lacking thiamine. In this context, we also detected a four-fold increase of the Mei2 protein in the *mot2* Δ mutant relative to wild type cells (Fig. 3b,c), while *mei2+* mRNA levels remained similar (Fig. 3d). Moreover, defective meiotic mRNA suppression was still observed in the absence of Mot2 (Supplementary Fig. 2d). These data demonstrate that increased Mei2 protein levels are not due to altered transcription and/or stability of *mei2+* mRNAs in *mot2* Δ cells.

We also examined whether the control of Mei2 abundance involves additional Ccr4-Not components. Deletion of the non-essential subunits of the complex other than Mot2, including the RNA deadenylases Ccr4 and Pop2, did not result in the accumulation of Mei2 (Supplementary Fig. 2e). This indicates that the regulation of Mei2 levels depends specifically on the integrity of the E3 ubiquitin ligase.

Our results strongly suggest that the Ccr4-Not complex suppresses the expression of meiotic RNAs in vegetative cells by limiting the steady state levels of the Mmi1 inhibitor Mei2. It is however possible that the Ccr4-Not complex affects the levels of meiotic RNAs independently of Mei2. To demonstrate that the two events are causally linked, we assessed the levels of meiotic transcripts in a *mot2* Δ *mei2* Δ double mutant. Crucially, deletion of *mei2+* restored degradation of meiotic mRNAs in the absence of Mot2 but not in *rrp6* Δ cells (Fig. 3e), indicating that loss of Mei2 does not induce degradation of these transcripts by another pathway. Importantly, over-expression of the *mei2+* gene in a wild type background led to a strong stabilization of meiotic RNAs (Fig. 3f), phenocopying the effect of a *mot2* Δ mutant. Thus, these results demonstrate that the Ccr4-Not complex is important to maintain low levels of Mei2 to ensure efficient Mmi1- and exosome-dependent degradation of meiotic transcripts during vegetative growth. The E3 ubiquitin ligase Mot2 is central to this regulation.

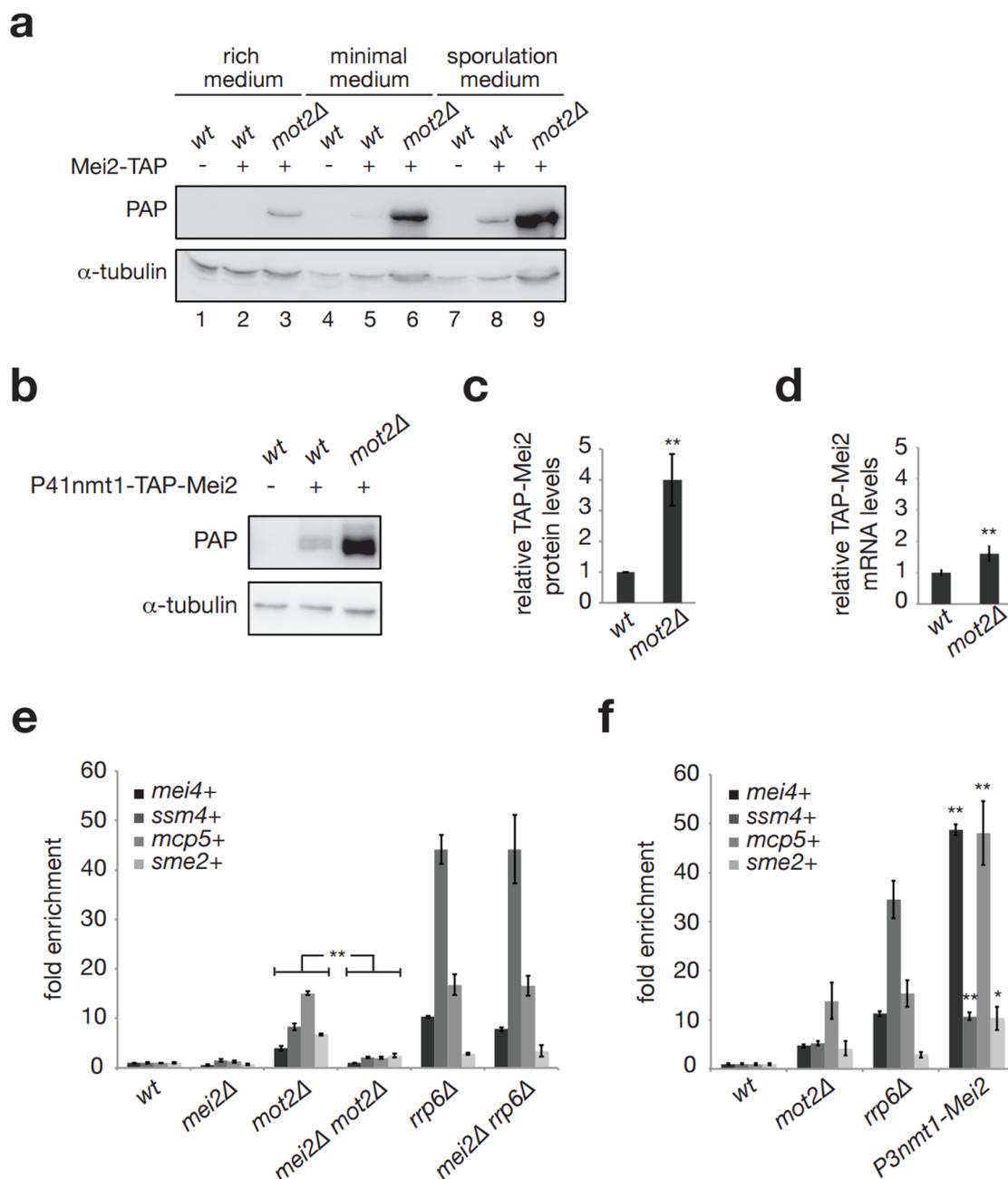


Figure 3. Ccr4-Not promotes meiotic mRNA suppression by limiting the accumulation of the Mmi1 inhibitor Mei2.

(a) Western blot showing total Mei2-TAP levels in wt and *mot2Δ* cells grown at 30°C to mid-log phase in rich (YE), minimal (EMM0.5X) and sporulation (ME) media. An anti-tubulin antibody was used as a loading control. Note that a fraction of wild type cells undergoes mating and meiosis in ME. (b) Western blot showing total TAP-Mei2 levels expressed from the P41nmt1 promoter in wt and *mot2Δ* cells grown in minimal medium (EMM0.5X). An anti-tubulin antibody was used as a loading control. (c) Quantification of TAP-Mei2 protein levels, normalized to tubulin and expressed relative to wt cells. Error bars represent the standard deviation from five experiments (t-test p-value=1.38E-3). (d) RT-qPCR analysis of *mei2+* mRNAs

levels expressed from the P41nmt1 promoter in wt and *mot2* Δ cells grown in minimal medium (EMM0.5X). Signals were normalized to *act1+* mRNA levels and expressed relative to the wild type strain. Error bars represent the standard deviation from at least three independent experiments (t-test p-value=8.5E-3). (e-f) RT-qPCR analyses of meiotic transcripts in cells of the indicated genetic backgrounds grown in minimal medium (EMM0.5X). Shown is the fold enrichment of RNAs levels normalized to *act1+* transcripts and expressed relative to the wild type strain. Error bars represent the standard deviation from three independent experiments. (e) Pairwise comparisons of four meiotic transcripts in *mot2* Δ versus *mei2* Δ *mot2* Δ mutants give p-values < 8E-3. (f) Stars denote statistical significance relative to wild type cells (see Supplemental Information).

Examination of exponentially growing cells by microscopy did not reveal the occurrence of ectopic meiosis in *mot2* Δ cells (Supplementary Fig. 3a), supporting the notion that meiotic transcripts accumulate in actively dividing cells and not in a fraction of cells undergoing meiosis. *mot2* Δ cells displayed a moderate growth defect in minimal medium that was at least partially dependent on the inappropriate expression of meiotic RNAs since the defect was suppressed by deletion of *mei2+* (Supplementary Fig. 3b). Consistent with this notion, although the *mot2* Δ mutant also grew slower than wild type cells in rich medium, this defect was Mei2-independent (Supplementary Fig. 3b) and presumably due to other functions of Mot2.

Mmi1 targets ubiquitination of Mei2 by Mot2

Our data are consistent with a model whereby Mmi1 recruits Ccr4-Not to limit the levels of Mei2. According to this model, the control of Mei2 abundance should depend on Mmi1. We indeed observed an accumulation of Mei2 in an *mmi1* Δ mutant, despite the presence of wild type Ccr4-Not (Fig. 4a). Instead, neither Red1 nor Rrp6 affected Mei2 levels. This indicates that Mmi1 and the Ccr4-Not complex are components of a cellular pathway that is distinct from the post-transcriptional regulation of meiotic mRNAs exerted by Mmi1, MTREC and the exosome.

We next wondered whether the regulation of Mei2 steady state levels requires the catalytic activity of Mot2. Expression of Mot2 variants lacking the RING domain (Mot2-RING Δ) or carrying single substitutions in residues involved in substrate ubiquitination (Mot2-C37A, -C45A and -C57A) did not restore wild type levels of Mei2 in a *mot2* Δ background (Fig. 4b). This demonstrates that the ubiquitination activity of Mot2 is required for suppressing expression of Mei2 in vegetative growth.

To address whether Mei2 is a substrate for ubiquitination by Mot2 *in vivo*, we purified total cellular ubiquitinated proteins from cells expressing His₆-tagged ubiquitin. The presence of ubiquitinated species of C-terminally 3xHA-tagged Mei2 was probed by immunoblotting in wild type and *mot2* Δ cells. Up to four specific bands corresponding to Mei2 ubiquitinated species (Ub-Mei2) could be observed in wild type cells (Fig. 4c, lane 6). Deletion of *mot2*⁺ resulted in the detection of similar levels of Ub-Mei2 in spite of the significantly higher amount of total Mei2, which corresponds to an overall four to five-fold decrease in the fraction of ubiquitinated species (Fig. 4d). This indicates that Mot2 ubiquitinates Mei2, presumably promoting its degradation.

Previous work showed that mutation of the E3 ubiquitin ligase Ubr1 also triggers accumulation of Mei2 in vegetative cells²⁹. To determine which of the two E3 ligases is responsible for the main Mei2 turnover pathway, we compared total and Ub-Mei2 levels in *ubr1* Δ and *mot2* Δ cells. Total Mei2 levels were strongly increased in the absence of Ubr1 relative to the *mot2* Δ mutant (Fig. 4c; Supplementary Fig. 4a,b). This was due to a major effect on the turnover rate of Mei2, as shown by cycloheximide chase experiments (Supplementary Fig. 4c). Consistent with a predominant role for Ubr1, ubiquitination of Mei2 was strongly decreased in *ubr1* Δ cells (Fig. 4c,d). Finally, increased Mei2 levels in the absence of Ubr1 resulted in a pronounced accumulation of Mmi1-targeted meiotic mRNAs (Supplementary Fig. 4d).

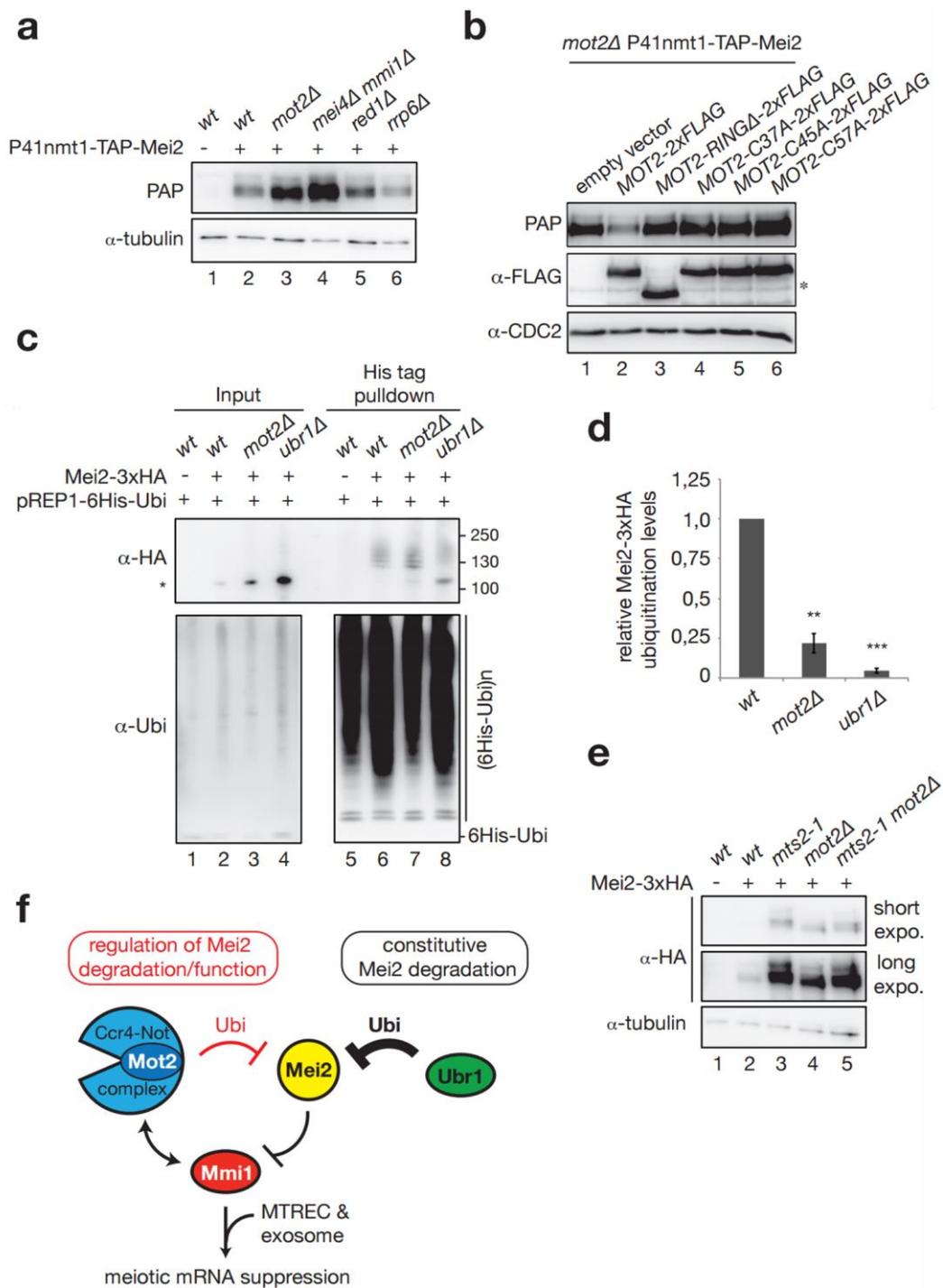


Figure 4. Mmi1 targets ubiquitination of Mei2 by Mot2.

(a-b) Western blot showing total TAP-Mei2 levels expressed from the P41nmt1 promoter in cells of the indicated genetic backgrounds and grown in minimal medium (EMM0.5X). Cells carrying vectors were grown in minimal medium lacking leucine (EMM-LEU0.5X). An anti-FLAG antibody was used to detect Mot2 variants expressed from the pREP41 vector. Anti-tubulin and anti-CDC2 antibodies were used as loading controls. (c) *In vivo* ubiquitination of Mei2-3xHA in wt, *mot2Δ* and *ubr1Δ* cells expressing His₆

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tagged-ubiquitin in minimal medium (EMM-LEU0.5X). Total and ubiquitinated Mei2 as well as ubiquitin conjugates were analyzed by immunoblotting using anti-HA and anti-ubiquitin antibodies respectively. An untagged wild type strain was used as negative control. The star denotes unmodified Mei2 molecules. **(d)** Quantification of Mei2 ubiquitinated species relative to total protein levels and expressed relative to Mei2-3xHA wild type cells. Error bars represent the standard deviation from four experiments. Stars denote statistical significance relative to Mei2-3xHA wild type cells (t-test p-values = 1.26E-4 for *mot2Δ*, and 1.53E-6 for *ubr1Δ*). **(e)** Western blot showing total Mei2-3xHA levels in cells of the indicated genetic backgrounds grown in minimal medium (EMM0.5X) following a temperature shift at 37°C for 1 hour. Shown are short and long exposition pictures for the anti-HA immunoblotting. An anti-tubulin antibody was used as a loading control. **(f)** Model depicting the regulatory circuit whereby Mmi1 recruits the Ccr4-Not complex to mediate Mot2-dependent ubiquitination of a pool of its own inhibitor Mei2. The E3 ubiquitin ligase Ubr1 has a major role in Mei2 ubiquitination and degradation. Both Mot2 and Ubr1 contribute to maintain low levels of Mei2, thereby preserving Mmi1 activity and sustaining meiotic mRNAs suppression.

To exclude the possibility that Mot2 represses translation of *mei2+* mRNAs, we first compared Mei2 protein levels in strains carrying the *mts2-1* thermosensitive mutation of the proteasome subunit Rpt2/Mts2 alone or in combination with the deletion of *mot2+*. If the absence of Mot2 leads to increased translation of *mei2+* transcripts, then Mei2 levels should be higher in an *mts2-1 mot2Δ* double mutant than in an *mts2-1* single mutant in which only protein degradation is impaired. Conversely, if Mot2 and the proteasome function in the same degradation pathway, Mei2 levels should not be affected by deletion of *mot2+* in *mts2-1* cells. As shown in Fig. 4e (compare lanes 3 and 5), Mei2 accumulates to similar levels in both *mts2-1* and *mts2-1 mot2Δ* mutants at the non-permissive temperature, indicating that Mot2 triggers Mei2 degradation via the proteasome pathway.

We also analyzed the levels of *mei2+* mRNAs that co-immunoprecipitate with the translation machinery in wild type and *mot2Δ* cells. If Mot2 represses translation of *mei2+* mRNAs, then the fraction of transcripts associated with translating ribosomes should increase in the *mot2Δ* mutant. Contrary to this scenario, both the 60S ribosomal subunit Rpl1601 and the elongation factor Tef3 did not pull down more *mei2+* transcripts in the absence of Mot2, despite identical protein levels (Supplementary Fig. 5a,c,e). Further indicating that *mei2+*

mRNA translation is not specifically increased in *mot2* Δ cells, the fraction of immunoprecipitated *mei2*⁺ mRNAs relative to *act1*⁺ transcripts was even slightly decreased in the mutant (Supplementary Fig. 5b,d).

From these results, we conclude that Mot2 is not responsible for the main turnover pathway of Mei2, which depends for its largest share on Ubr1. However, despite its modest impact, ubiquitination of Mei2 by Mot2 has an important role in fine-tuning the levels and/or the function of Mei2, allowing full repression of meiotic mRNAs during vegetative growth (Fig. 4f).

DISCUSSION

Fission yeast cells selectively eliminate meiosis-specific transcripts during the mitotic cell cycle to inhibit sexual differentiation and ensure robust vegetative growth. Here, we report the existence of a novel mechanism by which the evolutionarily conserved Ccr4-Not complex enforces the function of the YTH-family RNA-binding protein Mmi1 on the repression of the meiotic program during vegetative growth (Fig. 4f). Our results indicate that Mmi1 interacts with Ccr4-Not to promote Not4/Mot2-dependent ubiquitination and subsequent proteasomal degradation of a pool of its own inhibitor Mei2. This ensures the maintenance of Mmi1 in a functional state and the persistent suppression of meiotic mRNAs. Thus, Mmi1 has a dual role: in nuclear RNA surveillance, by targeting meiotic transcripts for degradation by the exosome, and in protein degradation, by recruiting Ccr4-Not to Mei2. These results also reveal a novel role for the Ccr4-Not complex in the control of sexual differentiation in fission yeast.

The existence of a regulatory circuit whereby a protein controls the levels of its own inhibitor might hamper alterations in the function of the protein (Mmi1) due to fluctuations in the levels of the inhibitor (Mei2), which might occur stochastically or under given growth conditions. This, in turn, is expected to prevent variations in the levels of meiotic RNAs and

their translation products, which might affect, directly or indirectly, the robustness of vegetative growth.

The need for buffering increased Mei2 production is the likely reason why the Ccr4-Not circuit only affects the levels of meiotic transcripts during growth in minimal medium. In yeast, meiosis occurs upon exposure to nutritional starvation, which activates signaling pathways that converge towards the induction of a complex transcriptional program finalized to sexual differentiation¹. Growth in minimal medium, when nutrients are available but limiting, might induce a partially analogous response, possibly anticipating the need for entry in meiosis. Indeed, we observed increased levels of Mei2 protein upon vegetative growth in minimal versus rich medium (Fig. 3a, Supplementary Fig. 2a), which might in principle be due to increased transcription, translation and/or protein stabilization. The low levels of Mei2 in rich medium even upon deletion of *mot2+* suggest that the expression of the *mei2+* gene is limited by transcription in these growth conditions. Conversely, the requirement for Mot2 ubiquitination activity to restrict Mei2 levels in minimal medium indicates that Mei2 is post-translationally controlled in this context.

Thus, the Ccr4-Not circuit has an important role in controlling the levels of Mei2 under conditions where entry into meiosis is to some extent transcriptionally prefigured, but for which a no-return commitment is premature.

Higher levels of Mei2 in *mot2Δ* cells could be due to increased Mei2 production, consistent with a known role of the Ccr4-Not complex in translational repression⁴³⁻⁴⁵. However, we believe this is unlikely because (1) the regulation of Mei2 by Mot2 requires Mmi1 (Fig. 4a), which localizes to the nucleus^{3,11,12,18,27}, (2) the absence of Mot2 does not further increase Mei2 levels upon proteasome inactivation (Fig. 4e), and (3) the fraction of *mei2+* mRNAs associated with translating ribosomes does not increase in the absence of Mot2 (Supplementary Fig. 5). Together with the absence of increased *mei2+* transcription in *mot2Δ* cells (Fig. 3b-d)

and a role for Mot2 ubiquitination activity in lowering Mei2 protein levels (Fig. 4b), this indicates that Mot2 affects the stability of Mei2.

The turnover of Mei2 mainly depends on the E3 ubiquitin ligase Ubr1. Considering the steady state levels of Mei2 in *ubr1Δ* and *mot2Δ* cells, ubiquitination by Mot2 is expected to contribute to roughly 15% of the overall degradation pathway. Accordingly, we could not detect a significant increase in Mei2 half-life by cycloheximide chase experiments, most likely because the predominant degradation pathway involving Ubr1 masks the effects of Mot2 absence. The limited role of Mot2 on the stability of the bulk of Mei2 is nonetheless necessary and sufficient to prevent inhibition of Mmi1. The marked disparity in the relative contributions of the two E3 ubiquitin ligases might suggest that Ubr1 mediates constitutive Mei2 turnover while Mot2 belongs to a regulatory pathway that controls the levels of Mei2 at its site of action or, alternatively, its function. Indeed, it is possible that Mot2 ubiquitinates Mei2, perhaps hindering its association with Mmi1 and favoring its subsequent polyubiquitination by Ubr1 for degradation. In this perspective, Mot2 may contribute to the inactivation of Mei2 rather than its degradation *per se*.

The control exerted by Ccr4-Not on Mei2 levels is only part of the mechanism that prevents meiosis onset in vegetative cells. Although Mei2 levels in *mot2Δ* cells largely exceed those observed in the wild type strain in sporulation medium (Fig. 3a, Supplementary Fig. 2a), this is not sufficient to induce ectopic entry in meiosis (Supplementary Fig. 3a). This observation suggests that additional events are required to activate meiosis and/or that another control exists on Mei2 that backs up the role of Ccr4-Not. Consistent with this latter hypothesis, the Pat1 and Tor2 protein kinases phosphorylate Mei2 to inhibit its activity and promote its degradation, and mutation of these enzymes or expression of a non-phosphorylatable form of Mei2 trigger ectopic entry into meiosis, even in haploid cells^{1,29-31}. Thus, it is possible that the

increased pool of Mei2 in *mot2Δ* cells might be partially inactivated by Pat1 and/or Tor2, thereby preventing full activation of the meiotic program.

A strict control on the inhibition of Mmi1 appears to be essential given the important role of this factor in suppressing mRNAs encoding meiosis-specific transcription factors that, in turn, control the expression of other transcription factors². On the other side, the onset of meiosis might require abrupt changes in physiology, which could benefit from a faster response associated to releasing the inhibition of factors already present in the cell as opposed to synthesizing these factors *de novo*. Whether this also requires regulation of expression or activity of the Ccr4-Not complex is an important question and matter for future studies.

The function of Ccr4-Not in sexual differentiation in fission yeast is analogous to the role played by the metazoan complex in controlling developmental decisions⁴⁷⁻⁵⁰. However, in these examples, the mechanism of action of the complex essentially relies on its deadenylation and translational repression activities, and a role for the conserved Not4/Mot2 E3 ubiquitin ligase has not been demonstrated. The control of Mei2 abundance by Mmi1 and Ccr4-Not adds another layer of complexity to the regulatory potential of the Ccr4-Not complex. Our work paves the way for addressing the important question of the conservation of this mechanism in other regulatory networks.

METHODS

Fission yeast strains and growth media

The *S. pombe* strains used in this study are listed in Supplemental Information. Strains were generated by transformation with a lithium acetate-based method or by random spore analysis. *mmi1Δ* cells were generated from a parental strain possessing a deletion of *mei4+*, since the absence of Mmi1 leads to severe growth and viability defects due to the deleterious expression of Mei4, a key meiosis-specific transcription factor. Growth media included

complete medium (YE), minimal medium (EMM) and sporulation medium (ME). All experiments in minimal medium were performed using EMM 0.5X (Formedium) supplemented with 1% glucose (2% final concentration) and 125 mg/L of each adenine, L-histidine, uracil, L-lysine and L-leucine. Standard molecular biology and biochemistry methods as well as mass spectrometry and transcriptomic analyses are described in Supplemental Experimental Procedures.

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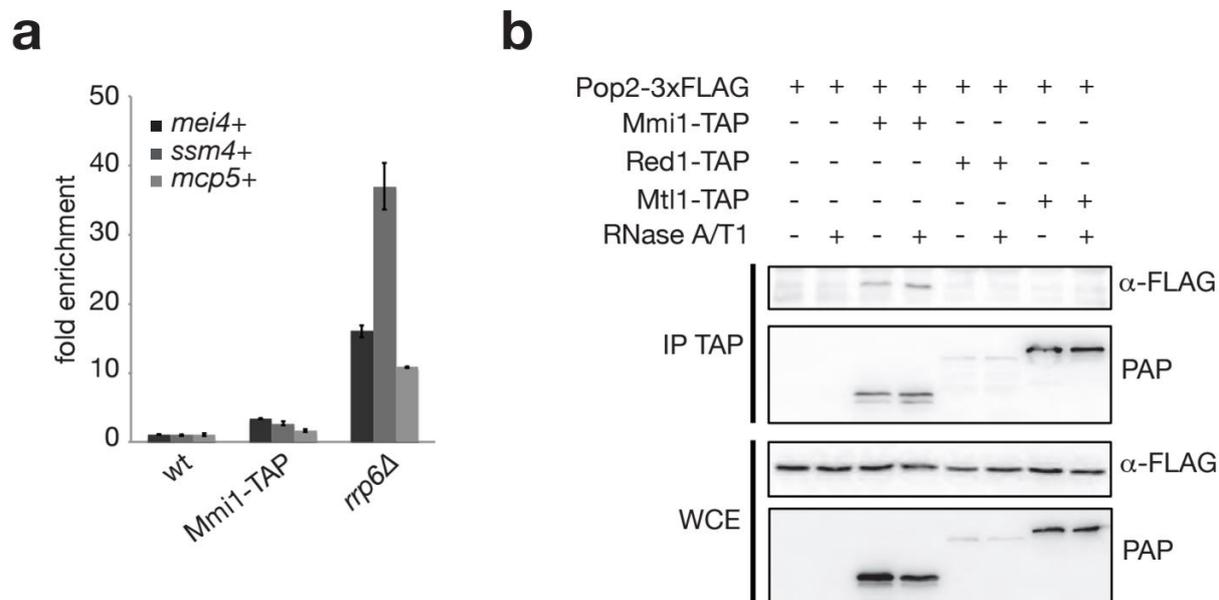
AUTHOR CONTRIBUTIONS

F.S. and S.L. performed experiments; T.C. performed bioinformatic analysis; D.L. designed experiments and wrote the paper; M.R. designed and performed experiments and wrote the paper. All authors have approved the manuscript.

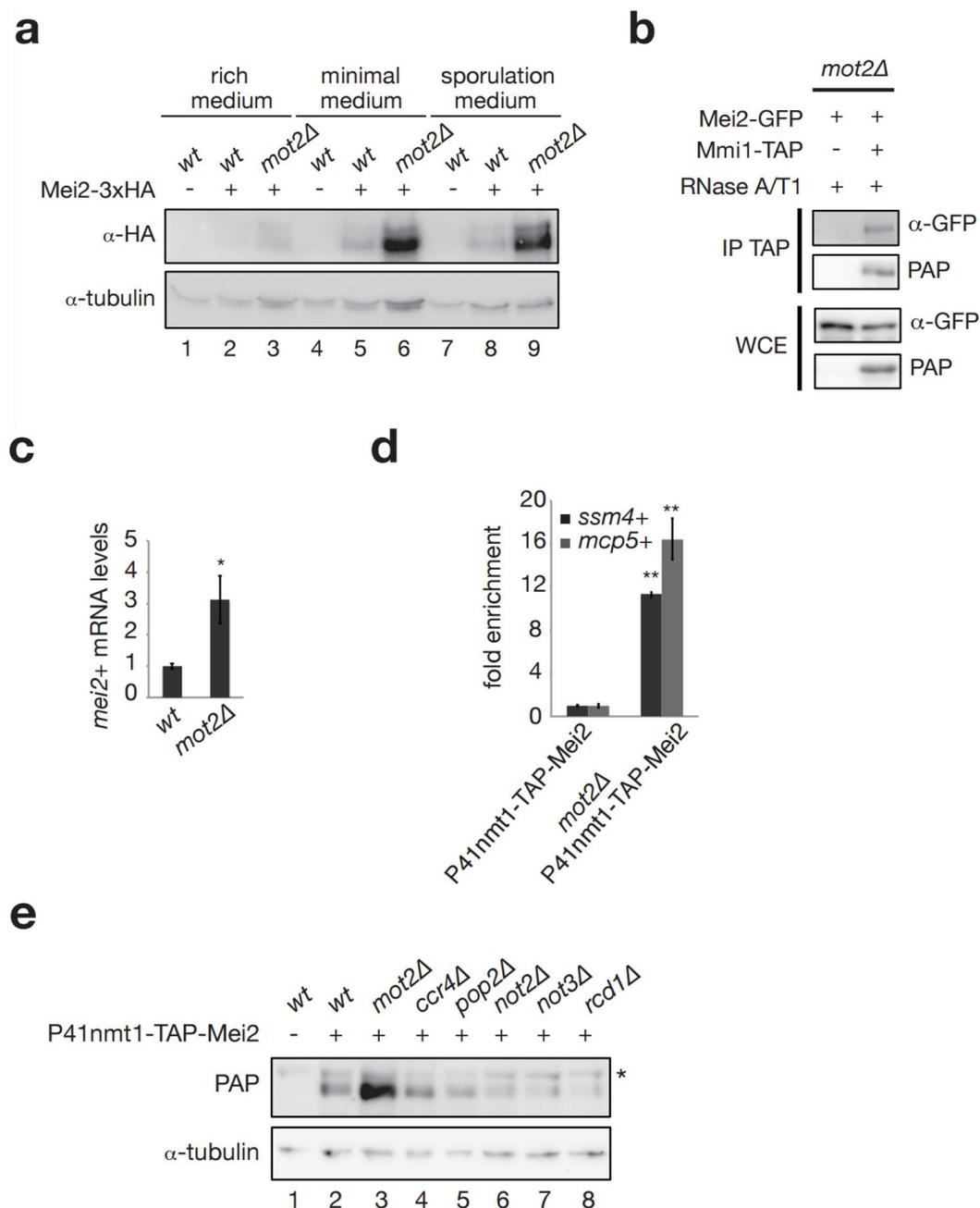
COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Supplemental Figures and Legends



Supplementary Figure 1. Mmi1 associates independently with the MTREC and Ccr4-Not complexes *in vivo*. (a) RT-qPCR analysis of the *mei4+*, *ssm4+* and *mcp5+* meiotic transcripts in Mmi1-TAP cells grown in minimal medium (EMM0.5X). Signals were normalized to *act1+* mRNA levels and expressed relative to the wild type strain. A strain deleted for Rrp6 was used as a control. Error bars represent the standard deviation from three independent experiments. Note that Mmi1-TAP cells display minor accumulation of meiotic transcripts. (b) Western blots showing that Pop2-3xFLAG co-immunoprecipitates with Mmi1-TAP in rich medium (YE), but not with Mtl1-TAP and Red1-TAP.



Supplementary Figure 2. Ccr4-Not promotes meiotic mRNA suppression by limiting the accumulation of the Mmi1 inhibitor Mei2. (a) Western blot showing total Mei2-3HA levels in wt and *mot2Δ* cells grown in rich (YE), minimal (EMM0.5X) and sporulation (ME) media. An anti-tubulin antibody was used as a loading control. (b) Western blots showing that Mei2-GFP co-immunoprecipitates with Mmi1-TAP in an RNA-independent manner in *mot2Δ* cells grown in minimal medium (EMM0.5X). (c) RT-qPCR analysis of *mei2+* mRNAs levels in wt and *mot2Δ* cells grown in minimal medium (EMM0.5X). Signals were normalized to *act1+* mRNA levels and expressed relative to the wild type strain. Error bars represent the standard deviation from at least three independent

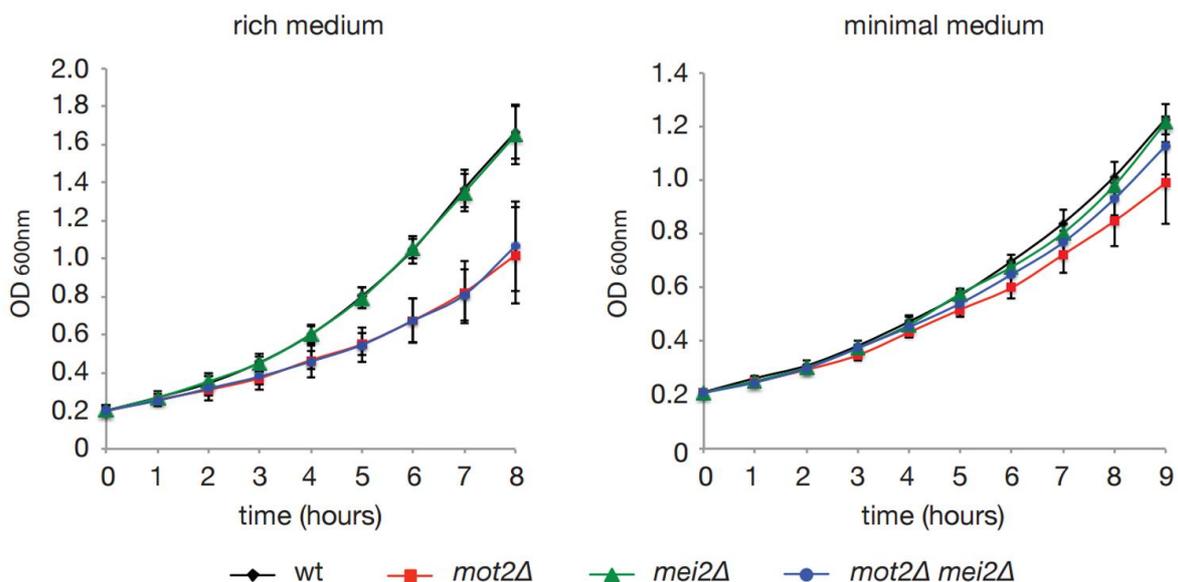
Results

experiments. The star denotes statistical significance relative to wild type cells (see Supplemental Experimental Procedures). **(d)** RT-qPCR analysis of meiotic transcripts in the indicated genetic backgrounds. Shown is the fold enrichment of RNAs levels normalized to *act1+* transcripts and expressed relative to the wild type strain. Error bars represent the standard deviation of four independent experiments. Stars denote statistical significance relative to P41nmt1-TAP-Mei2 cells (see Supplemental Experimental Procedures). **(e)** Western blot showing total TAP-Mei2 levels expressed from the P41nmt1 promoter in minimal medium (EMM0.5X) in wild type cells and strains deleted for non-essential subunits of the Ccr4-Not complex (i.e. all but Not1). An anti-tubulin antibody was used as a loading control. The asterisk denotes a non-specific band.

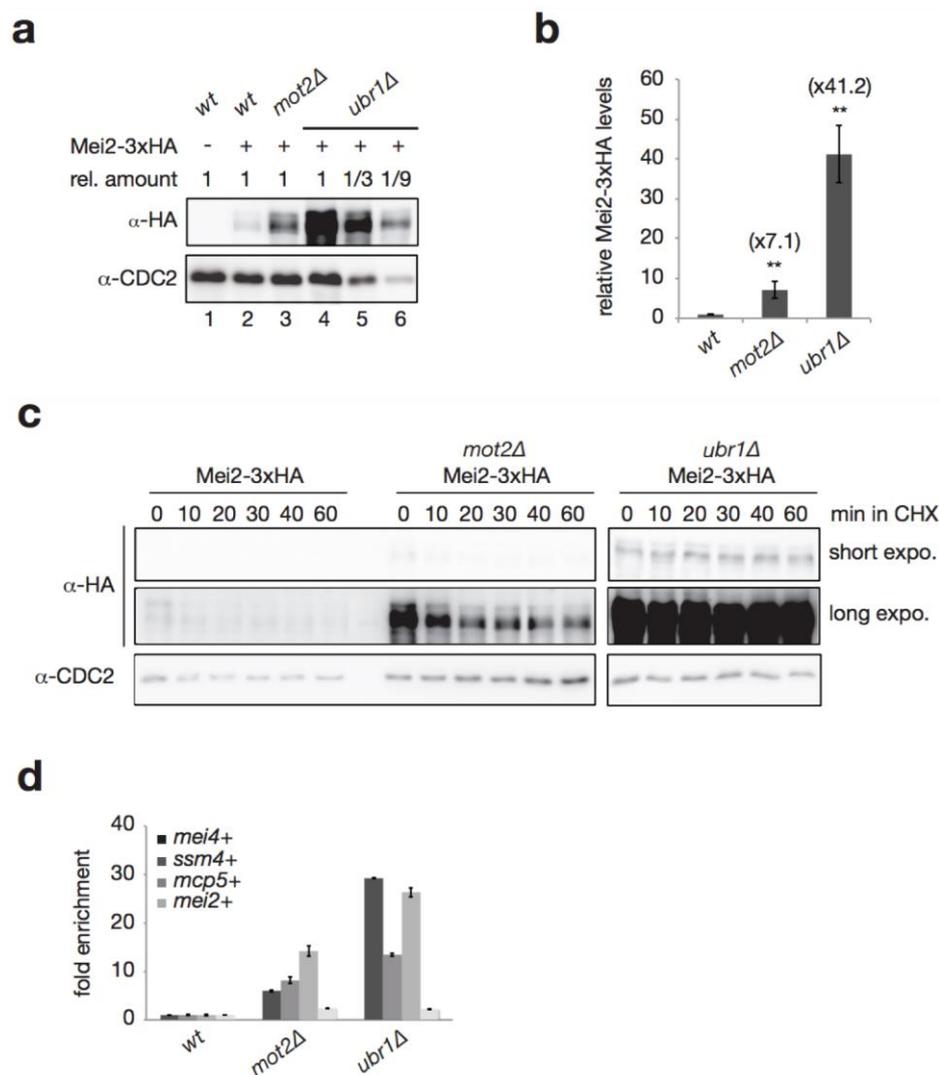
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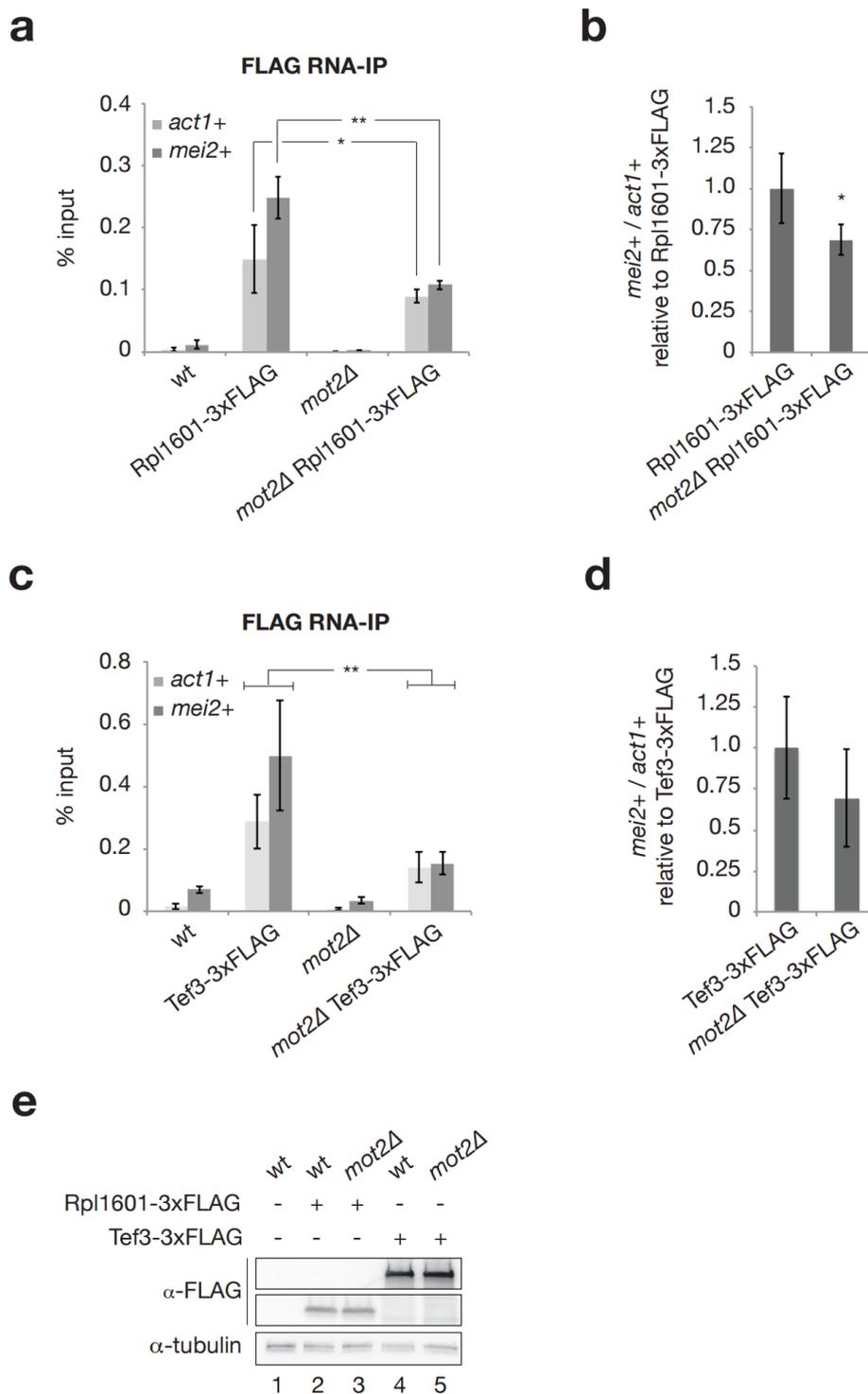
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Supplementary Figure 3. Phenotypic characterization of the *mot2Δ* mutant. **(a)** Phase contrast images of exponentially growing wild type, *mot2Δ*, *mei2Δ* and *mot2Δ mei2Δ* cells cultured in minimal medium (EMM0.5X). **(b)** Cells of the indicated genotypes were grown in rich (YE; left panel) and minimal (EMM0.5X; right panel) media, and the optical density (OD_{600nm}) was measured over time.



Supplementary Figure 4. Mot2 and Ubr1 differentially contribute to the degradation of Mei2 and meiotic mRNAs. (a) Representative Western blot showing total Mei2-3xHA levels in wt, *mot2Δ* and *ubr1Δ* cells grown in minimal medium (EMM0.5X) at 30°C. Mei2 was detected using an anti-HA antibody and an anti-CDC2 antibody was used as a loading control. (b) Quantification of total Mei2-3xHA levels, normalized to CDC2 and expressed relative to wild type cells. Error bars represent the standard deviation from five experiments. Stars denote statistical significance relative to Mei2-3xHA wild type cells (t-test p-values = 2.7E-3 for *mot2Δ*, and 2E-3 for *ubr1Δ*). (c) Cycloheximide chase experiment of Mei2-3xHA in wt, *mot2Δ* and *ubr1Δ* cells. Cells were grown in minimal medium (EMM0.5X) at 30°C and harvested at the indicated time points following addition of 100 μg/mL cycloheximide. Mei2 was detected by immunoblotting using an anti-HA antibody and an anti-CDC2 antibody was used as a loading control. (d) RT-qPCR analysis of meiotic transcripts in the indicated genetic backgrounds. Shown is the fold enrichment of RNAs levels normalized to *act1+* transcripts and expressed relative to the wild type strain. Error bars represent the deviation from the mean of biological duplicates.



Supplementary Figure 5. Mot2 does not repress *mei2+* mRNAs translation. (a,c) RNA-immunoprecipitation experiments in wild type and *mot2Δ* cells. Shown are the enrichments (% input) of *act1+* and *mei2+* mRNAs upon pulldown of the 3xFLAG-tagged 60S ribosomal subunit Rpl1601 (a) or the translation elongation factor Tef3 (c). Error bars represent the standard deviation of six independent immunoprecipitations from at least three biological replicates. Stars denote statistical

significance between samples (see Supplemental Experimental Procedures). **(b,d)** Quantification of *mei2+* mRNA levels normalized to *act1+* transcripts and expressed relative to the wild type tagged strains (Rpl1601-3xFLAG and Tef3-3xFLAG). Error bars represent the standard deviation of six independent immunoprecipitations from at least three biological replicates. Stars denote statistical significance between samples (see Supplemental Experimental Procedures). **(e)** Western blot showing that total levels of Rpl1601 and Tef3 are not affected by the deletion of *mot2+*. 3xFLAG-tagged proteins were detected with an anti-FLAG antibody and an anti-tubulin antibody was used as a loading control.

Supplementary Table 1. Complete lists of Mmi1 protein partners in wt and *mot2Δ* cells. Shown are lists of proteins co-purified with Mmi1-TAP in the presence or the absence of RNAs in wild type and *mot2Δ* cells. An untagged strain was used as a negative control. Protein scores (as provided by the Percolator algorithm), percentages of sequence coverage, numbers of peptides, numbers of peptide spectrum matches (PSM) as well as the sequence, q-value, Posterior Error Probability (PEP) and ion score for each peptide identified are indicated. Rows corresponding to Mmi1, subunits of the Ccr4-Not and MTREC complexes as well as Mei2 are highlighted in red, blue, green and yellow respectively.

Supplementary Table 2. Analysis of RNA-sequencing data. Shown are genes with a 1.5 fold increase (0.58496250072 on a log₂ scale) in expression in mutants relative to the wild type, the overlap between *mot2Δ* and *rrp6Δ* strains, and functional categories of genes upregulated in *mot2Δ* cells.

Supplemental Experimental Procedures

Affinity purification and mass spectrometry analysis

500 mL of cells were grown at 30°C in EMM 0,5X until $OD_{600nm} = 1.0 - 1.2$ and harvested by centrifugation. Cell pellets were resuspended in 5 mL lysis buffer (6 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , 150 mM $NaC_2H_3O_2$, 5 mM $MgC_2H_3O_2$, 0.25% NP-40, 2 mM EDTA, 1 mM EGTA, 5% glycerol, 1 mM AEBSF, 4 mM benzamidine and 2X Roche complete EDTA-free protease inhibitor cocktail) and slowly dropped into liquid nitrogen to form “pop-corn”. Lysis was performed using a Ball Mill (Retsch, MM400) for 15 min at a 10 Hz frequency. Extracts were cleared by centrifugation before precipitation with 7.5 mg of pre-washed rabbit IgG-conjugated M-270 Epoxy Dynabeads (Invitrogen) for 1 hour at 4°C. Lysates were incubated with or without 1 μ L RNaseA/T1 cocktail (Ambion) per mL of extract for 20 minutes at 4°C prior to immunoprecipitation. Beads were then washed twice with IPP150 (10 mM Tris pH8, 150 mM NaCl, 0.1% NP-40). Immunoprecipitated complexes were eluted over-night at 4°C by adding 50 units TEV protease (Invitrogen) in 200 μ L TEV cleavage buffer (10 mM Tris pH8, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT). 5 % of eluates were subjected to silver staining using the SilverQuest kit (Invitrogen) and the remaining samples were precipitated with methanol-chloroform. Briefly, TEV eluates were mixed sequentially with 4 volumes of methanol, 1 volume of chloroform and 3 volumes of water. Samples were centrifuged at 16000g for 30 min at 4°C and the upper phases were discarded. 3 volumes of methanol were added to the lower phases to extract chloroform and samples were vortexed, left at -20°C for 30 min and centrifuged at 16000g for 20 min at 4°C. Following removal of supernatants, protein precipitates were air dried and stored at -80°C prior to MS/MS analysis.

Precipitated proteins were digested overnight at 37°C by sequencing grade trypsin (12.5 μ g/ml; Promega Madison, Wi, USA) in 20 μ l of NH_4HCO_3 25 mmol/L. Digests were analyzed by an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a Thermo Scientific EASY-Spray nanoelectrospray ion source and coupled to an Easy nano-LC Proxeon 1000 system (Thermo Fisher Scientific, San Jose, CA). Chromatographic separation of peptides was performed with the following parameters: pre-column Acclaim PepMap100 (2 cm, 75 μ m i.d., 3 μ m, 100 Å), column Pepmap-RSLC Proxeon C18 (50 cm, 75 μ m i.d., 2 μ m, 100 Å), 300nl/min flow, gradient rising from 95 % solvent A (water, 0.1% formic acid) to 35% B (100 % acetonitrile, 0.1% formic acid) in 98 minutes. Peptides were

analyzed in the orbitrap in full ion scan mode at a resolution of 60000 (at m/z 400) and with a mass range of m/z 350-1550. Fragments were obtained with a Higher-energy Collisional Dissociation (HCD) activation with a collisional energy of 30%, and a quadrupole isolation width of 1.6 Da. MS/MS data were acquired in the linear ion trap in top-speed mode, with a dynamic exclusion of 50 seconds and a repeat duration of 60 sec. The maximum ion accumulation times were set to 250 ms for MS acquisition and 60 ms for MS/MS acquisition in parallelization mode. MS/MS data were processed with Proteome Discoverer 1.4 software (Thermo Fisher scientific, San Jose, CA) coupled to an in-house Mascot search server (Matrix Science, Boston, MA; version 2.5.1). The mass tolerance was set to 7 ppm for precursor ions and 0.5 Dalton for fragments. The following modifications were used in variable modifications: oxidation (M), phosphorylations (STY), acetylations (K, N-term), deamidations (N, Q), methylations (K), ubiquitylation (GG and LRGG motifs on K amino acids). The maximum number of missed cleavages by trypsin was limited to 2. MS/MS data were searched against SwissProt databases with the *Schizosaccharomyces pombe* taxonomy. False Discovery Rate (FDR) for peptides was calculated using the Percolator algorithm and peptides were considered identified under the 1% FDR threshold.

Coimmunoprecipitation

CoIPs were performed essentially as described in the previous section with the following modifications: 50 ODs of cells were grown at 30°C in EMM 0.5X and harvested by centrifugation. Cell pellets were resuspended in 2 ml lysis buffer to make “pop-corn”. 1 mg of pre-washed rabbit IgG-conjugated M-270 Epoxy Dynabeads (Invitrogen) was used for immunoprecipitation in the presence or the absence of RNaseA/T1 cocktail (Ambion). Following washes, precipitates and input fractions were boiled at 95°C for 10 min in the presence of sample loading buffer and analyzed by SDS-PAGE and Western blotting using 1:3000 peroxidase-conjugated antiperoxidase (PAP) (P1291, Sigma), 1:3000 monoclonal anti-FLAG antibody (F3165, Sigma) and 1:3000 monoclonal anti-GFP antibody (Roche). Detection was performed using ECL reagent (GE Healthcare) and a Fujifilm LAS-4000 imager.

Total protein analysis

2 to 5 ODs of cells grown at 30°C in YE, EMM 0.5X or ME were harvested and lysed on ice in the presence of 0.3M NaOH and 1% β -mercaptoethanol for 15 min with occasional vortexing. Extracts were treated with TCA (7% final concentration) for 15 min on ice before full speed centrifugation at 4°C. Pellets were then resuspended in loading buffer (200 mM phosphate buffer pH 6.8, 8 M urea, 5% SDS, 1 mM EDTA, 100 mM DTT, 0.08% bromophenol blue) and heat-denatured at 70°C for 10 min. Soluble fractions were recovered and samples were analyzed by standard immunoblotting procedures using 1:3000 peroxydase-conjugated antiperoxydase (PAP) (P1291, Sigma), 1:3000 monoclonal anti-HA antibody (12CA5, Sigma), 1:3000 monoclonal anti-FLAG antibody (F3165, Sigma), 1:3000 monoclonal anti-tubulin antibody (ab6160, Abcam) and 1:3000 anti-CDC2 antibody (ab5467, Abcam). For chase experiments, 100 μ g/mL cycloheximide (CHX, Sigma) was directly added to the cultures. Detection was performed using ECL reagents (GE Healthcare) and a Fujifilm LAS-4000 imager. The ImageQuant TL software (1D gel analysis) was used for signal quantification. Measurements were statistically compared using two-tailed t-tests with the following p-value cut-offs for significance: $5E-2 > * > 1E-2$; $1E-2 > ** > 1E-5$; $*** < 1E-5$.

Ubiquitin pulldown

50 ODs of cells grown at 30°C in EMM 0.5X lacking L-leucine were harvested after 1 hour in the presence of 10 mM N-ethylmaleimide. Cell pellets were lysed with the NaOH/TCA method. Following centrifugation, pellets were washed twice with ice-cold acetone and then resuspended in 1.5 mL buffer A (6 M guanidinium chloride, 100 mM NaH_2PO_4 , 150 mM Tris-base, 0.05% Tween-20), and incubated for up to 2 hours at room temperature on a nutator. Cell debris were removed by centrifugation and extracts were supplemented with 10 mM imidazole. 50 μ L of pre-washed His tag isolation and pulldown Dynabeads (Life Technology) were added and samples were incubated for up to 3 hours at room temperature on a nutator. Beads were then washed three times in buffer A containing 1 mM imidazole and four times in buffer C (8 M urea, 100 mM NaH_2PO_4 , 100 mM Tris-base, 0.05% Tween-20, 1 mM imidazole) before elution in 30 μ L sample loading buffer at 95°C for 10 min. For input samples, a fraction of extracts was precipitated with TCA, washed with acetone, air-dried and denatured in sample

loading buffer at 37°C for 15 min. Samples were analyzed by western blotting using 4-12% NuPAGE gels, 1:3000 anti-HA antibody (12CA5, Sigma) and 1:1000 anti-ubiquitin HRP-conjugated antibody (sc-8017 HRP, Santa Cruz Biotechnology). Detection was performed using ECL reagents (GE Healthcare) and a Fujifilm LAS-4000 imager. Measurements were statistically compared using two-tailed t-tests with the following p-value cut-offs for significance: $5E-2 > * > 1E-2$; $1E-2 > ** > 1E-5$; $*** < 1E-5$.

RNA extraction and RT-qPCR analyses

RNAs were prepared using the hot acid phenol method and treated with DNaseI (New England Biolabs). 4 μ g RNAs were used in reverse transcription reactions with 200 units of M-MLV RT (Invitrogen) and strand-specific primers. Following cDNA synthesis at 37°C for 50 min, the enzyme was inactivated at 80°C for 10 min. Samples were analyzed by qPCR with SYBR Green using a LightCycler LC480 apparatus (Roche) and quantification was performed using the $\Delta\Delta$ Ct method. Controls without reverse transcriptase were systematically run in parallel to estimate the contribution of contaminating DNA. Amplification efficiencies were measured for each primer pairs in every run. Measurements were statistically compared using two-tailed t-tests with the following p-value cut-offs for significance: $5E-2 > * > 1E-2$; $1E-2 > ** > 1E-5$; $*** < 1E-5$.

RNA-immunoprecipitation

50 ODs of cells were grown at 30°C in EMM 0.5X and harvested by centrifugation. Cell pellets were resuspended in 2 ml lysis buffer (6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 150 mM NaC₂H₃O₂, 5 mM MgC₂H₃O₂, 0.25% NP-40, 2 mM EDTA, 1 mM EGTA, 5% glycerol, 1 mM AEBSF, 4 mM benzamidine, 2X Roche complete EDTA-free protease inhibitor cocktail and 160 U Murine RNase inhibitor (New England Biolabs)) to make “pop-corn”. Lysis was performed using a Ball Mill (Retsch, MM400) for 15 min at a 10 Hz frequency. Extracts were cleared by centrifugation before precipitation with 40 μ L pre-washed anti-FLAG M2 affinity gel (A2220, Sigma) for 2 hours at 4°C. Beads were then washed twice with IPP150 (10 mM Tris pH8, 150 mM NaCl, 0.1% NP-40). Total and immunoprecipitated RNAs were extracted with

phenol:chloroform 5:1 pH4.7 (Sigma) and precipitated with ethanol. RNA samples were treated with DNase (AM1906, Ambion) prior to RT-qPCR analyses as mentioned above. Measurements were statistically compared using two-tailed t-tests with the following p-value cut-offs for significance: $5E-2 > * > 1E-2$; $1E-2 > ** > 1E-5$; $*** < 1E-5$.

Transcriptome analyses by RNA-sequencing

cDNA libraries were generated according to standard Illumina protocols. The RNA sequences reported in this paper have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE72327. Reads were trimmed with cutadapt and mapped to the ASM294v2.23 *S. pombe* genome using bowtie2. Read counts for every annotated transcript were calculated using HT-seq count and the ASM294v2.23 genome annotation. Differential expression was computed using the R bioconductor package DESeq2. Functional analysis was performed using the DAVID online tool.

Microscopy

Exponentially growing cells cultured in minimal medium (EMM0.5X) were imaged at room temperature with a motorized Olympus BX-61 fluorescence microscope equipped with an Olympus PlanApo 100× oil-immersion objective (1.40 NA), Nomarski optics, a QiClick cooled monochrome camera (QImaging, Surrey, BC, Canada) and the MetaVue acquisition software (Molecular Devices; Sunnyvale, CA, USA).

Table of *S. pombe* strains used in this study

Strain	Genotype	Source
PR040	h90, <i>ura4-DS/E</i> , <i>ade6-M210</i> , <i>leu1-32</i> , <i>mat3M::ura4+</i>	D. Moazed
PR167	h90, <i>ura4-DS/E</i> , <i>leu1-32</i> , <i>mat3M::gfp+::nat^RMX</i>	M. Bühler
PR206	PR167, <i>rrp6::kan^RMX</i>	This study
PR314	PR040, <i>Mmi1-TAP::hph^RMX</i>	This study
PR389	PR040, <i>Pop2-3xFLAG::kan^RMX</i>	This study
PR398	PR040, <i>Not1-3xFLAG::kan^RMX</i>	This study
PR403	PR040, <i>Mmi1-TAP::hph^RMX Pop2-3xFLAG::kan^RMX</i>	This study
PR404	PR040, <i>Mmi1-TAP::hph^RMX Not1-3xFLAG::kan^RMX</i>	This study
PR425	PR040, <i>pop2::nat^RMX</i>	This study
PR426	PR040, <i>ccr4::nat^RMX</i>	This study
PR430	PR040, <i>Mtl1-TAP::hph^RMX Not1-3xFLAG::kan^RMX</i>	This study
PR440	PR040, <i>red1::nat^RMX Mmi1-TAP::hph^RMX Not13xFLAG::kan^RMX</i>	This study
PR446	PR040, <i>Red1-TAP::hph^RMX Not1-3xFLAG::kan^RMX</i>	This study
PR469	PR040, <i>not3::kan^RMX</i>	This study
PR471	PR040, <i>rcd1::kan^RMX</i>	This study
PR483	PR040, <i>not2::nat^RMX</i>	This study
PR486	PR040, <i>rrp6::hph^RMX</i>	This study
PR523	PR040, <i>mot2::nat^RMX Mmi1-TAP::hph^RMX</i>	This study
PR524	PR040, <i>mot2::nat^RMX Pop2-3xFLAG::kan^RMX</i>	This study
PR526	PR167, <i>mot2::kan^RMX</i>	This study
PR637	PR040, <i>Mei2-3xHA::hph^RMX</i>	This study
PR638	PR040, <i>Mei2-TAP::hph^RMX</i>	This study
PR647	PR040, <i>mot2::nat^RMX Mei2-3xHA::hph^RMX</i>	This study
PR648	PR040, <i>mot2::nat^RMX Mei2-TAP::hph^RMX</i>	This study
PR652	PR040, <i>mei2::hph^RMX</i>	This study
PR657	PR040, <i>mot2::nat^RMX mei2::hph^RMX</i>	This study
PR658	PR040, <i>rrp6::nat^RMX mei2::hph^RMX</i>	This study
PR662	PR040, <i>pREP1-6His-Ubi::LEU2</i>	This study
PR667	PR040, <i>Mei2-3xHA::hph^RMX, pREP1-6His-Ubi::LEU2</i>	This study
PR668	PR040, <i>mot2::nat^RMX Mei2-3xHA::hph^RMX, pREP1-6His Ubi::LEU2</i>	This study
PR669	PR040, <i>kan^RMX::P3nmt1-3xFLAG-Mei2</i>	This study
PR675	PR040, <i>kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR687	PR040, <i>mot2::nat^RMX Mei2-GFP::kan^RMX</i>	This study
PR720	PR040, <i>mot2::nat^RMX kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR721	PR040, <i>red1::nat^RMX kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR722	PR040, <i>pop2::nat^RMX kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR723	PR040, <i>rrp6::nat^RMX kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR725	h-, <i>ura4-D18</i> , <i>leu1-32</i> , <i>mts2-1</i> , <i>Mei2-3xHA:: hph^RMX</i>	This study
PR726	PR040, <i>mei4::nat^RMX mmi1::hph^RMX kan^RMX::P41nmt1-TAP Mei2</i>	This study
PR728	h-, <i>ura4-D18</i> , <i>leu1-32</i> , <i>mts2-1</i> , <i>mot2::nat^RMX Mei2-3xHA:: hph^RMX</i>	This study
PR731	PR040, <i>mot2::nat^RMX kan^RMX::P41nmt1-TAP-Mei2 pREP41::LEU2</i>	This study
PR735	PR040, <i>ccr4::nat^RMX kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR736	PR040, <i>not2::nat^RMX kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR737	PR040, <i>not3::nat^RMX kan^RMX::P41nmt1-TAP-Mei2</i>	This study
PR753	PR040, <i>Mot2-GFP::kan^RMX</i>	This study
PR759	PR040, <i>red1::nat^RMX Mmi1-TAP::hph^RMX Pop2-3xFLAG::kan^RMX</i>	This study
PR785	PR040, <i>Mmi1-TAP::hph^RMX Mot2-GFP::kan^RMX</i>	This study
PR790	PR040, <i>red1::nat^RMX Mmi1-TAP::hph^RMX Mot2-GFP::kan^RMX</i>	This study

Results

PR792	PR040, mot2:: <i>nat^RMX</i>	This study
PR802	PR040, mot2:: <i>nat^RMX</i> Mmi1-TAP:: <i>hph^RMX</i> Mei2-GFP:: <i>kan^RMX</i>	This study
PR821	PR040, ubr1:: <i>kan^RMX</i> , Mei2-3xHA:: <i>hph^RMX</i>	This study
PR853	PR040, ubr1:: <i>kan^RMX</i> , Mei2-3xHA:: <i>hph^RMX</i> , pREP1-6His-Ubi:: <i>LEU2</i>	This study
PR860	PR040, rcd1:: <i>hph^RMX kan^RMX</i> ::P41nmt1-TAP-Mei2	This study
PR869	PR040, ubr1:: <i>hph^RMX</i>	This study
PR882	PR040, Tef3-3xFLAG:: <i>kan^RMX</i>	This study
PR883	PR040, Rpl1601-3xFLAG:: <i>kan^RMX</i>	This study
PR887	PR040, mot2:: <i>nat^RMX</i> Rpl1601-3xFLAG:: <i>kan^RMX</i>	This study
PR888	PR040, mot2:: <i>nat^RMX</i> Tef3-3xFLAG:: <i>kan^RMX</i>	This study
PR889	PR040, mot2:: <i>nat^RMX kan^RMX</i> ::P41nmt1-TAP-Mei2 pREP41-Mot2-2xFLAG:: <i>LEU2</i>	This study
PR890	PR040, mot2:: <i>nat^RMX kan^RMX</i> ::P41nmt1-TAP-Mei2 pREP41-Mot2-RINGΔ-2xFLAG:: <i>LEU2</i>	This study
PR904	PR040, mot2:: <i>nat^RMX kan^RMX</i> ::P41nmt1-TAP-Mei2 pREP41-Mot2-C37A-2xFLAG:: <i>LEU2</i>	This study
PR905	PR040, mot2:: <i>nat^RMX kan^RMX</i> ::P41nmt1-TAP-Mei2 pREP41-Mot2-C45A-2xFLAG:: <i>LEU2</i>	This study
PR906	PR040, mot2:: <i>nat^RMX kan^RMX</i> ::P41nmt1-TAP-Mei2 pREP41-Mot2-C57A-2xFLAG:: <i>LEU2</i>	This study

Table of oligonucleotides used in this study

Primers	Sequence
P249: <i>mei4+</i> fwd	5'-TGGATCAGATCCGTGGAATC-3'
P250: <i>mei4+</i> rev	5'-AACGCTCGATTAGAAGGCAT-3'
P253: <i>act1+</i> fwd	5'-AACCTCAGCTTTGGGTCTT-3'
P254: <i>act1+</i> rev	5'-TTTGCATACGATCGGCAATA-3'
P325: <i>ssm4+</i> fwd	5'-ACACAGTTTACGGGATTCTA-3'
P326: <i>ssm4+</i> rev	5'-GATTGTGATGAAAACCTGGGT-3'
P607: <i>mcp5+</i> fwd	5'-AGACGTATTCACCTTACCTC-3'
P608: <i>mcp5+</i> rev	5'-GTTTCCCATCATGACATGTT-3'
P645: <i>sme2+</i> fwd	5'-TTGCCGATTTACGAAGTT-3'
P646: <i>sme2+</i> rev	5'-ATCTGTCTGTTCTGCTGCT-3'
P855: <i>mei2+</i> fwd	5'-CCAACAAGGGTACCTATGAT-3'
P882: <i>mei2+</i> rev	5'-GAGTACCCACTCTAGCTTTG-3'
P1010: <i>mei2+</i> fwd	5'-GAGTTGGTGAACGGAAAGTA-3'
P881: <i>mei2+</i> rev	5'-GGGATTCTGAGAGAACAGAA-3'

3 Supplemental Results

3.1 meiRNA is not required for Mmi1-dependent Mei2 degradation

In order to switch from mitosis to meiosis, cells must inactivate Mmi1 to allow translation of meiotic mRNAs [54]. Upon nutritional starvation, Mmi1 is sequestered in an RNP complex, which includes the meiosis inducer Mei2 and the DSR-containing lncRNA meiRNA, encoded by the *sme2+* gene [59, 78, 79]. The Mei2-meiRNA complex sequesters Mmi1 at the *sme2+* locus, and cells defective for this function are not able to enter meiosis [54, 76, 78]. In light of these observations, we sought to investigate whether meiRNA might cooperate with Mmi1 and the Ccr4-Not complex to control the levels of Mei2.

Interestingly, we showed that deletion of *sme2+* did not affect Mei2 levels in exponentially growing cells, suggesting that meiRNA itself is not involved in the repression of Mei2 (**Fig. 24A**, lanes 2 and 4). Additionally, Western blotting and RT-qPCR experiments indicate that the accumulation of Mei2 and meiotic mRNAs in *mot2Δ* cells is not altered in the absence of meiRNA (**Fig. 24A**, lanes 3 and 5; **Fig. 24B**). This suggests that meiRNA is also dispensable for the inhibition of Mmi1 by high levels of Mei2.

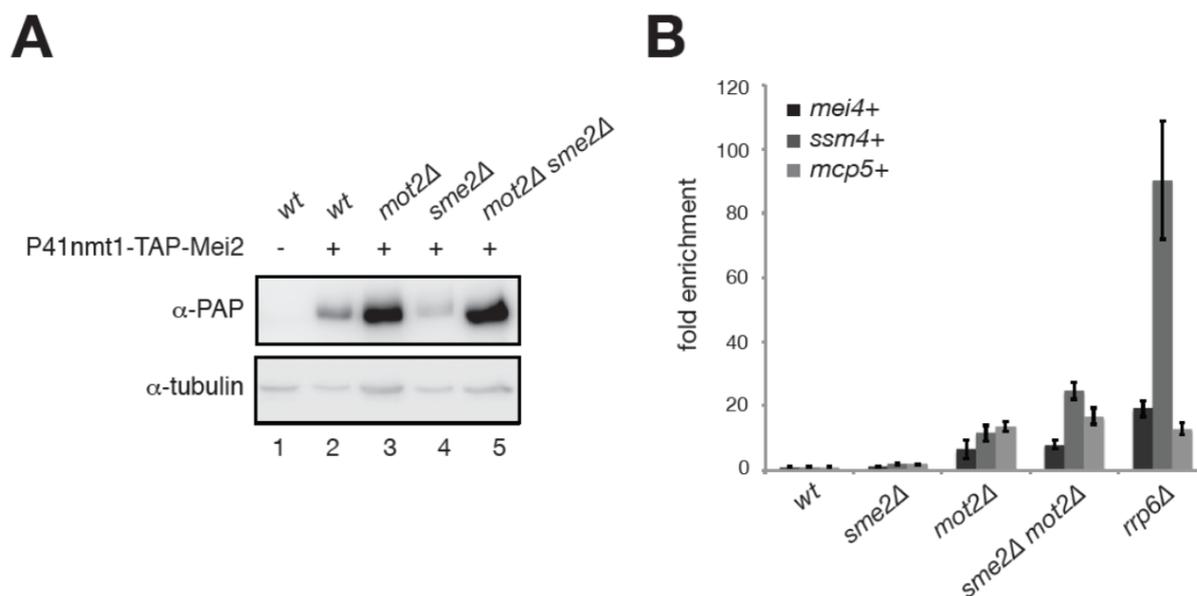


Figure 24. meiRNA is not involved in the repression of Mei2 and the inactivation of Mmi1 during vegetative growth.

(A) Western Blot showing that levels of TAP-Mei2 expressed from the nmt1 promoter are not affected by the deletion of *sme2+* in wt and *mot2Δ* cells grown in minimal medium (compare lanes 2 and 4, and lanes 3 and 5). An anti-tubulin antibody was used as a loading control. (B) RT-qPCR analyses of meiotic transcripts in cells of the indicated genetic backgrounds grown in minimal medium. Shown is the fold enrichment of RNAs levels normalized to *act1+* transcripts and expressed relative to the wild type strain. Error bars represent the standard deviation from three independent experiments. A strain deleted for the exosome subunit Rrp6 was used as a control.

Together, our results reveal that Mei2 can efficiently inhibit Mmi1 even in the absence of meiRNA, which implicates that the current model needs to be revisited.

3.2 The YTH domain of Mmi1 is required for degradation of Mei2

To determine whether the RNA-binding activity of Mmi1 is required for the regulation of Mei2, we constructed a strain expressing a version of Mmi1 lacking its C-terminal YTH domain and performed Western blot experiments to assess the levels of Mei2. We verified that this truncated form of Mmi1 is properly expressed (see below). Remarkably, we observed that the Mmi1-*YTH*Δ mutant displays increased levels of Mei2, similarly to cells deleted for *mot2*+ or *mmi1*+ (**Fig. 25**). The YTH-family RNA-binding domain of Mmi1 is therefore required for the Mei2 degradation.

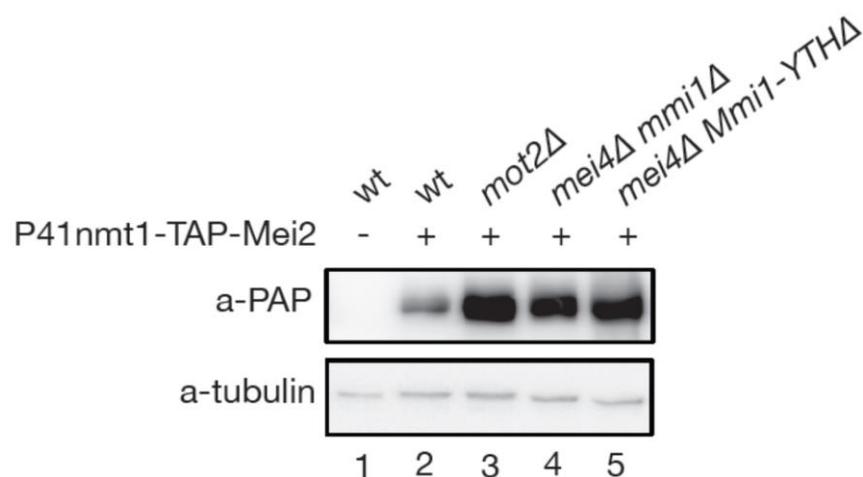


Figure 25. The YTH domain of Mmi1 is required for the repression of Mei2 levels in mitotic cells. Western Blot showing that TAP-Mei2 expressed from the *nmt1* promoter accumulates in cells expressing a variant of Mmi1 lacking its YTH domain (deletion of residues 350 to 488). Note that *mmi1*Δ cells were generated from a parental strain possessing a deletion of *mei4*+, since the absence of Mmi1 leads to severe growth defects due to the deleterious expression of Mei4. An anti-tubulin antibody was used as a loading control.

The requirement for the YTH domain of Mmi1, but not *mei*RNA, in the regulation of Mei2 is consistent with at least two scenarios: 1) The YTH domain of Mmi1 binds to an RNA species that is different from *mei*RNA, possibly bringing Mmi1 and Mei2 in close proximity,

or 2) the YTH domain of Mmi1 directly mediates the interaction with Mei2, raising the intriguing possibility that a protein-protein interaction would be sufficient for the inhibition of Mmi1 function in meiotic mRNAs suppression. Importantly, the association of Mei2 with the RNA-binding domain of Mmi1 could prevent the latter from recognizing meiotic mRNAs, thereby rationalizing the inhibitory function of Mei2.

3.3 The YTH domain of Mmi1 is required for interaction with Mei2

Previous work showed that Mmi1 and Mei2 directly associate *in vitro* [54], although the specific regions involved in the interaction are not known. Based on this evidence and our data, we investigated the possibility that the two proteins physically interact through the YTH domain.

To this aim, we affinity purified a truncated version of Mmi1 that lacks its YTH domain (**Fig. 26A**). Mass spectrometry analyses of co-eluting proteins revealed that Mmi1-*YTH* Δ still interacts with the MTREC core components (Red1, Mtl1 and Iss10) and the whole Ccr4-Not complex (**Fig. 26B**). In the absence of the YTH domain, the amount of Mei2 bound to Mmi1 decreases substantially. Note that Mei2 levels from the Mmi1-*YTH* Δ mutant should be directly compared to the ones in the *mot2* Δ Mmi1-TAP strain, since total Mei2 levels are identical in these cells. These results strongly support the notion that Mmi1 interacts with Mei2 via its YTH domain.

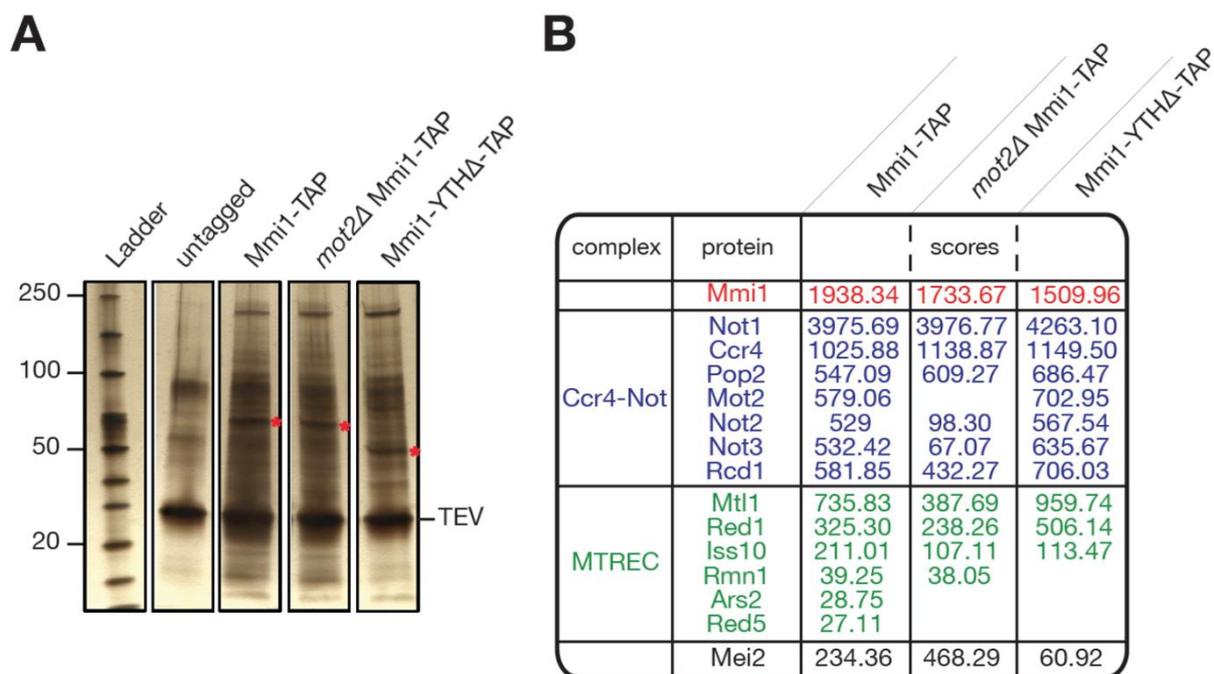


Figure 26. The YTH domain of Mmi1 is important for the interaction with Mei2.

(A) Silver-stained SDS polyacrylamide gel showing proteins co-eluting with full-length or *YTHΔ* TAP-tagged Mmi1 in minimal medium after one-step affinity purification. Extracts were treated with RNaseA/T1 before immunoprecipitation and TEV cleavage. As a control, extracts from cells expressing untagged protein were used. A red asterisk denotes the position of the bait protein (Mmi1-CBP or Mmi1-*YTHΔ*-CBP).

(B) Results of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of Mmi1-TAP and Mmi1-*YTHΔ*-TAP associated proteins. The scores (i.e. significance of the identified peptides represented as the $-\log_{10}$ of the Posterior Error Probability provided by the Percolator algorithm) of a subset of identified proteins (i.e. subunits of the Ccr4-Not and MTREC complexes, and Mei2) are indicated. Note that the scores for the Rmn1, Ars2 and Red5 subunits of MTREC are low and might not reflect stable interactions. Data for Mmi1-TAP and *mot2Δ* Mmi1-TAP are those included in the manuscript (Simonetti et al.).

To validate mass spectrometry results, we verified interactions of interest using different tagged strains in co-immunoprecipitation experiments. Consistent with previous data, we observed that the YTH domain is dispensable for the interaction of Mmi1 with the scaffolding subunit Not1 of Ccr4-Not and the core component Red1 of MTREC (**Fig. 27A-B**,

lanes 4 and 6). Importantly, however, the interaction between Mmi1 and Mei2 was lost upon removal of the YTH domain (**Fig. 27C**, lanes 2 and 4).

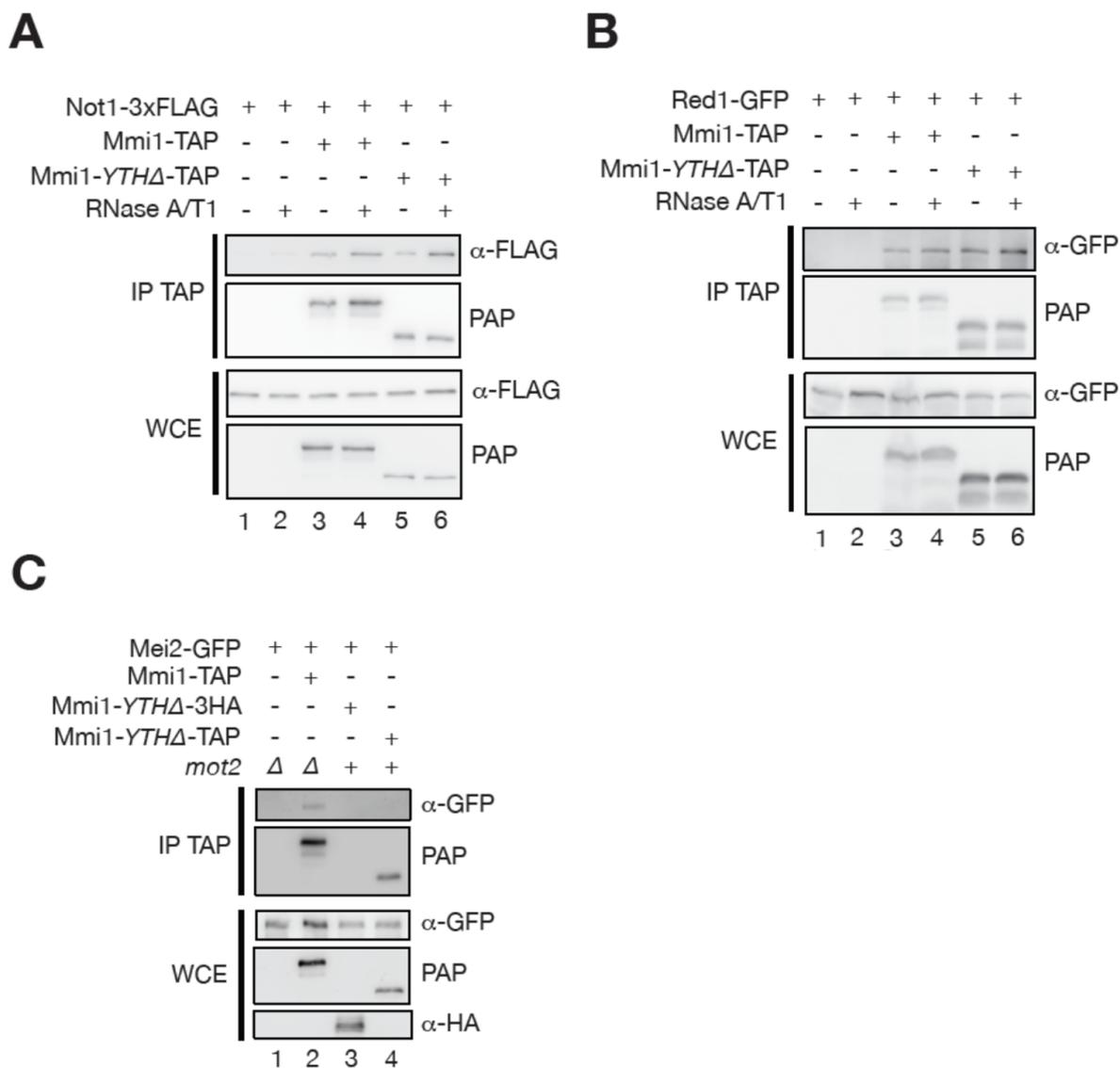


Figure 27. The YTH domain of Mmi1 is required for the interaction with Mei2, but not with Ccr4-Not and MTREC.

(A-B) Western blots showing that Not1-3xFLAG (A) and Red1-GFP (B) co-immunoprecipitate with both Mmi1-TAP and Mmi1-YTHΔ-TAP in an RNA-independent manner in minimal medium. (WCE) Whole Cell Extract; (IP) Immunoprecipitation.

(C) Western blots showing that the interaction between Mei2-GFP and Mmi1-TAP is lost in the absence of the YTH domain. Note that, because Mei2 levels are low in the wild type strain, *mot2Δ* cells were used for direct comparison with Mmi1-YTHΔ mutants. All extracts were treated with RNaseA/T1 prior to immunoprecipitation.

In conclusion, our *in vivo* approaches point to a crucial role for the YTH domain of Mmi1 in the interaction with its own inhibitor Mei2. They also have important implications about the mechanism by which Mei2 inhibits the activity of Mmi1, which will be discussed in the next section.

3.4 The N-terminal region of Mmi1 mediates the interactions with the MTREC and Ccr4-Not complexes

Our work indicates that Mmi1, but not MTREC (e.g. Red1 and Mtl1), stably associates with the Ccr4-Not complex *in vivo* during vegetative growth. This suggests that Mmi1 might interact independently with each effector complex.

To get more clues about the regions of Mmi1 involved in the interactions with MTREC and Ccr4-Not, we constructed several C-terminally truncated versions of the protein (**Fig. 28A**) and tested their ability to associate with Not1 or Red1, respectively.

As shown in **Fig. 28B-C**, the full-length and truncated versions of Mmi1 (e.g. Mmi1-TAP, Mmi1-YTH Δ -TAP, Mmi1-(265-488) Δ -TAP and Mmi1-(177-488) Δ -TAP) efficiently pulled down both Not1-3xFLAG and Red1-GFP in the presence or the absence of RNaseA/T1. These results indicate that the first 176 amino acid residues of Mmi1 are sufficient to maintain stable interactions with Ccr4-Not and MTREC. Whether this N-terminal portion of Mmi1 contains independent or overlapping binding sites for both complexes remains to be investigated. The implications of these alternative hypotheses are discussed below.

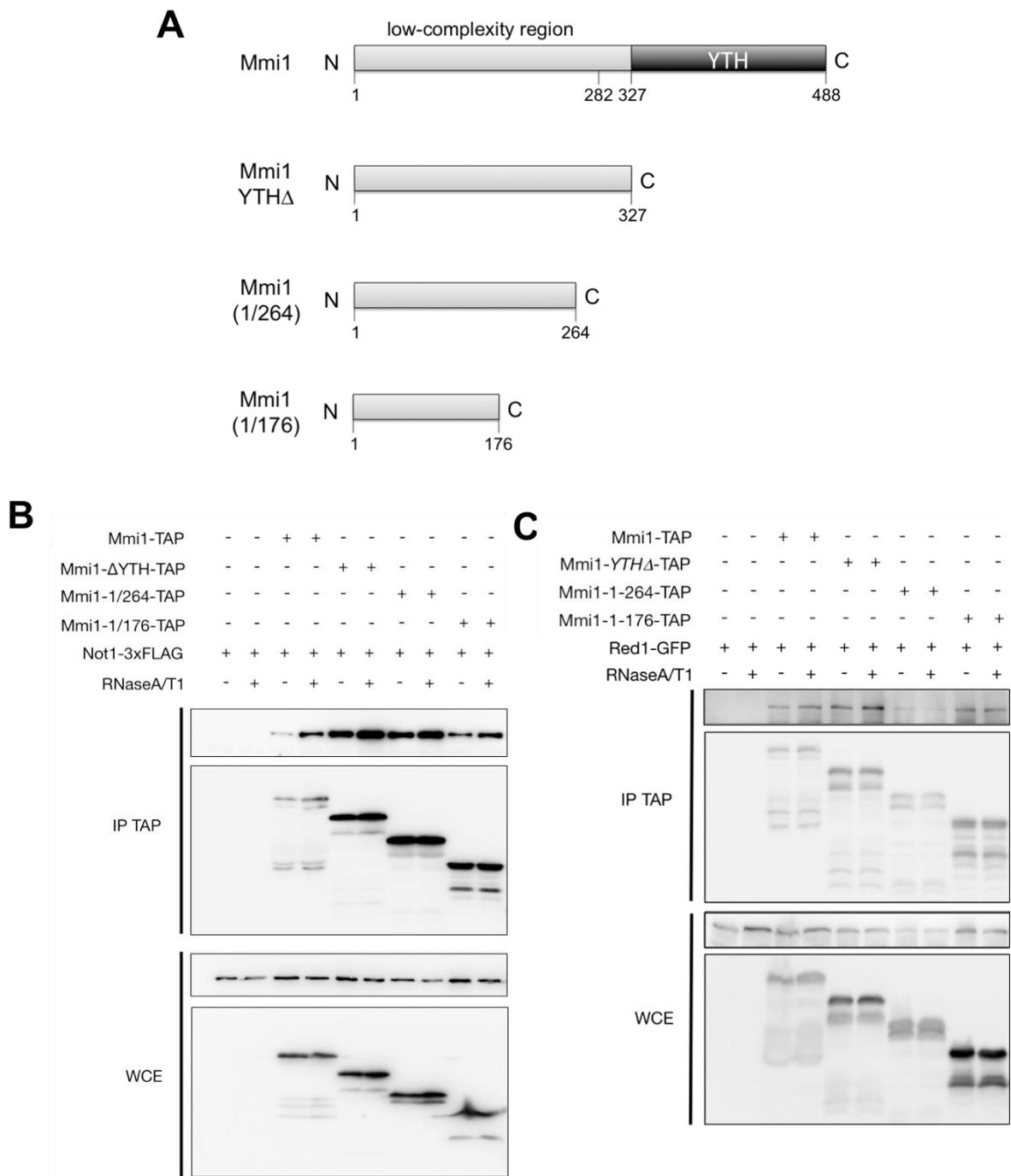


Figure 28. Analysis of the regions in Mmi1 involved in the interaction with Ccr4-Not and MTREC
(A) Scheme showing the full-length and truncated versions of Mmi1 used in the co-immunoprecipitation experiment described in **(B)**.
(B-C) Western blots showing that the full-length and truncated versions of Mmi1 all coimmunoprecipitate Not1-3xFLAG **(B)** and Red1-GFP **(C)**. Immunoprecipitations were performed from extracts treated or not with RNaseA/T1.

3.5 N-terminal residues of Mmi1 are important for efficient meiotic mRNAs suppression

Our results indicate that the N-terminal residues (1 to 176) of Mmi1 are sufficient for its association with the Ccr4-Not and MTREC complexes. We next sought to determine which part of the N-terminal region of Mmi1 is important for the degradation of meiotic transcripts. For this purpose, we constructed strains expressing different versions of Mmi1 truncated at its N-terminus. These include mutants of Mmi1 lacking the first 29, 65, 100 and 140 amino acids: Mmi1-(1-29) Δ , Mmi1-(1-65) Δ , Mmi1-(1-100) Δ , Mmi1-(1-140) Δ (**Fig. 29A**). The full-length and N-terminal truncated versions of Mmi1 were expressed from the *nmt1* promoter and the levels of specific meiotic mRNAs were analyzed in RT-PCR assays. Wild type and *mmi1* Δ strains were used as controls to estimate the relative accumulation of transcripts in the different strains.

As shown in **Fig. 29B**, the levels of *ssm4+*, *spo5+* and *mcp5+* meiotic mRNAs as well as the lncRNA *meiRNA* were substantially increased upon deletion of *mmi1+*, as expected. Deletions of the first 29 or 65 N-terminal residues of Mmi1 resulted in a minor accumulation of meiotic transcripts, suggesting that the regions removed from the protein participate only partially in the interactions with MTREC and/or Ccr4-Not. Conversely, the absence of the first 100 or 140 N-terminal residues triggered a significant increase in meiotic mRNA levels, similar to the deletion of *mmi1+* itself. These results indicate that the region located between residues 65 and 100 is important for the activity of Mmi1. Whether this underlies the presence of motifs dedicated to the interaction with MTREC and/or Ccr4-Not is still elusive and future work is needed to distinguish between distinct or overlapping binding sites in Mmi1.

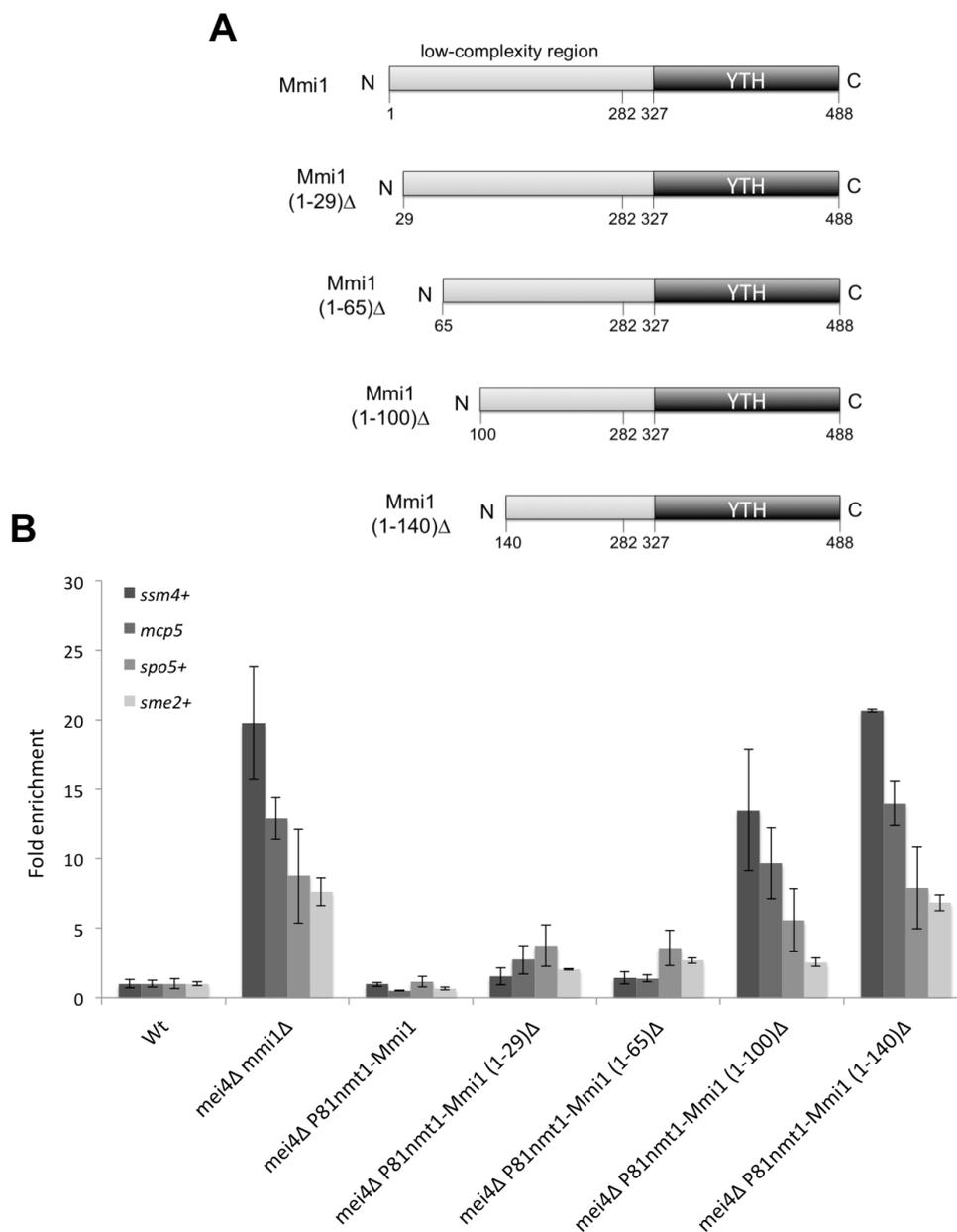


Figure 29. Study of the impact of the Mmi1 N-terminal region in meiotic mRNAs degradation.

(A) Scheme showing the full-length and truncated versions of Mmi1 used in the RT-qPCR assays described in (B).

(B) RT-qPCR analyses of meiotic transcripts in cells of the indicated genetic backgrounds and grown in minimal medium. Shown is the fold enrichment of RNAs levels normalized to *act1+* transcripts and expressed relative to the wild type strain. Error bars represent the standard deviation from three independent experiments. Strains deleted for Mmi1 or expressing the full-length protein from the *nmt1* promoter were used as controls. Note that all truncated versions of Mmi1 were constructed in a parental strain deleted for *mei4*, a key meiosis-specific transcription factor whose ectopic expression in the absence of Mmi1 leads to severe growth defects.

DISCUSSION

1 Mechanisms of sexual differentiation by the Mmi1-Mei2 system

1.1 YTH proteins and the Ccr4-Not complex: RNA deadenylation and/or protein ubiquitination?

Our results indicate that the YTH-family RNA-binding protein Mmi1 has a dual role in the regulation of sexual differentiation in fission yeast: in nuclear mRNA surveillance, by targeting meiotic transcripts for degradation by the nuclear exosome, and in protein ubiquitination and degradation, by recruiting the Ccr4-Not complex to its substrate Mei2. We have proposed that this latter mechanism reinforces the function of Mmi1 in meiotic mRNAs suppression by preventing the accumulation of its own inhibitor Mei2 during vegetative growth.

Interestingly, we have shown that the deadenylation activity of Ccr4-Not is dispensable for the degradation of DSR-containing meiotic transcripts. This observation is consistent with previous studies indicating that the RNA deadenylases Ccr4 and Pop2 do not affect the turn over and the translation of meiotic mRNAs [113, 218]. However, recent work in the Passmore lab indicates that Mmi1 stimulates the deadenylation activity of Ccr4-Not *in vitro* in a sequence-dependent manner [84]. The deadenylation kinetics of RNA substrates carrying a UUAAC sequence, but not a mutated DSR motif, is indeed considerably improved. These observations raise the question of whether Mmi1 might also promote Ccr4-Not-dependent deadenylation and degradation *in vivo*. So far, work from ours and other laboratories clearly argue against this possibility [113, 218] (our results). The reason for this discrepancy is not completely understood but it is possible that the Mmi1/Ccr4-Not complex inherently favors deadenylation of specific RNA substrates in *in vitro* reconstituted systems. Nonetheless, it can

be envisioned that a subset of DSR-containing transcripts is subjected to deadenylation, perhaps to facilitate subsequent degradation by the nuclear exosome.

Previous studies demonstrated that budding yeast and human YTH proteins (e.g. Mrb1/Pho92 and YTHDF2, respectively) also associate with the Ccr4-Not complex [255, 256], indicating that the interaction between these factors is evolutionary conserved. In both cases, it was shown that YTH homologues promote deadenylation and degradation of RNA substrates *in vivo*. Whether the ubiquitination activity of Ccr4-Not would also be involved in specific regulatory processes, as in case of fission yeast, is currently unknown. In this perspective, our work posits the bases to address such a possibility. Alternatively, and given the requirement for the nuclear exosome in degradation of Mmi1-targeted meiotic transcripts, *S. pombe* may have evolved an alternative function for Ccr4-Not.

1.2 Molecular basis for the inactivation of Mmi1 by Mei2

We have shown that the E3 ubiquitin ligase Mot2 of the Ccr4-Not complex has a crucial role in maintaining low levels of the Mmi1 inhibitor Mei2 in mitotic cells. Importantly, our analyses indicate that Mot2 targets only a pool of Mei2, whose constitutive degradation relies on another E3 ligase, Ubr1. We have suggested that Mot2 fine-tunes the abundance of Mei2, possibly at its sites of action, to avoid fluctuations in its levels that might cause a partial inhibition of Mmi1 during vegetative growth. This post-translational control of Mei2 maintains the function of Mmi1, thereby preventing untimely expression of the meiotic program. However, and despite these recent progresses, a full understanding of the regulation of sexual differentiation by the Mmi1-Mei2 system is still lacking. Indeed, our initial results raise several questions: How does Mmi1 bring the Ccr4-Not complex to its target Mei2? What is the molecular basis for the inactivation of Mmi1 by Mei2?

Because Mei2 forms an RNP complex with meiRNA to inactivate Mmi1 upon entry into meiosis, we investigated the role of the lncRNA in the regulation of Mei2. Interestingly, we found that meiRNA is dispensable for the repression of Mei2 and the inactivation of Mmi1 by high levels of Mei2 (**Fig. 24A-B**). Instead, the YTH domain of Mmi1 is crucial for the downregulation of Mei2 (**Fig. 25**). These results might underlie the existence of an unknown ncRNA that might bring Mmi1 and Mei2 in close proximity or a direct interaction between the two proteins that would depend on the integrity of the YTH domain itself.

To address this latter possibility, we assessed the capacity of Mmi1 lacking its YTH domain to associate with Mei2. Remarkably, Mmi1-*YTH* Δ cells failed to interact with Mei2 in mitotic cells (**Fig. 26B** and **Fig. 27C**). Importantly, this effect was specific since the associations with the MTREC and Ccr4-Not complexes were preserved in this context (**Fig. 27A-B**). Together, these experiments raise the unprecedented possibility that the YTH domain of Mmi1 serves as a binding platform not only for RNA molecules but also for protein(s).

A key prediction from the above-mentioned results is that Mei2 should directly affect the RNA-binding capacity of Mmi1. Accordingly, it is tempting to speculate that meiotic transcripts and Mei2 might compete for binding to the YTH domain of Mmi1 (**Fig. 30**). Such a scenario would rationalize the inhibitory function of Mei2 and provide important information about the mechanisms at play in the regulation of sexual differentiation by the Mmi1-Mei2 system.

To directly test this hypothesis, one could perform RNA-immunoprecipitation experiments using Mmi1 as a bait protein in wild type and *mot2* Δ strains. Following RNA extraction, a few selected meiotic transcripts could be analyzed in RT-qPCR assays. Should our model be correct, one would expect that Mmi1 bind less efficiently to meiotic mRNAs in the absence of Mot2, as a consequence of increased Mei2 levels. High-throughput sequencing

of the isolated RNA samples could then provide a global view of the effect of Mei2 on Mmi1 binding to its targets.

To determine whether Mmi1 binds to meiotic transcripts and Mei2 in a mutually exclusive manner, *in vitro* electromobility shift assays (EMSA) using recombinant proteins and a synthetic radiolabeled DSR-containing RNA could be envisioned. This should allow assessing whether the formation of an Mmi1-RNA complex can be challenged by the presence of Mei2. If our hypothesis is correct, increasing levels of recombinant Mei2 should displace the equilibrium towards the formation of the Mmi1-Mei2 complex. Overall, these approaches should enable us to unveil the precise mechanism by which Mei2 inhibits the activity of Mmi1.

Our data strongly support the notion that the YTH domain of Mmi1 is required for the interaction with Mei2. However, the region(s) or domains within Mei2 that are involved in the interaction are currently unknown. *In vitro* pulldown experiments using different fragments of Mei2 produced from bacteria could be performed to localize and narrow down the region(s) of interaction with Mmi1. This should permit to determine the major elements that dictate the interaction between Mmi1 and Mei2. Interestingly, Mei2 contains three RNA Recognition Motifs (RRM) and the possibility that one of them participates in the association with the YTH domain of Mmi1 is fascinating.

Regardless of the precise regions involved, these experiments could set the basis for future structural studies of both proteins or fragments thereof, alone or in complex. This would allow the identification of the amino acids directly involved in the interaction, which could then be substituted by targeted mutagenesis to test their impact *in vivo*. One can predict that point mutants of Mei2 that fail to associate with Mmi1 should escape Ccr4-Not-dependent ubiquitination and degradation while preserving meiotic mRNAs suppression, which could be assessed by western blotting and RT-qPCR respectively.

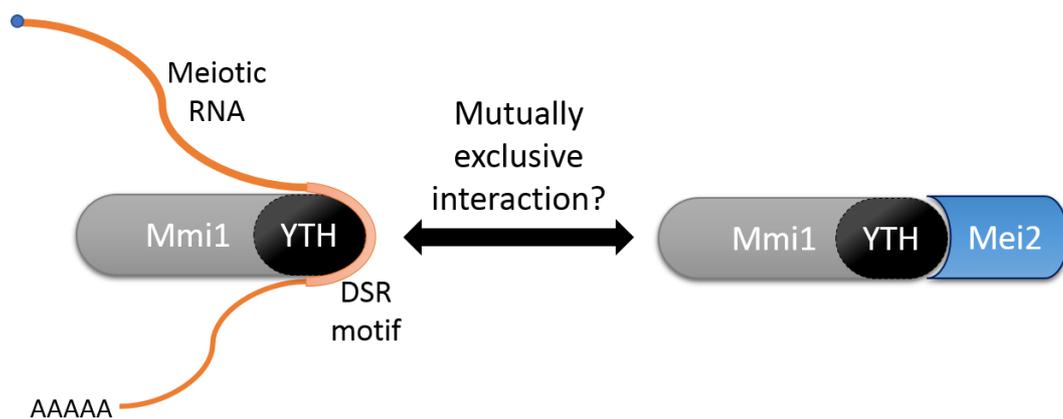


Figure 30. Scheme of the possible roles of the YTH domain of Mmi1 in protein-RNA and/or protein-protein interaction.

The YTH domain of Mmi1 might directly mediate the association with Mei2. In this perspective, the inhibitory action of Mei2 on Mmi1 would consist in occluding the YTH domain, hampering its RNA-binding ability. Meiotic mRNAs and Mei2 might therefore associate with the YTH domain of Mmi1 in a mutually exclusive manner.

1.3 Distinct Mmi1-containing complexes for specific functions?

Affinity purification and mass spectrometry analyses revealed that Mmi1 is engaged in a number of protein-protein interactions. Interestingly, we have found that Mmi1 associates with the Ccr4-Not complex even when the integrity of MTREC is compromised (e.g. in *red1Δ* cells). Moreover, our co-immunoprecipitation assays indicate that MTREC does not stably interact with Ccr4-Not *in vivo*. Together, these results suggest that different Mmi1-containing complexes assemble in the nucleus of fission yeast cells.

To determine whether the associations of Mmi1 with MTREC and Ccr4-Not reflect the existence of distinct or overlapping binding sites, we performed pulldown experiments using various truncated versions of Mmi1 and observed that an N-terminal portion of the protein encompassing residues 1 to 176 was sufficient to maintain the interactions with both complexes (**Fig. 28B-C**). We also uncovered that the first 100 amino acid residues in Mmi1 are important

for meiotic mRNA suppression (**Fig. 29B**). Both datasets highlight a crucial function for the N-terminal region of Mmi1 in protein interactions and RNA degradation. Interestingly, it has been recently shown that the first 56 residues of Mmi1 are important, although not essential, for interaction with Ccr4-Not [84]. Further dissection of the Mmi1 sequences involved in the interactions with MTREC and Ccr4-Not is needed to determine the presence of independent or overlapping binding sites (**Fig. 31**).

To address whether the two complexes associate with Mmi1 in a mutually exclusive manner, the direct partners should be known. Recent cryoEM-based structural analyses suggested that Mmi1 directly contacts the N-terminal part of the scaffolding subunit Not1 of Ccr4-Not and possibly its RNA deadenylase Pop2 [166]. Other biochemical studies showed that Iss10 contribute to bridge Mmi1 to MTREC [101, 108, 109]. Taking advantage of these studies, we could test the functional consequences of overexpressing Not1 or Iss10 *in vivo* on meiotic mRNAs suppression and Mei2 degradation. If the interactions are mutually exclusive, over-expression of both factors in mitotic cells should result in the accumulation of meiotic transcripts: increased Iss10 levels should reduce the association of Mmi1 with Ccr4-Not, thereby precluding degradation of Mei2 and as a consequence meiotic mRNA suppression. Conversely, over-expression of Not1 should displace MTREC from Mmi1 and result in a defect in the degradation of meiotic transcripts.

To determine the exact nature of the interactions at play, *in vitro* reconstitution with purified proteins is ultimately required. Should Iss10 and Not1 be the direct Mmi1 partners, recombinant proteins or fragments could be used in competition assays. If the two proteins compete for binding to Mmi1, then addition of recombinant Iss10 or Not1 to preassembled Mmi1-Not1 or Mmi1-Iss10 complexes, respectively, could displace the equilibrium towards the formation of the alternative complexes (Mmi1-Iss10 and Mmi1-Not1, respectively).

Alternatively, formation of larger Mmi1-Iss10-Not1 complexes would be indicative of distinct regions within Mmi1 that are required for interaction with each partner.

Future biochemical work should provide clues about the assembly and the dynamics of distinct Mmi1-containing complexes. It is fascinating to imagine that Mmi1 may act as a hub to recruit different effector complexes (MTREC and Ccr4-Not) to their respective substrates (meiotic transcripts and Mei2).

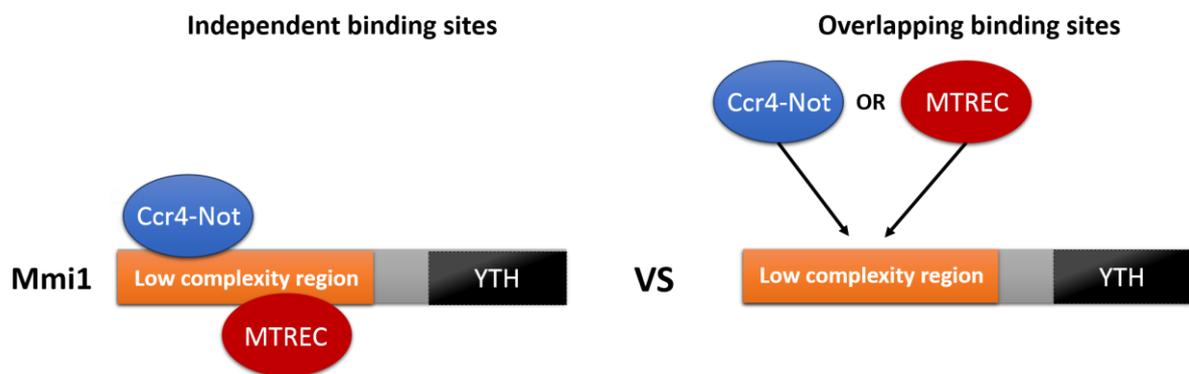


Figure 31. Models for the association of the Ccr4-Not and MTREC complexes to Mmi1.

According to current evidence and our results, two possible scenarios can be envisioned: the Ccr4-Not and MTREC complexes might contact Mmi1 on two distinct sites (**left**) or the two complexes might associate with Mmi1 in a mutually exclusive manner, sharing overlapping binding sites (**right**).

1.4 A general model for the regulation of meiotic mRNAs suppression

During the mitotic cell cycle, Mmi1 localizes to one or several scattered nuclear foci [54, 76]. These subnuclear structures have been shown to also contain several factors involved in meiotic mRNAs suppression, including components of the 3'-end processing and polyadenylation machinery, MTREC subunits as well as the nuclear exosome [101, 102, 105, 106]. It was proposed that the nuclear bodies serve as RNA processing and/or decay factories that bring effectors in close proximity, but whether RNA degradation is effectively occurring at these sites is still elusive [257].

Evidence for the colocalization of Mmi1 and Ccr4-Not in nuclear foci is currently lacking. Nonetheless, because Mmi1 recruits the complex to its RNA targets *in vivo* [218], it is conceivable that Ccr4-Not might be, at least transiently, a component of these structures. In this perspective, different Mmi1-containing complexes (i.e. Mmi1/MTREC and Mmi1/Ccr4-Not) may colocalize in nuclear bodies, raising the possibility that they bind simultaneously to the same DSR-containing meiotic mRNAs, possibly using distinct UNAAAC motifs. One fascinating scenario predicts that RNA-bound Mmi1/Ccr4-Not complexes may preserve the activity of neighboring Mmi1/MTREC complexes by targeting surrounding Mei2 molecules for ubiquitination and degradation (**Fig. 32**). A local environment free from Mei2 would thus sustain the function of Mmi1/MTREC in meiotic mRNA suppression during vegetative growth. One corollary is that, upon nutritional starvation, increased Mei2 levels would overcome the Ccr4-Not ubiquitination activity and evict Mmi1-containing complexes from meiotic transcripts by occluding YTH domains.

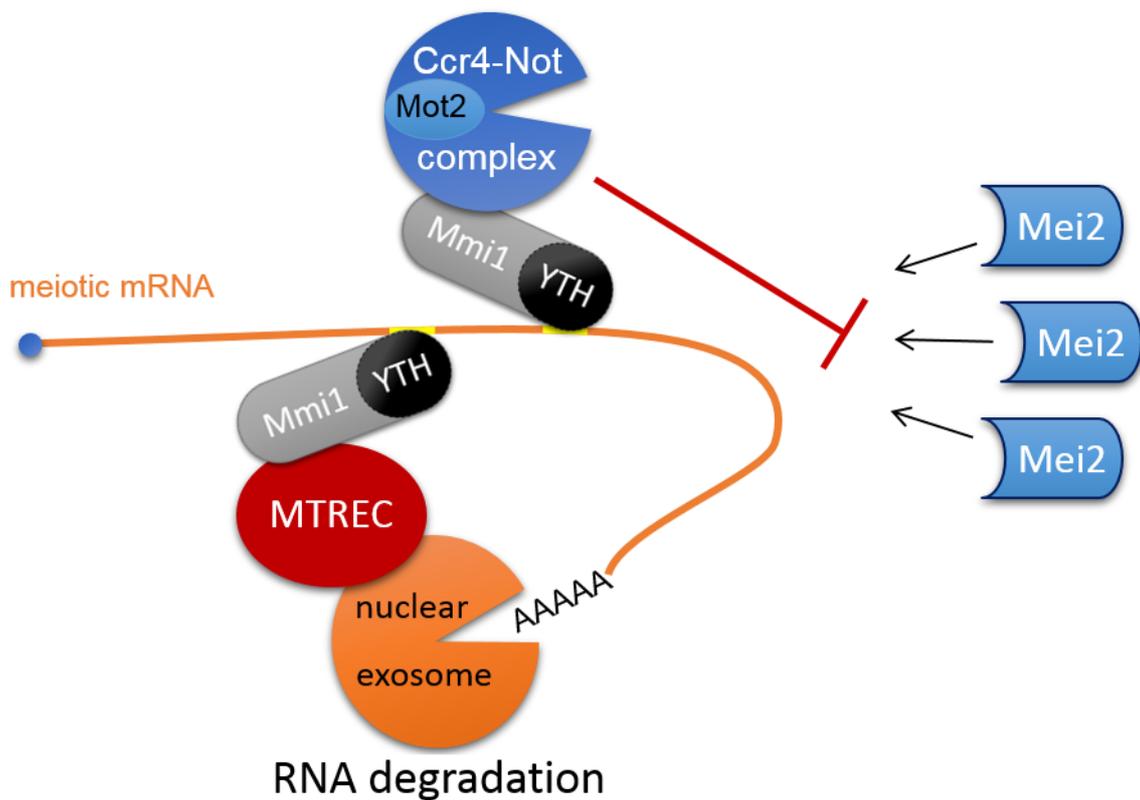


Figure 32. Model for the regulation of meiotic mRNA degradation by Mei2 and the Ccr4-Not complex.

Putative model in which more than one Mmi1-containing complex can associate to a single meiotic mRNA (containing several DSR motifs). In this perspective, the ubiquitination activity of the Mot2 subunit of the Ccr4-Not complex might efficiently avoid the YTH domain of Mmi1 to be invaded by Mei2. This will sustain the meiotic mRNA degradation process.

Upon meiosis onset, scattered Mmi1 nuclear bodies converge to a single dot, which localized to the *sme2+* locus and contains both meiRNA and Mei2 [54, 76]. This RNP complex has been proposed to sequester and inactivate Mmi1, thereby allowing meiotic mRNAs to escape nuclear degradation and enter a cytoplasmic phase for translation. How Mmi1 and Mei2 assemble on meiRNA in the dot is not completely understood, but previous studies suggested that Mei2 associates preferentially with the 5'-end of the transcript while Mmi1 binds to its 3'-end where DSR motifs are enriched [76]. However, expression of the 3' part of meiRNA was sufficient for cells to sporulate [76], indicating that Mei2 is still functional in these conditions,

possibly because it can also bind the 3' part of meiRNA *in vivo*. Based on the literature and our results, we suggest a model in which Mei2 may associate directly and simultaneously with both meiRNA and the YTH domain of Mmi1 (**Fig. 33**). In this view, the inactivated form of Mmi1 may not directly interact with meiRNA since Mei2 occludes its YTH domain. This is consistent with our findings that meiRNA is dispensable for the inactivation of Mmi1 by high Mei2 levels (i.e. in the absence of Mot2). An important aspect of the model is that the nature and the composition of the meiRNA dot change during the switch from mitosis to meiosis: in mitotic conditions, Mmi1, MTREC, the exosome and possibly Ccr4-Not assemble in larger complexes to mediate meiRNA (and possibly other meiotic transcripts) degradation at the *sme2+* locus, while a tripartite Mmi1-Mei2-meRNA complex may represent one molecular facet of the meiRNA dot observed upon meiosis onset. Future work should help distinguishing between different scenarios to better understand the regulatory mechanisms of sexual differentiation involving the Mmi1-Mei2 system.

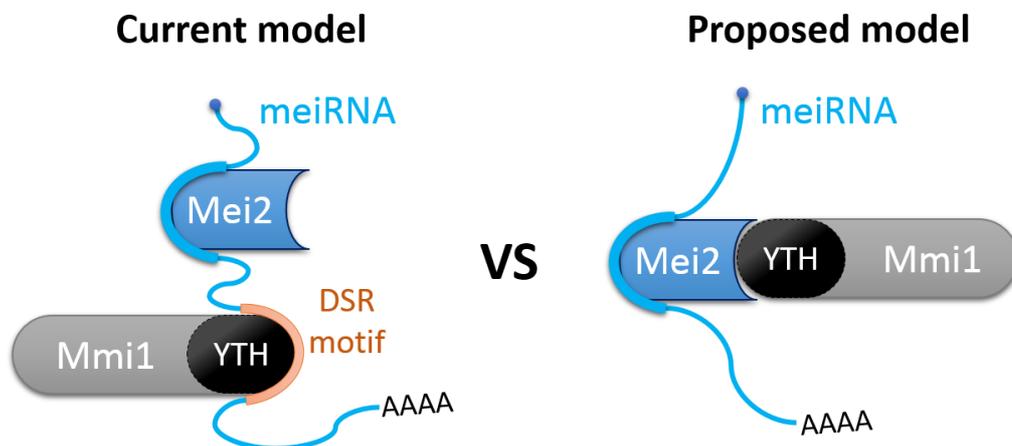


Figure 33. Model for the possible association of Mei2 with meiRNA and Mmi1 within the Mei2-dot.

The Mei2-dot is composed by the RNA-binding protein Mmi1, the RNA-binding protein Mei2 and the lncRNA meiRNA. The current model (**left**) provides that Mmi1 and Mei2 both associate with the DSR-containing lncRNA meiRNA. We propose a model for which Mei2 associate with meiRNA and also with the YTH domain of Mmi1, impairing its RNA-binding ability (**right**).

1.5 The assembly/disassembly of nuclear foci

Another important unresolved issue in the field concerns the mechanisms and the dynamics of assembly/disassembly of the Mmi1-containing nuclear foci in both mitosis and meiosis. The structures resemble nuclear factories that facilitate processing/degradation of RNA molecules such as P-bodies in yeast and mammalian stress granules [257]. Interestingly, the N-terminal half of Mmi1 contains low complexity sequences (serin and prolin rich), which are believed to favor the formation of subcellular structures by promoting reversible structural rearrangements of proteins from a soluble to a polymeric state [258]. Importantly, RNA molecules have been shown to aid the assembly of such structures [259]. In light of these considerations, it is tempting to speculate that RNA-bound Mmi1 may itself contribute to the assembly of nuclear foci, facilitating a local gathering of the many factors involved in meiotic mRNA suppression. Conversely, increased Mei2 levels upon meiosis onset could contribute to the dismantling of the nuclear bodies (but not the meiRNA dot) by precluding the association of Mmi1 with its RNA targets. Noteworthy, MTREC nuclear foci observed during vegetative growth disappear when cells enter meiosis [106]. Biochemical and biophysical studies are needed to test these attractive hypotheses.

2 Beyond meiotic mRNA suppression: additional roles for the RNA-binding protein Mei2 and the E3 ubiquitin ligase Mot2?

2.1 Towards a comprehensive view of Mei2 RNA targets

Beside its involvement in the inactivation of Mmi1, Mei2 has also been shown to promote pre-meiotic DNA synthesis and telomere clustering at the Spindle Pole Body during meiotic prophase [18, 59]. The molecular details underlying these phenotypes are currently unknown but it has been proposed that they might underlie the association of Mei2 with specific RNA species, different from meiRNA. Mei2-RNA complexes may act as molecular glue for the recruitment of specific factors involved in chromosome-related processes. Intriguingly, recent work showed that the accumulation of meiRNA at the *sme2+* locus mediates robust pairing of homologous chromosomes during meiotic prophase [77]. By analogy, one can imagine that additional RNA species cooperate with Mei2 to regulate chromosomal associations, like telomere clustering. However, the catalogue of transcripts that associate with Mei2 is currently missing, meiRNA being the only RNA target described so far.

To determine the full repertoire of Mei2 RNA targets, one could perform a CRAC analysis (UV CRosslinking and Analysis of cDNA), which allows the genome-wide identification of RNA molecules associated with a protein of interest. The procedure relies on UV-based crosslinking of protein-RNA complexes in living cells followed by two-step affinity purification of the given protein, isolation of the associated RNAs and high-throughput sequencing of the corresponding cDNAs. Bioinformatic analyses would identify RNA species that are significantly enriched, using meiRNA as a reference for the efficiency of the overall

procedure. In a best-case scenario, a subset of additional lncRNAs could be uncovered and their role in Mei2-dependent meiotic processes would be systematically studied.

2.2 The E3 ligase Mot2 is required for the repression of Ste11 target genes

Our results indicate that the E3 ubiquitin ligase subunit Mot2 of the Ccr4-Not complex targets Mei2 for ubiquitination and degradation to preserve the function of Mmi1 in meiotic mRNA suppression during vegetative growth. Consistent with this, transcriptome analyses of *mot2Δ* cells by RNA-sequencing revealed a substantial accumulation of DSR-containing mRNAs. Moreover, another subset of genes, encoding various factors involved in the mating and conjugation processes, was also up-regulated in the absence of Mot2. Interestingly, several of these are known targets of the meiosis-specific transcriptional regulator Ste11. Remarkably, we found that Mmi1 and Rrp6 do not participate in the downregulation of these RNA species, indicating that they are not targeted to the Mmi1/exosome degradation pathway. This is also consistent with the absence of DSR motifs in the targets.

The biological relevance of this phenotype is not yet understood but it is tempting to speculate that it might reflect a role for Mot2 in the regulation of Ste11 levels. Similarly to Mei2, Ste11 is inhibited by phosphorylation through the action of the Pat1 and Tor2 kinases. This causes the binding of 14-3-3 proteins to Ste11, which inhibits its transcriptional activity by preventing its nuclear accumulation [45]. It has also been proposed that these phosphorylation events may stimulate the ubiquitin-dependent degradation of Ste11 [46], but experimental evidence supporting this is currently lacking. The possibility that Mot2 participates to the ubiquitination and possibly the degradation of Ste11 is particularly attractive, providing a molecular basis for the accumulation of Ste11 targets in the *mot2Δ* mutant. This scenario could be tested experimentally by determining the steady state levels, stability and

ubiquitination of the protein in wild type and *mot2Δ* cells. Of note, Ste11 controls the expression of its own gene [34], suggesting that even small changes in its stability could convey to a substantial response (i.e. increased expression of Ste11 target genes). Regardless of the precise mechanistic details, it is intriguing to imagine that Mot2 may also regulate earlier steps in sexual differentiation.

Several different scenarios could explain the requirement for Mot2 in the regulation of Ste11 target genes (**Fig. 34**). For example, Mot2 may inactivate and/or induce the degradation of a positive regulator of *ste11+* expression, possibly via its ubiquitination activity. These include the Rst2, Atf1 and Pcr1 transcription factors [26, 34, 260], the transcriptional coactivator SAGA complex [41] as well as the Sty1 and Spk1 signaling kinases that phosphorylate Ste11 to activate it [18, 261]. Moreover, it has been suggested that Mei2, whose expression depends on Ste11, in turn stimulates the transcription of *ste11+* in a complex positive feedback loop [28]. Considering our results, it is possible that the increased levels of Mei2 in *mot2Δ* cells indirectly promote expression of *ste11+* and its target genes. We cannot exclude additional indirect effects that may convey expression of this set of meiotic genes in the absence of Mot2.

In conclusion, the role of Ccr4-Not in sexual differentiation seemingly goes beyond its function in suppressing the accumulation of Mei2 in vegetative cells. How the E3 ligase Mot2 regulates early steps of the meiotic program, including mating and conjugation, is an intriguing topic for future research aimed at deciphering the mechanisms involved in the control meiosis onset in fission yeast.

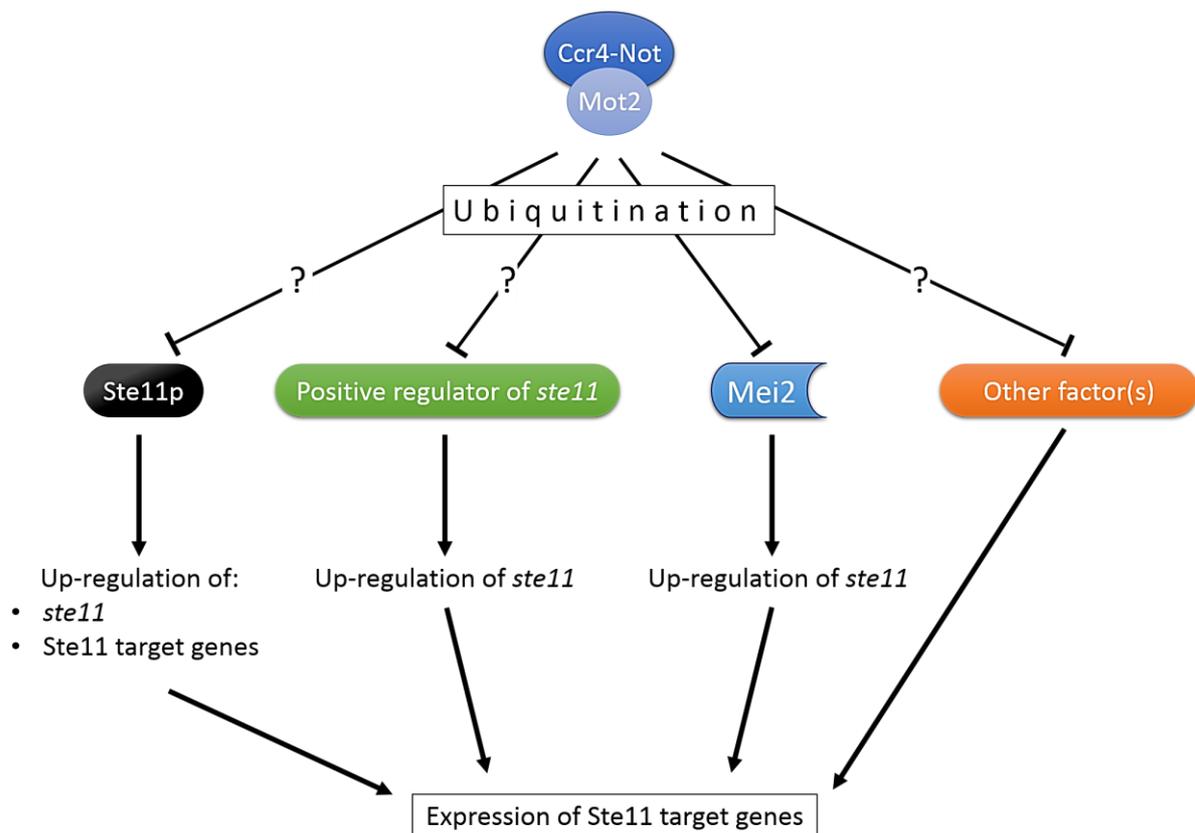


Figure 34. Scheme for the role of Mot2 in downregulating the expression of some Ste11 target genes.

It can be envisioned that the downregulation of Ste11 targets by Mot2 may be achieved through different ways. Mot2, possibly via its ubiquitination activity, might directly affect Ste11p, or a positive regulator of the *ste11+* gene, or other, yet unidentified factors. This would induce a down-regulation of Ste11 target genes. Additionally, given the role of Mot2 in down-regulating Mei2p (our results), the decrease in Mei2p levels would lead to a lower expression of *ste11+*. This might lead to a subsequent decrease in the expression of Ste11 target genes. (? : no currently available evidence supporting the mechanism).

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RÉSUMÉ DE LA THÈSE EN FRANÇAIS

Le passage du cycle cellulaire de la phase de mitose à celle de méiose est associé à des changements profonds dans l'expression des gènes. Dans la levure, en particulier, l'entrée en méiose se déclenche en condition d'absence de substances nutritives. Elle dépend aussi d'une voie de signalisation très spécifique. Plusieurs gènes sont exprimés grâce à des facteurs de la transcription, qui définissent les étapes du programme méiotique.

Chez *S. pombe*, il a précédemment été montré l'existence d'un mécanisme additionnel qui contrôle l'entrée en méiose. Un système de dégradation de l'ARN supprime des transcrits spécifiques à la méiose qui se produisent pendant le cycle cellulaire mitotique. Ce système de dégradation interdit donc la différentiation sexuelle. La protéine de liaison à l'ARN Mmi1 (meiotic mRNA interceptor factor 1), membre de la famille YTH, est essentielle pour ce processus. Elle est localisée dans le noyau et reconnaît, grâce à son domaine YTH C-terminale, des répétitions de motifs spécifiques (UNAAAC) au sein des transcrits, et dirige ces derniers vers la dégradation par l'exosome nucléaire. Mmi1 est aussi associé à plusieurs

ARN codant et noncodant, avec moins de motifs UNAAAC, qui indiquent une flexibilité dans la reconnaissance des cibles.

Plusieurs facteurs identifiés par des études génétiques ainsi que par des analyses biochimiques, coopèrent avec Mmi1 afin de promouvoir la suppression des ARNm méiotiques pendant la croissance végétative. Etudes précédentes ont montré un rôle du composant de la machine de traitement du 3'-end, aussi que de la polymérase des Poly(A) canonique Pla1, et de la protéine de liaison aux Poly(A) Pab2. Mécaniquement c'a été d'abord proposé que Mmi1 promeut l'hyperadenylation des transcrits ciblés et la liaison de Pab2 sur les queues de Poly(A) des ARNm. Pab2 donc recrute la sous-unité Rrp6 de l'exosome nucléaire pour la dégradation.

Des études ont plus tard montré que Mmi1 s'associe et coopère avec les complexes MTREC afin de promouvoir la suppression des ARNm. Les composants principaux de ce complexe, incluent la protéine doigt à zinc Red1 et l'hélicase de l'ARN Mtl1. MTREC est essentiel à la dégradation des ARN méiotiques, et il a été proposé qu'il sert de médiateur dans l'interaction entre Mmi1 lié aux ARN, et l'exosome nucléaire. Des études récentes ont aussi montré

une interaction entre Mmi1 et le complexe Ccr4-Not, qui constitue la majeure deadenylase des ARN cytoplasmiques des levures aux humaines. En revanche, malgré Mmi1 recruté Ccr4-Not à ses ARN cibles *in vivo* et promu son activité de deadenylation *in vitro*, le complexe n'est pas nécessaire à la dégradation et à la traduction des ARN méiotiques.

En condition d'absence de substances nutritives, Mmi1 est séquestré dans un complexe RNP, qui permet la traduction des ARN méiotiques et l'avancement de la cellule dans le processus de méiose. Ce complexe inhibiteur inclut la protéine de liaison à l'ARN Mei2, et l'ARN long non codant, meiRNA, qui est codé par le gène *sme2+*. La séquestration de Mmi1 par le complexe Mei2-MeiRNA, arrive au locus génétique de *sme2+*, et l'échec dans l'assemblage de cette structure empêche l'entrée en méiose, ce qui met en évidence sa relevance biologique dans le contexte de la différentiation sexuelle.

Malgré les progrès récents, une compréhension totale de la façon avec laquelle Mmi1 coopère avec ses cofacteurs, pendant la croissance végétative, dans le but d'empêcher l'initiation du programme méiotique, c'est encore quelque chose de pas complètement acquis.

En utilisant une technique de purification par affinité et des expérimentations de co-immunoprécipitation, nous avons montré que Mmi1, mais pas MTREC, s'associe *in vivo* stablement avec le complexe Ccr4-Not. Cette interaction est importante d'un point de vue fonctionnel, car il est requis à la suppression des ARN méiotiques pendant la croissance végétative. Nous avons aussi montré que la ligase de l'ubiquitine Mot2 du complexe Ccr4-Not a un rôle primordial dans la dégradation des ARNm méiotiques. Cette fonction consiste à maintenir bas le niveau de l'inhibiteur de Mmi1, Mei2, dans les cellules mitotiques.

Fait d'importance considérable, nos analyses indiquent que Mot2 cible seulement un sous-ensemble de Mei2, dont la dégradation constitutive dépend d'une autre ligase, Ubr1. Nous avons suggéré que Mot2 règle finement la quantité de Mei2, éventuellement chez ses sites cible d'action. Cela afin d'éviter fluctuations dans son niveau, fluctuations qui pourraient produire une inhibition partielle de Mmi1, pendant la croissance végétative. Ce contrôle post-traductionnel de Mei2 maintient la fonction de Mmi1, ainsi en prévenant une expression inopportune du programme méiotique.

Nos résultats suggèrent que Mmi1 a un double rôle dans la régulation de la différenciation sexuelle de la levure fissipare: au niveau de la surveillance nucléaire des ARNm, en ciblant les transcrits méiotiques à la dégradation par l'exosome nucléaire, et au niveau de l'ubiquitination et dégradation des protéines, en recrutant le complexe Ccr4-Not aux substrats de Mei2. Nous avons proposé que ce dernier mécanisme renforce la fonction de Mmi1 dans la suppression des ARN méiotiques, en empêchant l'accumulation de son propre inhibiteur Mei2, pendant la croissance végétative.

Dans ce contexte on a pu remarquer un aspect très intéressant: l'activité de deadenylation de Ccr4-Not ne semble pas être nécessaire à la dégradation des transcrits méiotiques. Cette observation est en ligne avec certaines études faites en précédente, qui en particulier ont indiqué que les deadenylases de l'ARN Ccr4 et Pop2 n'affectent pas le turnover et la traduction des ARN méiotiques. De l'autre côté, les travaux du laboratoire de Passmore suggèrent que Mmi1 stimule l'activité de deadenylation de Ccr4-Not *in vitro*, d'une façon dépendante de la séquence de l'ARN. Ces observations font penser à la possibilité que Mmi1 puisse aussi promouvoir la deadenylation dépendante de Ccr4-Not, et donc une

suivante dégradation *in vivo*. Jusqu'à présent, les travaux faits par nous, ainsi que par d'autres laboratoires, soulèvent des forts doutes par rapport à cette possibilité.

La raison de cette divergence n'est pas tout à fait comprise. Cependant il se peut que le complexe Mmi1/Ccr4-Not promut la deadenylation des substrats ARN spécifiques, dans des systèmes reconstitués *in vitro*. Néanmoins, on peut envisager qu'un sous-ensemble des transcrits est sujet à la deadenylation, ce qui pourrait faciliter la dégradation suivante, faite par l'exosome nucléaire.

Des études précédentes ont montré que les protéines YTH de la levure bourgeonnante, ainsi que dans les humaines, s'associent aussi au complexe Ccr4-Not, ce qui indique que l'interaction entre ces facteurs est conservée au cours de l'évolution. Dans les deux cas, il a été montré que les homologues YTH promeuvent la deadenylation et la dégradation des substrats ARN *in vivo*. Si l'activité d'ubiquitination de Ccr4-Not ait, ou pas, aussi un rôle dans des processus réglementaires, comme dans le cas de la levure fissipare, ce n'est pas clair à l'heure actuelle.

A cet égard, notre travail jette les bases à l'investigation de cette possibilité. Autrement, vue la nécessité de la présence de l'exosome nucléaire dans la dégradation des transcrits méiotiques ciblés par Mmi1, c'est possible que *S. pombe* ait développé une fonction alternative pour Ccr4-Not.

Cependant, malgré ces progrès récents, il faut reconnaître qu'une compréhension complète de la régulation de la différenciation sexuelle par le système Mmi1-Mei2, n'est pas encore acquise. En effet nos résultats préliminaires ont soulevé plusieurs points d'interrogation: entre autres, comment ça se fait que Mmi1 amène le complexe Ccr4-Not chez sa cible Mei2? Ou bien, quel est le mécanisme moléculaire pour l'inactivation de Mmi1 par Mei2?

Etant donné que Mei2 forme un complexe RNP avec MeiRNA, pour désactiver Mmi1, lors de l'entrée en méiose, nous avons investigué le rôle du long ARN non codant dans la régulation de Mei2. Dans ce contexte nous avons réussi à montrer que MeiRNA n'est pas nécessaire à la répression de Mei2 et à la désactivation de Mmi1, en présence des hauts niveaux de Mei2. En plus nous avons aussi montré que le domaine YTH de Mmi1 est essentiel pour la régulation négative de Mei2. Ces résultats pourraient sous-entendre l'existence d'un ARN

non codant inconnu, qui pourrait rapprocher Mmi1 et Mei2 ou une interaction directe entre les deux protéines, qui dépendrait de l'intégrité du domaine YTH même.

Remarquablement nous avons montré que Mmi1 sans son domaine YTH, n'est pas capable d'interagir avec Mei2 dans les cellules mitotiques. C'est aussi important de remarquer que cet effet est spécifique, parce que les associations avec les complexes MTREC et Ccr4-Not étaient dans ce contexte préservées. Dans l'ensemble, ces expérimentations font penser à la possibilité, sans précédent, que les domaines YTH de Mmi1 se comportent comme des plateformes d'interaction non seulement pour les molécules d'ARN, mais aussi pour les protéines.

En conséquence il est tentant de spéculer que les transcrits méiotiques et Mei2 puissent être en concurrence pour interagir avec le domaine YTH de Mmi1. Un tel scénario pourrait donner une explication à la fonction inhibitrice de Mei2, ainsi que fournir des informations cruciales par rapport au mécanisme impliqué dans la régulation de la différenciation sexuelle faite par le système Mmi1-Mei2.

Digne d'être remarqué c'est que Mmi1 est localisé dans un ou plusieurs points nucléaires. Il a été montré que ces structures sous-nucléaires contiennent aussi plusieurs facteurs jouant un rôle dans la suppression des ARN méiotiques, parmi lesquels on peut mentionner certains composants du mécanisme de traitement du 3'-end et de polyadénylation, ainsi que des sous-unités MTREC et l'exosome nucléaire.

Il a été proposé que ces corpuscules nucléaires jouent le rôle de centre de traitement de l'ARN et/ou centre de dégradation, qui rapprochent des effecteurs. Si la dégradation se passe dans cet endroit ou pas, c'est toujours quelque chose d'inconnu. Actuellement la littérature ne montre pas d'indices de co-localisation de Mmi1 et Ccr4-Not, aux spots nucléaires. Néanmoins, étant donné que Mmi1 recrute le complexe aux cibles d'ARN *in vivo*, nous avons proposé que Ccr4-Not pourrait être, au moins de façon temporaire, un composant de ces structures. A cet égard, plusieurs complexes contenant Mmi1 (c'est-à-dire Mmi1/MTREC et Mmi1/Ccr4-Not), pourraient être co-localisés dans les spots nucléaires. Ils évoqueraient donc la possibilité que ces mêmes complexes se lisent au même

temps, au même ARN méiotique, éventuellement en utilisant des motifs UNAAAC différents.

Un scénario vraisemblable arrive à prédire que le complexe Mmi1/Ccr4-Not lié à l'ARN pourrait préserver l'activité des complexes Mmi1/MTREC à proximité, en ciblant des molécules Mei2 aux alentours, pour leur ubiquitination et dégradation. An environnement local en absence de Mei2, soutiendrait donc la fonction de Mmi1/MTREC dans la suppression des ARN méiotiques, pendant la croissance végétative.

Un des corollaires qui viendrait de se produire serait que, en condition d'absence de substance nutritive, une augmentation des niveaux de Mei2 dépasserait l'activité d'ubiquitination de Ccr4-Not. En plus elle expulserait les complexes qui contiennent Mmi1 des transcrits méiotiques, en obstruent les domaines YTH.

En conclusion, le contrôle de la quantité de Mei2 opérée par Mmi1 et Ccr4-Not, bâtie un ultérieur niveau de complexité au mécanisme de régulation médié par le complexe Ccr4-Not et ce travail ouvre la voie à l'investigation de la

question importante qui concerne la conservation de ces mécanismes dans le contexte d'autres réseaux réglementaires.

Titre : Etude des mécanismes de la différenciation sexuelle chez la levure fissipare *Schizosaccharomyces pombe*

Mots clés : Méiose, switch du cycle cellulaire, Mmi1, Mei2, complexe Ccr4-Not

Résumé : Chez la levure fissipare *S. pombe*, certains gènes méiotiques sont exprimés de façon constitutive pendant la croissance végétative. Cependant, pour empêcher le déclenchement prématuré de la méiose, la cellule a mis en place un système de dégradation sélective des ARN messagers correspondant. La protéine de liaison à l'ARN Mmi1, de la famille YTH, reconnaît des répétitions de motifs spécifiques (UNAAAC) au sein des transcrits et dirige ces derniers vers la dégradation par l'exosome nucléaire. Lors de l'entrée en méiose, Mmi1 est séquestré par un complexe ribonucléoprotéique comprenant la protéine de méiose Mei2 et l'ARN noncodant meiRNA, ce qui permet aux ARNm méiotiques d'être exportés et traduits. Au cours de ma thèse, je me suis intéressé au rôle de Mmi1 dans la dégradation des transcrits méiotiques pendant la croissance végétative. En accord avec des études récentes, nos travaux montrent que Mmi1 interagit étroitement avec le complexe Ccr4-Not de déadénylation des ARNm. Cette interaction est fonctionnelle car Ccr4-Not est requis pour la dégradation des ARNs méiotiques. De façon surprenante, cependant, l'activité de déadénylation n'est pas requise. Nos analyses génétiques et biochimiques suggèrent que la sous-unité E3 ubiquitin ligase Mot2 de

Ccr4-Not ubiquitine un pool de l'inhibiteur de Mmi1 protéine Mei2, pour faciliter sa dégradation par le protéasome. Cette voie de régulation permet de maintenir la fonction de Mmi1 et donc la répression des ARNm méiotiques dans les cellules mitotiques. Ainsi, Mmi1 a une double fonction: cibler les ARNm méiotiques vers la dégradation par l'exosome nucléaire, et recruter Ccr4-Not pour ubiquitiner et dégrader son propre inhibiteur Mei2. Ces résultats mettent également en avant un nouveau rôle pour la sous-unité E3 ligase du complexe Ccr4-Not dans le contrôle de la différenciation sexuelle. Des expériences supplémentaires indiquent que le domaine YTH de liaison à l'ARN de Mmi1, mais pas l'ARN noncodant meiRNA, est requis pour la dégradation de Mei2. De façon importante, nos données révèlent aussi que le domaine YTH de Mmi1 a un rôle clé dans l'interaction avec Mei2. Ceci suggère fortement que le domaine YTH agit comme un module bifonctionnel, permettant la liaison non seulement aux ARNs méiotiques mais aussi aux protéines comme Mei2. Nous discutons ces résultats dans le contexte de la littérature actuelle et proposons un nouveau modèle du contrôle de la différenciation sexuelle par le système Mmi1-Mei2.

Title : Study of the mechanisms of sexual differentiation in the fission yeast *S. pombe*

Keywords : meiosis, cell cycle switch, Mmi1, Mei2, Ccr4-Not complex

Abstract : In the fission yeast *S. pombe*, several meiotic genes are constitutively expressed during the mitotic cell cycle. In order to avoid untimely entry into meiosis, cells have adopted a degradation system that selectively eliminates the corresponding mRNAs. The YTH family RNA-binding protein Mmi1 recognizes specific sequence motifs within these transcripts (UNAAAC) and delivers them to the nuclear exosome for degradation. Upon entry into meiosis, Mmi1 is sequestered in a ribonucleoprotein complex, made of the meiotic protein Mei2 and the non-coding RNA meiRNA, thereby allowing meiotic mRNAs to be exported and translated. During my PhD studies, I focused my work on the role of Mmi1 in the degradation of meiotic transcripts during vegetative growth. Consistent with recent studies, we showed that Mmi1 stably interacts with the mRNA deadenylation complex Ccr4-Not. This interaction is functionally relevant because Ccr4-Not is required for the degradation of meiotic mRNAs. Surprisingly, however, the deadenylation activity of the complex is not involved. Rather, our genetic and biochemical analyses indicate that the E3 ubiquitin ligase subunit Mot2, ubiquitinates a pool of

the Mmi1 inhibitor, Mei2, to promote its degradation by the proteasome. This regulatory mechanism ensures the maintenance of Mmi1 in a functional state, leading to the persistent repression of meiotic mRNAs in mitotic cells. Thus, Mmi1 has a dual role: in nuclear mRNA surveillance, by targeting meiotic transcripts for degradation by the exosome, and in protein degradation, by recruiting Ccr4-Not to its own inhibitor Mei2. These results have also revealed a novel role for the ubiquitin ligase activity of the Ccr4-Not subunit Mot2 in the control of sexual differentiation in fission yeast. Further experiments indicate that the YTH RNA-binding domain of Mmi1, but not the non-coding RNA meiRNA, is required for the degradation of Mei2. Intriguingly, our results support the notion that the YTH domain of Mmi1 mediates the interaction with Mei2. This strongly suggests that the YTH domain acts as a bifunctional module, binding not only to meiotic RNAs but also to proteins. We discuss these results within the context of the current literature and we propose a novel model for the control of sexual differentiation by the Mmi1-Mei2 system.

