

# Mmi1 RNA surveillance machinery directs RNAi complex RITS to specific meiotic genes in fission yeast

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**RNA interference (RNAi) silences gene expression by acting both at the transcriptional and post-transcriptional levels in a broad range of eukaryotes. In the fission yeast *Schizosaccharomyces pombe* the RNA-Induced Transcriptional Silencing (RITS) RNAi complex mediates heterochromatin formation at non-coding and repetitive DNA. However, the targeting and role of RITS at other genomic regions, including protein-coding genes, remain unknown. Here we show that RITS localizes to specific meiotic genes and mRNAs. Remarkably, RITS is guided to these meiotic targets by the RNA-binding protein Mmi1 and its associated RNA surveillance machinery that together degrade selective meiotic mRNAs during vegetative growth. Upon sexual differentiation, RITS localization to the meiotic genes and mRNAs is lost. Large-scale identification of Mmi1 RNA targets reveals that RITS subunit Chp1 associates with the vast majority of them. In addition, loss of RNAi affects the effective repression of sexual differentiation mediated by the Mmi1 RNA surveillance machinery. These findings uncover a new mechanism for recruiting RNAi to specific meiotic genes and suggest that RNAi participates in the control of sexual differentiation in fission yeast.**

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## Introduction

RNAi is an RNA-based gene silencing process widespread among eukaryotes with functions in the regulation of cell fate and differentiation, the neutralization of viruses and the maintenance of genome stability (Ghildiyal and Zamore, 2009; Malone and Hannon, 2009; Lau, 2010; Ketting, 2011). RNAi exerts these functions by acting in the cytoplasm as well as in the nucleus in the vicinity of, or directly on, chromatin. For example, RNAi-based processes targeting chromatin contribute to the silencing of transposons during gametogenesis in animals and plants (Klenov *et al*, 2007; Aravin *et al*, 2008; Bourc'his and Voynnet, 2010; Lau, 2010), DNA elimination in *Tetrahymena thermophila* (Mochizuki *et al*, 2002), DNA methylation in plants (Chan *et al*, 2004; Matzke *et al*, 2009), and epigenetic modification of chromatin in animals and the fission yeast *Schizosaccharomyces pombe* (Volpe *et al*, 2002; Pal-Bhadra *et al*, 2004; Fagegaltier *et al*, 2009; Verdel *et al*, 2009; Burkhart *et al*, 2011; Lejeune and Allshire, 2011).

In *S. pombe*, RNAi mediates formation of heterochromatin and gene silencing at the non-coding and repeated DNA found at the pericentromeric, subtelomeric and mating type regions (Volpe *et al*, 2002; Noma *et al*, 2004; Cam *et al*, 2005). Double-stranded RNA (dsRNA) produced from pericentromeric DNA repeats activates RNAi (Verdel and Moazed, 2005; Buhler and Moazed, 2007; Grewal and Jia, 2007). The ribonuclease III Dicer (Dcr1) digests the dsRNAs into small interfering RNA (siRNA) molecules of 21–23 nucleotides that load onto the Ago1-containing RNAi complex, called RNA-Induced Transcriptional Silencing (RITS) complex (Reinhart and Bartel, 2002; Verdel *et al*, 2004). RITS is composed of Ago1, the unique member of the Argonaute protein family in *S. pombe* and onto which the siRNA binds, Chp1, a chromodomain protein, and Tas3, a WG/GW containing protein, that bridges Ago1 to Chp1 (Verdel *et al*, 2004; Partridge *et al*, 2007). Transcription of the repeats by RNA polymerase II has been proposed to produce a nascent transcript that serves as a recruiting platform onto which RITS binds thanks to the base-pairing of an Ago1-bound siRNA with the nascent transcript platform (Motamedi *et al*, 2004; Djupedal *et al*, 2005; Kato *et al*, 2005; Buhler and Moazed, 2007). In addition, Chp1 chromodomain specifically recognizes the methylated lysine 9 of histone H3 (H3K9me) residue (Partridge *et al*, 2002), and anchors RITS to chromatin (Noma *et al*, 2004; Partridge *et al*, 2007). H3K9 methylation is catalysed by the histone methyltransferase Clr4/Suv39h (Nakayama *et al*, 2001). RITS and Clr4 complex (CLRC) physically interact (Zhang *et al*, 2008; Bayne *et al*, 2010; Gerace *et al*, 2010), and both complexes are engaged in a positive self-reinforcing loop, whereby RITS RNA-mediated association with chromatin would stimulate Clr4-dependent H3K9 methylation, which in turn would promote siRNA production that enables more RITS to bind the nascent transcript platform and to

participate in heterochromatin formation (Verdel and Moazed, 2005; Buhler and Moazed, 2007; Grewal and Jia, 2007).

Genome-wide mappings have localized several RNAi components to a few genomic sites outside of the major heterochromatin regions in fission yeast. Dcr1 localizes and participates in the silencing of transposon long terminal repeats (Woolcock *et al*, 2011). In addition, Ago1 together with the H3K9me repressive mark were detected at two meiotic genes, *mei4* and *ssm4* (Cam *et al*, 2005). Noticeably, *mei4* encodes a master transcription factor that is required for the transcriptional activation of several hundred meiotic genes during sexual differentiation (Horie *et al*, 1998; Mata *et al*, 2007). Proper silencing of *mei4* is critical to avoid untimely and deleterious expression of these meiotic genes (Harigaya *et al*, 2006; Mata *et al*, 2007; Nakase *et al*, 2009). However, whether Ago1 association with the meiotic genes *mei4* and *ssm4* reflects a direct role of RNAi in sexual differentiation control is unclear. Moreover, the mechanism recruiting RNAi to *mei4* and *ssm4* is unknown. During vegetative growth *mei4* and *ssm4* genes are transcribed, but an RNA surveillance machinery guided by the protein Mmi1 silences their expression by degrading their mRNAs (Harigaya *et al*, 2006; St-Andre *et al*, 2010; Yamanaka *et al*, 2010; Cremona *et al*, 2011). Mmi1 is an RNA-binding protein that belongs to the YTH protein family (Stoilov *et al*, 2002). The nuclear exosome component Rrp6, the poly(A)-binding protein Pab2 and the zinc finger protein Red1 are components of the Mmi1 RNA surveillance machinery and important for Mmi1-mediated silencing (Harigaya *et al*, 2006; St-Andre *et al*, 2010; Yamanaka *et al*, 2010; Sugiyama and Sugioka-Sugiyama, 2011).

In this study, we provide evidence that Mmi1 RNA surveillance machinery recruits RITS to *mei4* and *ssm4* chromatin and mRNAs, during mitotic growth. In addition, upon sexual differentiation induction, RITS localization to *mei4* and *ssm4* is lost while these genes become active. Thanks to a large-scale identification of Mmi1 RNA targets we also show that Chp1 interacts with the vast majority of them and that these targets accumulate preferentially before meiosis I. Furthermore, we find that inactivation of RNAi alleviates the efficient repression of sexual differentiation mediated by Mmi1. These findings reveal that Mmi1 RNA surveillance machinery directs RNAi to selective meiotic genes. They also suggest that RNAi directly participates in sexual differentiation regulation in fission yeast.

## Results

### Localization of RITS to *mei4* gene and mRNA

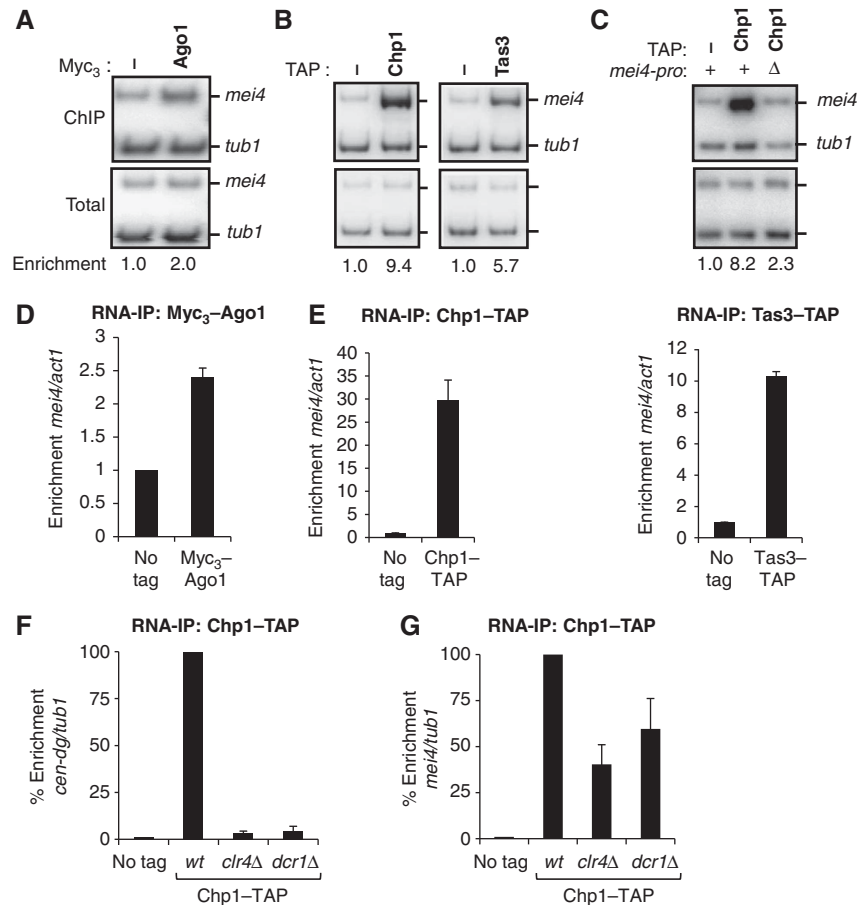
Following on a genome-wide chromatin immunoprecipitation (ChIP) analysis that detected Ago1 at the meiotic gene *mei4* (Cam *et al*, 2005), we investigated whether *mei4* might represent a new genomic target for the Ago1-containing complex RITS. As shown in Figure 1A and B (and Supplementary Figure 1A and B), we found that RITS subunits, Ago1, Chp1 and Tas3 specifically localized to *mei4*, using ChIP experiments. Ago1 immunoprecipitation consistently gave lower enrichment but, as reported previously (Motamedi *et al*, 2004; Verdel *et al*, 2004), this is likely due to the less efficient immunoprecipitation using the Myc<sub>3</sub> tag, which is imposed by the fact that Ago1 function is disrupted by its fusion to larger tags. Given that at centromeres RITS

association with chromatin involves the transcription of pericentromeric DNA by RNA polymerase II (Djupeal *et al*, 2005; Kato *et al*, 2005) and its binding to pericentromeric RNA (Motamedi *et al*, 2004), we checked whether the process of transcription and *mei4* mRNA could participate in recruiting RITS to *mei4* chromatin. Deleting *mei4* promoter led to a considerable loss of Chp1 binding to *mei4* (Figure 1C and Supplementary Figure 1C). Moreover, we found that all subunits of RITS interacted with *mei4* mRNA, using RNA-IP experiments (Figure 1D and E). Since the histone methyltransferase Clr4 and the ribonuclease Dcr1 are both essential for Chp1 binding to pericentromeric RNA (Figure 1F; Motamedi *et al*, 2004), we next tested whether they were also necessary for Chp1 interaction with *mei4* mRNA. Quite unexpectedly, Chp1 could bind *mei4* mRNA in *clr4Δ* or *dcr1Δ* cells, although in a less pronounced manner (Figure 1G; ~40 and ~60% of the enrichment was kept at *mei4* in *clr4Δ* or *dcr1Δ* cells, respectively). This binding was still observed in *clr4Δ dcr1Δ* double mutant cells (Supplementary Figure 1D), thus ruling out the possibility that a Clr4-dependent mechanism was responsible for the Chp1 binding to *mei4* observed in *dcr1Δ* cells and *vice versa*. Hence, these findings show that RITS localizes to both *mei4* gene and mRNA. Furthermore, they reveal that RITS association with *mei4* mRNA presents similarities but also clear distinctions compared to its binding to pericentromeric RNA, suggesting the possibility of a distinct mechanism for recruiting RITS to *mei4*.

### Mmi1 protein is required for RITS localization to *mei4* and *ssm4* mRNAs

The Clr4- and Dcr1-independent binding of RITS to *mei4* transcript led us to investigate if a specific sequence in *mei4* mRNA could play a role in recruiting RITS. For this purpose, we made increasing truncations of *mei4* open reading frame, starting from its 3' end (Figure 2A, left panel). Truncations were made by introducing a GFP cassette in frame with the remaining 5' part of *mei4* coding sequence. Using RNA-IP experiments, we examined Chp1 binding to a region of *mei4* mRNA present in each deletion mutant, at the 5' end of its coding sequence. Deletions of the 3' untranslated region (UTR) or of the 3' half of *mei4* transcript did not impact on Chp1 binding (Figure 2A, right panel, compare histograms *mei4-fl* to *mei4-1551* and *mei4-777*). However, deletion of an additional 389 nucleotide region led to a near complete loss of Chp1 association with *mei4* mRNA (Figure 2A, right panel, compare histograms *mei4-777* and *mei4-388*). Importantly, this region overlapped with most of *mei4* determinant of selective removal (DSR) sequence (Figure 2A), the region responsible for *mei4* mRNA degradation in mitotic cells (Harigaya *et al*, 2006). A deletion of only the DSR sequence also led to a considerable loss of Chp1 binding (Figure 2B), thereby demonstrating the critical role of this sequence in recruiting RITS to *mei4* mRNA.

Since *mei4* DSR sequence is bound by the RNA-binding protein Mmi1 (Harigaya *et al*, 2006), we next evaluated the importance of Mmi1 for RITS association with *mei4* mRNA. Because the deletion of *mmi1* strongly affects cell growth and viability, due to the deleterious untimely expression of Mei4 transcription factor (Harigaya *et al*, 2006), we combined *mmi1* deletion with a mutation in *mei4* that disrupts its function but preserves the DSR sequence, which we called *mei4-828* (see experimental procedure for further details).



**Figure 1** Localization of RITS to *mei4* gene and mRNA. (A, B) ChIP analyses of Myc<sub>3</sub>-Ago1, Chp1-TAP and Tas3-TAP association with the *mei4* gene, relative to the control gene tubulin (*tub1*). <sup>32</sup>P-labelled PCR fragments were run on polyacrylamide gel and quantified with a phosphorimager. (C) ChIP analysis of Chp1-TAP association with *mei4* wild type gene or *mei4* deleted of its promoter (deletion of nucleotides -307 to -7), relative to *tub1*. Panels A, B and C show each time the result of one ChIP experiment. The average enrichments obtained from three independent experiments are shown in Supplementary Figures 1A, B and C. (D, E) RNA-IP analyses of Myc<sub>3</sub>-Ago1, Chp1-TAP and Tas3-TAP association with *mei4* mRNA, relative to actin (*act1*) control mRNA, normalized to an untagged strain. Quantification was done using qRT-PCR. (F, G) RNA-IP analysis of Chp1-TAP association with pericentromeric RNA (*cen-dg*) or *mei4* mRNA, relative to *tub1* mRNA. Enrichments are expressed as a percentage of the relative enrichment monitored in wild type. Error bars represent standard deviations (s.d.) from at least three independent biological replicates. Figure source data can be found with the Supplementary data.

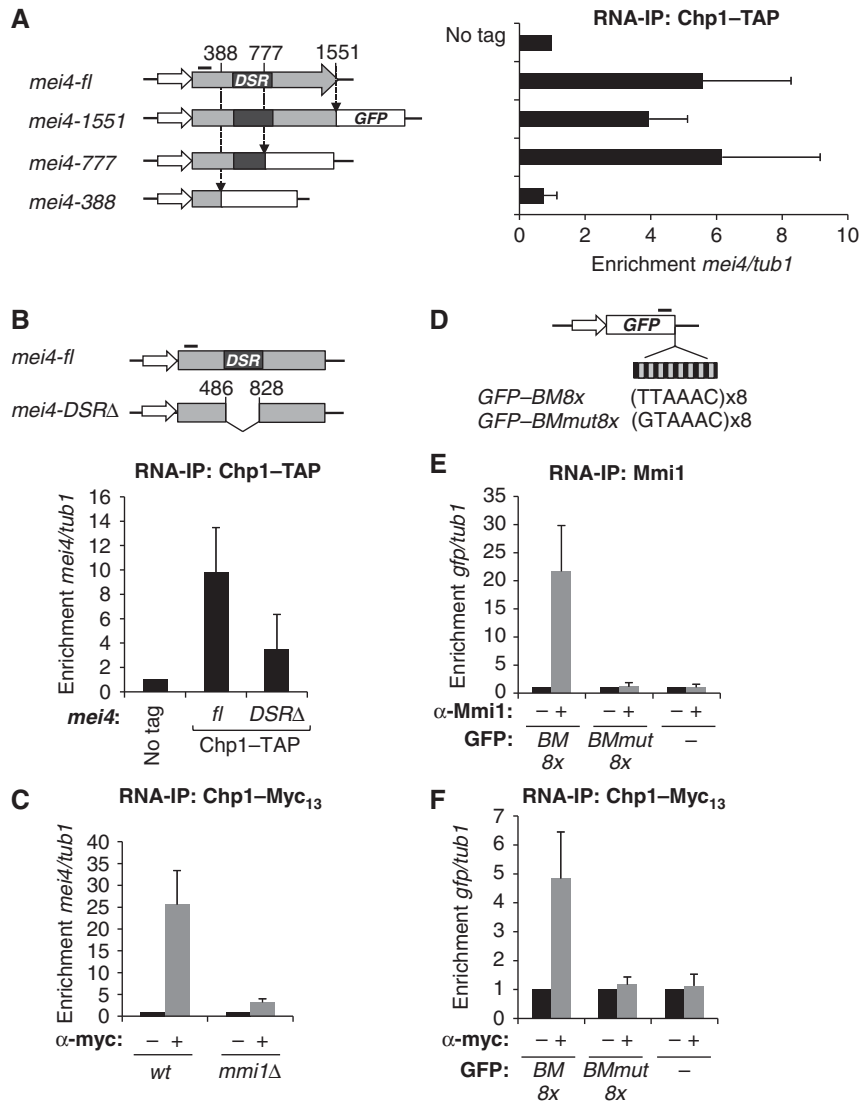
Using RNA-IP experiments, we showed that Chp1 binding to *mei4*-828 mRNA was reduced to near background level in *mmi1Δ* cells (Figure 2C). This loss was not the result of a destabilization of Chp1 protein in *mmi1Δ* cells as shown by a western blot analysis (Supplementary Figure 2A), indicating that Mmi1 is required for recruiting RITS to *mei4* mRNA.

In addition to *mei4*, Ago1 had been detected at a second meiotic gene, *ssm4* (Cam *et al*, 2005). Interestingly, *ssm4* mRNA had also been independently identified as a direct target of Mmi1 (Harigaya *et al*, 2006). RNA-IP experiments showed that RITS subunits associated with *ssm4* mRNA (Supplementary Figure 2B and C). Moreover, Chp1 binding to *ssm4* mRNA was dependent on Mmi1 (Supplementary Figure 2D). Based on these results, we conclude that Mmi1 is required for recruiting RITS to at least two of its targets, *mei4* and *ssm4* mRNAs.

#### Targeting Mmi1 to a GFP mRNA is sufficient to induce the recruitment of Chp1

Because Mmi1 protein binds directly to *mei4* and *ssm4* mRNA (Harigaya *et al*, 2006), we hypothesized that Mmi1 may act as

a specificity factor guiding RITS to these meiotic mRNAs. To test this hypothesis, we assessed Chp1 association with a GFP mRNA fused to Mmi1 RNA-binding motifs. Initially, the identity of Mmi1 RNA-binding motif was unknown but a bioinformatic approach (described in the experimental procedure) led us to identify the UNAAAC RNA sequence as a potential Mmi1-binding site, with the UUAAC motif being the most represented motif (Supplementary Figure 3). Mmi1 specific binding to the UNAAAC motif was demonstrated and analysed in detail in another study (Yamashita *et al*, 2012). As anticipated, RNA-IP experiments showed that Mmi1 associated *in vivo* with a GFP mRNA containing 8 repeats of the UUAAC binding motif (GFP-BM8x), but not with the GFP mRNA alone or fused to the mutated binding motif GUAAAC repeated 8 times (GFP-BMmut8x; Figure 2D and E). We next examined Chp1 binding to the same GFP mRNA derivatives. Importantly, Chp1 interacted with the GFP-BM8x mRNA, but not with the other two GFP mRNAs (Figure 2F). These results strongly support the possibility that Mmi1 binding to a target RNA is sufficient to promote the recruitment of RITS to this RNA.

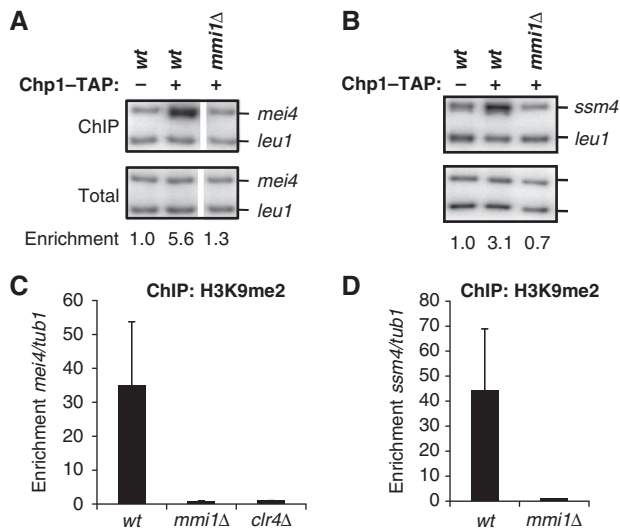


**Figure 2** RITS association with *mei4* mRNA requires *mei4* DSR sequence and the RNA-binding protein Mmi1. (A) Diagram (left panel and not to scale) depicting the three insertion sites made in *mei4* gene to gradually delete *mei4* open reading frame (at nucleotides +1551, +777 and +388, relative to *mei4* start codon). Deletions were made by inserting a GFP cassette (schematized by the white box) composed of the GFP coding sequence fused to the terminator of *adh1* followed by the kanamycin resistance gene. RNA-IP analysis (right panel) of Chp1-TAP binding to *mei4* mRNA full-length (*mei4-fl*) or to the three truncated *mei4* mRNAs (*mei4-1551*, *mei4-777* and *mei4-388*), relative to *tub1* mRNA. Chp1-TAP binding was checked at a region located in the 5' part of *mei4* mRNA, represented by a thick black line on the diagram. Black box highlights the position of the determinant of selective removal (DSR) sequence. (B) Diagram (upper panel and not to scale) of *mei4* gene deleted of just its DSR sequence with no foreign DNA sequence left and RNA-IP experiment (lower panel) of Chp1-TAP binding to the 5' part of *mei4-DSRΔ* mRNA, as in (A). Relative enrichments were measured by qRT-PCR and compared to an untagged control strain. (C) RNA-IP analysis of Chp1-Myc<sub>13</sub> binding to *mei4* mRNA in wild type and *mmi1Δ*, relative to *tub1*. (D) Diagram of the GFP coding sequence fused to 8 repeats of the binding motif (BM) TTAAAC (GFP-BM8x) or to 8 repeats of the mutated binding motif GTAAAC (GFP-BMmut8x). The thick black line highlights the region of the GFP mRNA where the association with Mmi1 and Chp1 was examined. (E, F) RNA-IP analyses of Mmi1 and Chp1-Myc<sub>13</sub> association with GFP mRNA or the recombinant GFP mRNAs described in (D), relative to *tub1*. All error bars represent s.d. from at least three independent replicates.

### RITS associates with *mei4* and *ssm4* genes in a Mmi1- and Clr4-dependent fashion

Given that RITS localized to *mei4* chromatin (Figure 1A and B), we next investigated whether Mmi1 was also important for recruiting RITS to chromatin. Using ChIP experiments, we observed a dramatic reduction of Chp1 localization to *mei4* in *mmi1Δ* cells, indicating that Mmi1 is critical for RITS association with the *mei4* gene (Figure 3A and Supplementary Figure 4A). Similarly, Chp1 associated with the *ssm4* gene in a Mmi1-dependent manner (Figure 3B and Supplementary

Figure 4B). Because Chp1 stable binding to chromatin requires its interaction with the H3K9me mark (Partridge *et al*, 2007) and this mark had been detected at the *mei4* and *ssm4* genes (Cam *et al*, 2005), we examined whether H3K9 methylation was affected in *mmi1Δ* cells. Indeed, H3K9 methylation was completely lost in *mmi1Δ* and *clr4Δ* cells (Figure 3C and D), suggesting that Mmi1 mediates the Clr4-dependent H3K9 methylation, which is then recognized by the chromodomain of Chp1 to stably anchor RITS to the chromatin of *mei4* and *ssm4*.



**Figure 3** RITS associates with *mei4* and *ssm4* genes in a Mmi1-dependent fashion. (A, B) ChIP analysis of Chp1-TAP binding to *mei4* and *ssm4* chromatin in wild type or *mmi1Δ*, relative to the control gene *leu1*. Panel A and B are the results of one experiment. The average enrichments obtained from three independent experiments are shown in Supplementary Figure 4A and B. Note that in panel A the white separation between the second and third lane is to indicate that the lanes come from the same gel but were non-adjacent and that the gel was cropped to juxtapose them. (C) ChIP analysis of H3K9me2 deposition on *mei4* gene in wild type, *mmi1Δ* and *clr4Δ*, relative to *tub1*. (D) ChIP analysis of H3K9me2 deposition on *ssm4* gene in *mmi1Δ*, relative to *tub1*. Error bars represent s.d. from three independent experiments. Figure source data can be found with the Supplementary data.

In parallel, we analysed the possible implication of Mmi1 in recruiting RITS to the major heterochromatic sites. However, in contrast to the *mei4* and *ssm4* genes, Chp1 localization to all heterochromatin regions tested in *mmi1Δ* cells was similar to wild type cells (Supplementary Figure 4C). Moreover, pericentromeric transcriptional gene silencing of a *ura4* reporter gene inserted at the inner most repeat of centromere 1 (*imr1*) was not impaired in *mmi1Δ* cells (Supplementary Figure 4D), indicating that Mmi1 is not essential for gene silencing at constitutive heterochromatin regions.

#### Mmi1-mediated repression of sexual differentiation is affected in RNAi deficient cells

The fact that RITS was recruited to *mei4* and *ssm4* chromatin and mRNAs by a Mmi1-dependent mechanism suggested that RNAi may directly participate in Mmi1-mediated gene silencing. In support of this possibility, we observed that upon sexual differentiation induced in diploid cells by nitrogen starvation, Chp1 was gradually lost from both the chromatin and mRNAs of *mei4* and *ssm4*, while both genes became activated (Figure 4A). To test the contribution of RNAi to Mmi1-mediated gene silencing more directly we analysed *mei4* and *ssm4* mRNA levels in different RNAi deficient cells, by quantitative reverse transcription PCR (qRT-PCR). We observed a reproducible ~1.5 to 2.5 fold increase in *mei4* and *ssm4* mRNA levels in *chp1Δ*, *tas3Δ*, *ago1Δ* and *dcr1Δ* cells (Figure 4B). This increase appeared specific as the levels of *cdc2* and *tub1* control RNAs did not vary significantly under the same conditions. However, when compared to

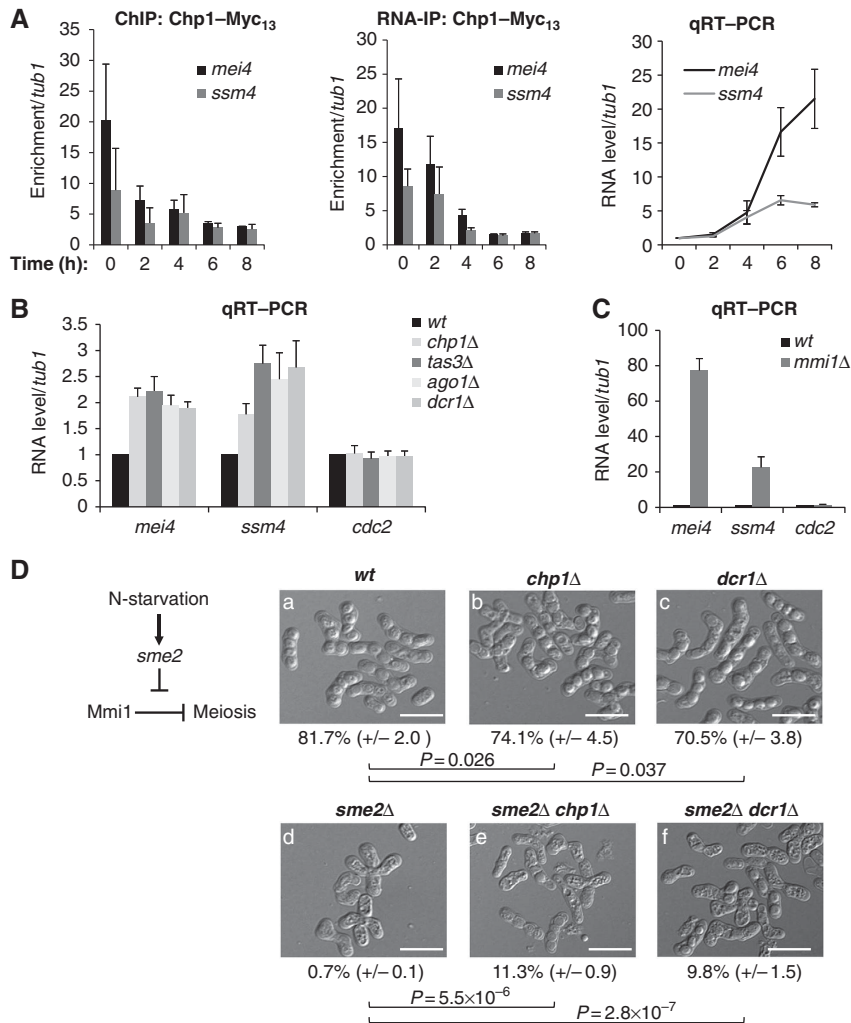
*mei4* and *ssm4* mRNA accumulation in *mmi1Δ* cells, the accumulation observed in RNAi deficient cells appeared relatively weak (Figure 4C). This result may reflect important redundancy between RNAi and other RNA degradation activities acting in Mmi1 RNA surveillance machinery (see further below).

In parallel, we assessed the role of RNAi on the overall Mmi1-mediated repression of sexual differentiation by measuring sporulation efficiency. For this purpose, we used an established read out assay based on the rescue of the *sme2Δ*-dependent meiotic arrest (Yamashita *et al*, 1998; Harigaya *et al*, 2006; St-Andre *et al*, 2010; Yamanaka *et al*, 2010). Upon sexual differentiation, *sme2* non-coding locus participates in Mmi1 inhibition by sequestering it (Figure 4D, left panel; Harigaya *et al*, 2006; Yamashita *et al*, 2012). In *sme2Δ* cells induced to differentiate, Mmi1 is not sequestered and not inhibited, Mmi1 target genes are thus kept silenced, and sexual differentiation eventually arrests before meiosis I. Measure of the sporulation efficiency in *chp1Δ* or *dcr1Δ* cells showed that it was consistently lower than in wild type cells (Figure 4D, right panel, compare images b and c to a), probably because of chromosome segregation defects caused by poor heterochromatin formation at pericentromeric repeats, as suggested previously (Hall *et al*, 2003). In contrast, we observed a higher sporulation frequency in *sme2Δ chp1Δ* or *sme2Δ dcr1Δ* cells compared to *sme2Δ* cells (Figure 4D, right panel, compare images e and f to d, respectively). Collectively, these results support a role for RNAi in Mmi1-mediated repression of sexual differentiation.

#### Differential contribution of Mmi1 RNA surveillance factors to RITS localization to mei4

The 3' to 5' exonuclease Rrp6 plays a major role in Mmi1-mediated degradation of *mei4* and *ssm4* mRNAs (Harigaya *et al*, 2006; St-Andre *et al*, 2010; Yamanaka *et al*, 2010; Sugiyama and Sugiyama-Sugiyama, 2011). To further dissect the mechanism by which Mmi1 RNA surveillance machinery recruits RNAi to *mei4* and *ssm4*, we analysed the contribution of Rrp6 to this recruitment. RNA-IP and ChIP experiments showed that, like Mmi1, Rrp6 is critical for recruiting Chp1 to *mei4* mRNA and gene, respectively (Figure 5A and B). H3K9 methylation at *mei4* was also completely dependent on Rrp6 (Supplementary Figure 5A). In contrast, Mmi1 still bound *mei4* mRNA in *rrp6Δ* cells (Figure 5C). Furthermore, loss of Chp1 binding to *mei4* mRNA and chromatin was not caused by a destabilization of Chp1 protein, as shown by western blot analysis (Supplementary Figure 5B). From these results we conclude that Rrp6 acts downstream of Mmi1 albeit it is also required for RITS association with *mei4* mRNA and gene.

Red1 and Pab2 are two other proteins recently found to be part of Mmi1 mRNA surveillance machinery (St-Andre *et al*, 2010; Yamanaka *et al*, 2010; Sugiyama and Sugiyama-Sugiyama, 2011). RNA-IP experiments showed that while Chp1 association with *mei4* mRNA remained relatively high in *red1Δ* cells, with approximately 50% of the enrichment observed in wild type cells, it was completely lost in *pab2Δ* cells (Figure 5D). At the chromatin level, ChIP experiments showed the opposite tendency, with Chp1 localization to *mei4* completely lost in *red1Δ* cells but only partially lost in *pab2Δ* cells (Figure 5E). We hypothesized that these opposite effects of deleting *red1* and *pab2* might come from the fact that Mmi1 RNA surveillance machinery recruits RITS by two

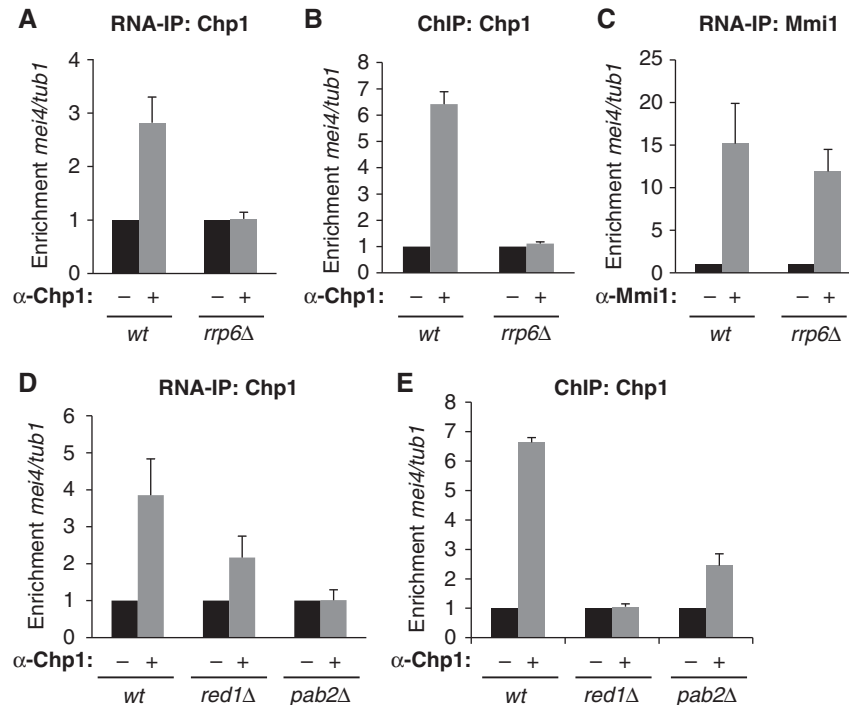


**Figure 4** Effective Mmi1-mediated silencing activity is impaired in RNAi deficient cells. (A) Time course analysis from diploid cells induced to undergo sexual differentiation by nitrogen starvation. ChIP (left panel) and RNA-IP (middle panel) analyses of Chp1-Myc<sub>13</sub> association with respectively the chromatin and mRNAs of *mei4* and *ssm4*, relative to *tub1*. qRT-PCR analysis (right panel) of *mei4* and *ssm4* RNA levels, relative to *tub1* RNA, during the time course analysis. (B) qRT-PCR analysis of *mei4*, *ssm4* and *cdc2* RNA levels in *chp1Δ*, *tas3Δ*, *ago1Δ* or *dcr1Δ*, relative to *tub1* RNA and normalized to the wild type. (C) qRT-PCR analysis of *mei4*, *ssm4* and *cdc2* RNA levels in *mmi1Δ*, relative to *tub1* RNA and normalized to the respective RNA levels in wild type. All error bars represent s.d. from three independent biological replicates. (D) Diagram depicting the *sme2*-dependent inhibition of Mmi1 upon sexual differentiation (left panel). Representative images of cells counted after sexual differentiation induction to measure sporulation frequency of the wild type (a), *chp1Δ* (b), *dcr1Δ* (c) and *sme2Δ* (d) single mutants, and *sme2Δ chp1Δ* (e) and *sme2Δ dcr1Δ* (f) double mutants (right panel). Sporulation frequency was determined by counting ascospores and the total number of cells under a microscope. An ascospore was counted as the product of two cells. Sporulation frequency expressed in percentage is represented as a mean ( $\pm$  s.d.) calculated from three independent biological replicates. *P* values were calculated using the Student's *t*-test. White scale bars = 10  $\mu$ m. Figure source data can be found with the Supplementary data.

non-exclusive mechanisms. The first one would act at the RNA level and preferentially involve Pab2 whereas the second one acting at the chromatin level would rather implicate Red1. Consistent with this possibility, H3K9 methylation at *mei4* was only reduced in *pab2Δ* cells whereas it was completely lost in *red1Δ* cells (Supplementary Figure 5A). In agreement with Pab2 playing an important role in recruiting RITS to Mmi1 RNA targets, no significant effect on *mei4* mRNA accumulation was observed in *ago1Δ pab2Δ* double mutant cells, compared to the *pab2Δ* cells (Supplementary Figure 5C). Altogether, these results show that factors of the Mmi1 mRNA surveillance machinery contribute in distinct manners and degrees to the recruitment of RITS to Mmi1 target genes or mRNAs.

#### Widespread binding of Chp1 to Mmi1 RNA targets

Finally, in order to investigate RITS binding to Mmi1 target mRNAs in a comprehensive manner, we conducted a transcriptome analysis of *mmi1Δ* cells to first identify Mmi1 RNA targets, and then to examine Chp1 binding to these targets. As indicated before (and further described in the experimental procedure section) to alleviate the severe growth defects associated with *mmi1* deletion we conducted the transcriptome analysis on the *mmi1Δ mei4-828* double mutant cells. From the genome-wide transcriptomic analysis, we found a total of 212 protein-coding genes whose mRNA level was upregulated at least 1.5 fold in *mmi1Δ* cells, compared to wild type or to *mei4-828* cells (Figure 6A and Supplementary Figure 6A and B). Noticeably, more than 70% of these



**Figure 5** Mmi1 RNA surveillance factors, Rrp6, Red1 and Pab2 show distinct contributions to RITS association with *mei4*. (A) RNA-IP analysis of Chp1 association with *mei4* mRNA in wild type or *rrp6Δ*, relative to *tub1* mRNA. Relative enrichment was normalized to IP done without antibody. (B) ChIP analysis of Chp1 association with *mei4* gene in wild type or *rrp6Δ*, relative to *tub1*. (C) RNA-IP analysis of Mmi1 binding to *mei4* mRNA in wild type or *rrp6Δ*, relative to *tub1* mRNA. (D) RNA-IP analysis of Chp1 association with *mei4* mRNA in wild type, *red1Δ* or *pab2Δ*, relative to *tub1* mRNA. (E) ChIP analysis of Chp1 association with *mei4* gene in wild type, *red1Δ* or *pab2Δ*, relative to *tub1*. All error bars represent s.d. from three independent experiments.

mRNAs accumulate during sexual differentiation (Figure 6B), according to the classification described by Mata and co-workers (Mata *et al*, 2002). This result confirmed that Mmi1 likely recognizes and triggers degradation of sexual differentiation-specific mRNAs, as proposed in the initial characterization of Mmi1 (Harigaya *et al*, 2006). 90 mRNAs were also found to be less abundant in *mmi1Δ* cells compared to wild type or *mei4-828* cells, but gene ontology analysis showed no enrichment for a given regulatory function or a specific cellular process, suggesting that their down-regulation is probably an indirect effect of *mmi1Δ* deletion.

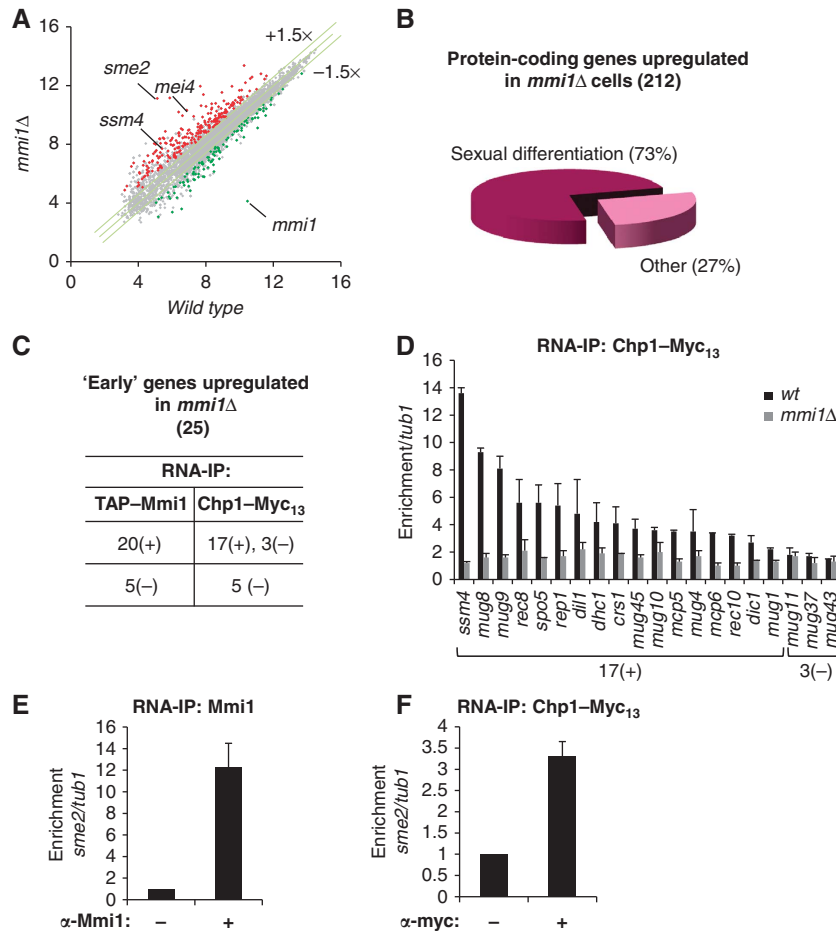
*S. pombe* sexual differentiation gene expression programme is composed of four successive waves of transcription, called N-starvation, early, middle and late phases (Mata *et al*, 2002). A search for the overrepresentation of Mmi1 RNA-binding motifs among the mRNAs accumulating in *mmi1Δ* cells revealed a strong bias towards mRNAs accumulating in the phase ‘early’ of sexual differentiation (Supplementary Table I). Using RNA-IP experiments, we undertook to test Mmi1 binding to all ‘early’ mRNAs accumulating in *mmi1Δ* cells. Due to the low abundance of some of these mRNAs we were able to test Mmi1 binding to 25 of the 29 ‘early’ mRNAs accumulating in *mmi1Δ* cells. We found that 20 of them showed at least 4 fold enrichments over background after Mmi1 immunoprecipitation, whereas the other 5 mRNAs did not show any significant enrichment (Figure 6C and Supplementary Table II). Strikingly, Chp1 bound to 17 out of the 20 Mmi1 RNA targets but not to any of the other 5 ‘early’ mRNAs not targeted by Mmi1 (Figure 6C). Furthermore, Chp1 binding to all 17 mRNAs

was lost in *mmi1Δ* cells (Figure 6D). In parallel, we examined Chp1 and H3K9me localization to the 20 genes expressing the Mmi1 RNA targets, by ChIP experiments. Remarkably, in contrast to the widespread association of Chp1 with Mmi1 RNA targets, Chp1 and H3K9 methylation were detected respectively at only 3 and 4 of the corresponding genes (Supplementary Figure 6C and D). From these results, we conclude that Chp1 binding to Mmi1 RNA targets is rather systematic, whereas its binding to the chromatin of the corresponding genes may be more restricted.

Since our transcriptome analysis was performed with a tiling array, which includes the intergenic regions, we also examined the level of non-coding RNAs in *mmi1Δ* cells. Surprisingly, the most upregulated RNA in the whole transcriptome analysis was expressed from *sme2*, the non-coding locus that sequesters Mmi1 during sexual differentiation. RNA-IP experiments showed that Mmi1 and Chp1 bound *sme2* non-coding RNA *in vivo* (Figure 6E and F). 26 other non-coding RNAs accumulated in *mmi1Δ* cells (Supplementary Table III). However, none of them showed an overrepresentation of Mmi1 RNA-binding motifs, supporting the idea that they were not direct targets of Mmi1. Thus, the transcriptome analysis of *mmi1Δ* cells combined with RNA-IP experiments revealed a widespread association of Chp1 with Mmi1 RNA targets, including a non-coding RNA with a key function in the control of sexual differentiation.

## Discussion

Our findings provide evidence that the Mmi1 RNA surveillance machinery guides RNAi to specific meiotic mRNAs and



**Figure 6** Transcriptome of *mmi1*Δ cells and large-scale identification of RNAs bound by Mmi1 and Chp1. (A) Scatter plot of *S. pombe* genome-wide gene expression measured from hybridization signals of RNAs isolated from *mmi1*Δ cells (Y axis) and from wild type cells (X axis). Red and green dots represent genes up and down regulated, respectively. Grey dots represent genes with no change in their RNA level in *mmi1*Δ compared to wild type and *mei4-828* cells. Each hybridization signal is an average of two independent biological replicates and is plotted using a log<sub>2</sub> scale. Note that the *mmi1*Δ cells used for the transcriptome analysis also contain the *mei4-828* mutation (further described in the experimental procedure section). Transcriptome of *mei4-828* cells is highly similar to wild type cells as shown in Supplementary Figure 6A. (B) Pie chart representing the distribution of the protein-coding genes upregulated in *mmi1*Δ cells according to their expression during sexual differentiation (Mata *et al*, 2002). (C) Table summarizing the RNA-IP analyses of TAP-Mmi1 and Chp1-Myc<sub>13</sub> association with 25 mRNAs specific of the 'early' phase of sexual differentiation and accumulating in *mmi1*Δ, relative to *tub1* mRNA control. An RNA was considered significantly associated with Mmi1 or Chp1 when enriched at least two fold after RNA-IP. Enriched and non-enriched mRNAs are classified as (+) and (-), respectively. (D) RNA-IP analysis of Chp1-Myc<sub>13</sub> association with Mmi1 mRNA targets in wild type or *mmi1*Δ, relative to *tub1*. 17(+) and 3(-) denote respectively RNAs that are and are not significantly enriched in Chp1-Myc<sub>13</sub> RNA-IP in (D). (E, F) RNA-IP analyses of Mmi1 or Chp1-Myc<sub>13</sub> association with *sme2* RNA, relative to *tub1*. Error bars represent s.d. from at least three independent experiments.

genes. They also suggest for the first time a direct implication of RNAi in the control of sexual differentiation in yeast. The major findings are as follow: first, RITS localizes to the chromatin and mRNAs of two meiotic genes, *mei4* and *ssm4*, during vegetative growth, and this association is lost upon sexual differentiation when both genes are activated. Second, localization of RITS to *mei4* and *ssm4* genes and their respective mRNAs requires the Mmi1 RNA surveillance machinery. Furthermore, Mmi1 binding to a recombinant mRNA containing Mmi1 hexameric RNA-binding motifs is sufficient to induce the recruitment of Chp1, consistent with Mmi1 being the specificity factor for recruiting RITS to selective meiotic genes and mRNAs. Third, a large-scale identification of Mmi1 targets reveals that Chp1 binds to the vast majority of them in a Mmi1-dependent manner. Fourth, in agreement with a role of RNAi in Mmi1-mediated repression of sexual

differentiation, a loss of RNAi partially suppresses the sexual differentiation block caused by a constitutive activation of Mmi1. Below we discuss the implications of these findings for the targeting of RITS and for the role of RNAi in sexual differentiation.

#### RITS association with meiotic genes and (m)RNAs

In *S. pombe* the association of RITS with major heterochromatic regions has been extensively studied. The mechanism of RITS recruitment to chromatin is best understood at the pericentromeric heterochromatin. At this genomic region, RITS binding to pericentromeric chromatin involves an RNA platform produced by transcription of the DNA repeats by RNA polymerase II (Verdel and Moazed, 2005; Buhler and Moazed, 2007; Grewal and Jia, 2007). RITS binding to pericentromeric RNA requires Clr4 and Dcr1, which

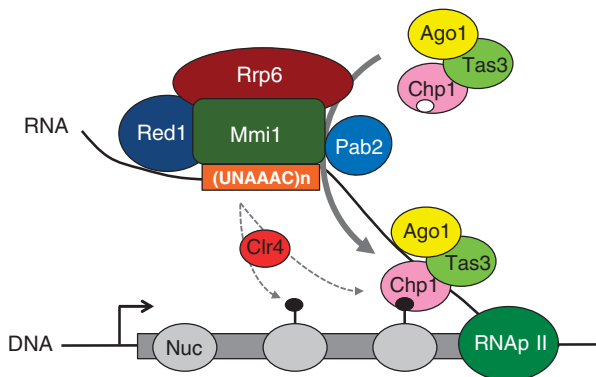


respectively methylates H3K9 and produces siRNAs that load onto RITS (Motamedi *et al*, 2004). Based on this Clr4 and Dcr1 dependency and other results, it has been proposed that the binding of Chp1 chromodomain to H3K9me and of Ago1-loaded siRNA to a nascent transcript are key events in recruiting RITS to pericentromeric heterochromatin. In this study, we identify a new mechanism that involves the Mmi1 RNA-binding protein and its associated RNA degradation machinery in recruiting RITS to specific meiotic genes and mRNAs. From our findings, we propose a model for the recruitment of RITS to these meiotic mRNAs and genes (see Figure 7 for further details). Mmi1 RNA-binding protein recognizes the hexameric motif UNAAAC that we and others have identified (Chen *et al*, 2011; Yamashita *et al*, 2012). Thanks to its sequence-specific binding Mmi1 acts as a specificity factor for recruiting RITS to selective meiotic mRNAs and chromatin during vegetative growth. Once sexual differentiation is induced, Mmi1 is inhibited and RITS loses its localization to the meiotic targets. Several factors of the Mmi1 RNA surveillance machinery differentially promote RITS binding to chromatin or mRNAs. Like Mmi1, the Rrp6 protein is required for RITS binding to both the mRNA and gene of *mei4*. In contrast, Pab2 and Red1 proteins are probably part of two non-exclusive mechanisms recruiting RITS preferentially at the mRNA and chromatin, respectively. By using a combination of RNA-IP and ChIP experiments, we show that Chp1 associates with most Mmi1 RNA targets whereas it is only detected in a small proportion of their corresponding genes. H3K9 methylation is also detected in only a limited fraction of Mmi1-silenced genes. Very recently, it was similarly reported

that H3K9 methylation at *mei4*, *ssm4* and other meiotic genes is dependent on Mmi1 RNA surveillance machinery, which interacts with Clr4 H3K9 methyltransferase (Zofall *et al*, 2012). In agreement with our findings, H3K9 methylation was detected at only 6 out of the 22 Mmi1-silenced genes identified by Harigaya and co-workers (Harigaya *et al*, 2006) and in our study. From these data, we postulate that the more restricted localization of Chp1 and H3K9me to chromatin may be because Red1-dependent mechanism acts on chromatin of only a subset of genes silenced by Mmi1, conversely to the Pab2-dependent mechanism, which would act on most, if not all, Mmi1 RNA targets. It will be important to address this possibility in future studies. However, it should be noted that testing the specific contribution of Pab2- and Red1-dependent mechanisms is challenging as these mechanisms are overlapping and reinforce RITS recruitment to both chromatin and RNA targets. Also, at this point we cannot completely exclude that another possibility such as a poorer detection limit for the ChIP compared to the RNA-IP experiment is responsible for this difference. Importantly, regardless of the mechanisms, our findings provide evidence that an RNA degradation machinery plays a critical role in recruiting the RNAi effector complex RITS to mRNAs and chromatin of cell fate-regulated genes.

A role for an RNA-binding protein in recruiting an Argonaute-containing complex to selective RNAs has been described in other eukaryotes. The HuR AU-rich RNA-binding protein recruits or stabilizes the binding of a RISC complex to two different mRNAs, in animal cell lines (Kim *et al*, 2009; Glorian *et al*, 2011). Similarly, in *A. thaliana* the RNA-binding protein KTF1, which participates in the RNAi-dependent process called RNA-directed DNA methylation (RdDM), interacts with the Argonaute protein AGO4 and directs or stabilizes AGO4 binding to nascent transcripts (He *et al*, 2009; Rowley *et al*, 2011). Together with our results, these findings set a paradigm for the promoted or assisted recruitment of Argonaute-containing complexes to RNA and chromatin targets in a wide range of eukaryotes, including unicellular organisms such as fission yeast.

In addition, our findings reveal that the nuclear exosome component Rrp6 is essential for the targeting of RNAi to chromatin regions and mRNAs. In this regard, it is interesting to note that a genome-wide transcriptome analysis found that RNAs emanating from genomic regions targeted by the RNAi-based RdDM accumulate in several mutants of the nuclear exosome in *A. thaliana* (Chekanova *et al*, 2007). Thus, it is tempting to speculate that the connection between exosome-mediated RNA degradation, RNAi and epigenetic modification of chromatin uncovered in fission yeast might also exist in plants and other eukaryotes. Furthermore, the finding that Mmi1 and Chp1 target a non-coding RNA expressed from *sme2* locus suggests that Mmi1/exosome-dependent recruitment of RNAi may extend to non-coding RNAs implicated in the control of cellular differentiation (Harigaya *et al*, 2006; Yamashita *et al*, 2012). Recently multiple non-coding RNAs identified in several organisms were found to exert important regulatory functions, including the control of cellular differentiation (Pauli *et al*, 2011). Whether some of these non-coding RNAs could be regulated by a similar mechanism remains to be determined.



**Figure 7** Model for the Mmi1 RNA surveillance-driven recruitment of RITS to meiotic (m)RNAs and genes. Mmi1 binds to a target meiotic RNA through the recognition of its hexameric RNA-binding motif (UNAAAC) and recruits the RNA surveillance machinery. Rrp6, Pab2 and Red1 proteins are factors important for Mmi1 RNA surveillance machinery. Recruitment of this machinery enables or stabilizes the binding of RITS to the target RNA. RITS can bind both meiotic RNAs and their corresponding genes. Pab2 protein assists mostly RITS binding to the RNA, whereas Red1 would favour the recruitment of RITS to chromatin by mediating Clr4-dependent H3K9 methylation. These two mechanisms working side-by-side would further reinforce the recruitment of RITS to chromatin and mRNAs. Furthermore, a selective regulation of only one of the two mechanisms may be a mean to modulate RITS binding to the RNA versus chromatin of the target gene. Nuc: nucleosome; Black lollipop: H3K9me repressive histone mark; White circle on Chp1 depicts its chromodomain; RNAP II: RNA polymerase II; (UNAAAC)<sub>n</sub>: cluster of Mmi1 hexameric binding motifs.

### RNAi and sexual differentiation

Although RNAi has been clearly involved in sexual differentiation in multiple eukaryotes its implication in yeast sexual differentiation has remained elusive. Recently, numerous antisense RNAs differentially expressed during vegetative growth and sexual differentiation have been identified (Bitton *et al*, 2011; Chen *et al*, 2012). These antisense RNAs could potentially produce double stranded RNAs that would activate RNAi upon or during sexual differentiation (Bitton *et al*, 2011). In this study, we now show that RNAi is linked to the control of sexual differentiation by the Mmi1 mRNA surveillance system. This system imposes a robust gene silencing essential for the proper inhibition of sexual differentiation gene expression programme during mitotic growth in fission yeast but no connection to RNAi had been found initially (Harigaya *et al*, 2006). We show that the Mmi1 RNA surveillance machinery plays a critical role in recruiting RNAi to meiotic mRNAs and genes, and that RITS may interact with most Mmi1 RNA targets. In addition, the partial rescue of the *sme2Δ*-induced sexual differentiation arrest in the absence of Dcr1 or Chp1 further supports the implication of RNAi in Mmi1-mediated inhibition of sexual differentiation. Noticeably, the silencing defects observed in RNAi deficient cells are weak compared to *mmi1Δ* cells. We believe this may be due to redundancy between RNAi and the nuclear exosome RNA degradation activities. In this regard, inactivation of Dis3, another nuclease acting together with Rrp6 in Mmi1-mediated gene silencing leads to only minor silencing defects (St-Andre *et al*, 2010). The implication of diverse nucleases, including Ago1 slicing and Dcr1 dicing activities, in Mmi1 RNA surveillance system may in fact be essential for the proper regulation of sexual differentiation programme under specific environmental conditions. In addition, an intriguing possibility is that RNAi might have another function at Mmi1 target genes. In *C. elegans*, similarly to Ago1 in *S. pombe*, the Argonaute protein CSR1 targets sexual differentiation-specific genes (Claycomb *et al*, 2009; van Wolfswinkel *et al*, 2009). However, it does not seem to play a role in silencing their expression. Instead, it has been proposed that CSR1 contributes to efficient chromosome segregation during mitotic and meiotic cell divisions, possibly by recruiting to meiotic genes chromatin modifiers, such as the cohesins. Intriguingly, an RNAi-dependent recruitment of the Rad21 cohesin has been described at *mei4* (Gullerova and Proudfoot, 2008). Hence, we cannot exclude that RITS recruitment to Mmi1 silenced genes may have other regulatory functions, such as to contribute to genome stability by loading for example cohesins to specific chromosomal regions.

In animals, RNAi is essential to sexual differentiation and is directly involved in the regulation of this cellular process by acting at several levels (Lau, 2010; Suh and Belloch, 2011). We present evidence that RNAi is also directly involved in the regulation of sexual differentiation in fission yeast. Thanks to the identification of Mmi1 RNA-binding motif and the transcriptome of *mmi1Δ* cells, our findings indicate that RITS may bind to a set of mRNAs and genes silenced in mitotic cells and expressed relatively early during sexual differentiation. Importantly, among the mRNAs targeted we identified *rep1* and *mei4*, which code for two key transcription factors of the sexual differentiation gene expression programme. If their silencing is defective during

vegetative growth, as when Mmi1 function is impaired, a potentially large proportion of this sexual differentiation programme is expressed at the wrong time, which ultimately provokes severe growth defects and death of the cell (Harigaya *et al*, 2006). In this regard, it is interesting to note that similarly in several other eukaryotes RNAi is critical for silencing the expression of sexual differentiation-specific genes, which maintains the germline stem cell (GSC) self-renewal and proliferation capacity (Lau, 2010; Suh and Belloch, 2011). Together these results argue that the implication of RNAi in the control of sexual differentiation and possibly its function in regulating this cellular process may well be conserved in numerous eukaryotes, including fission yeast.

## Material and methods

### Strain, media and plasmid construction

*S. pombe* strains used in this study are listed in Supplementary Table IV. Standard procedures were used for growth and manipulations of *S. pombe* cells (Moreno *et al*, 1991; Sabatino and Forsburg, 2010). Gene deletion and epitope tagging were made following the PCR-based methodology (Bahler *et al*, 1998). The *mmi1Δ* cells were generated from a parental strain possessing an inactive form of *mei4* (named *mei4-828*), as deletion of *mmi1Δ* leads to severe growth and viability defects due to the deleterious expression of Mei4. *mei4-828* mutant strains were made by inserting at nucleotide 829 of *mei4* coding sequence a GFP cassette, composed of the GFP coding sequence fused to *adh1* terminator and the kanamycin resistance gene. The recombinant *mei4-gfp* gene still possesses *mei4* DSR sequence but has lost the sequence coding for the activation domain of Mei4. The transcriptome analysis, as all other experiments involving *mmi1Δ* cells, was conducted with the double mutant *mmi1Δ mei4-828* cells. Sporulation of diploid cells was induced by nitrogen starvation and carried out as described (Moreno *et al*, 1991; Mata *et al*, 2002). Sporulation efficiency of h<sup>90</sup> cells was measured after growing cells on synthetic sporulation agar (SPA) for 2 days at 30 °C, and was calculated as the number of ascospores formed over the total number of cells counted. pRGT1 plasmids (derived from pREP1) express, under the control of the *adh* promoter, the GFP mRNA alone (pRGT1-GFP), in fusion to 8 repeats of the motif UAAAC (pRGT1-GFP-TTAAAC8x) or in fusion to 8 repeats of the mutated motif GUAAAC (pRGT1-GTAAAC8x). Construction of the plasmids is detailed elsewhere (Yamashita *et al*, 2012).

### Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed mainly as described previously (Motamedi *et al*, 2004). For ChIP done with antibodies, immunoprecipitation (IP) was carried out for 2 h using 2 μg of anti-H3K9me2 (Abcam, ab1220-100), anti-Myc 9E10 (Covance, MMS-150P) or anti-Chp1 (Abcam, ab18191), followed by 1 h incubation with 20 μl 1/1 slurry protein A-sepharose (GE Healthcare). Quantitative PCR was conducted with 4 μl of a 1/8 dilution of the IP DNA and 4 μl of a 1/50 dilution of the input. Relative enrichment was calculated as the ratio of signal of interest to signal of control in IP over input. Primers used are listed in Supplementary Table V.

### RNA-immunoprecipitation (RNA-IP)

RNA-IP experiments were mainly as described (Motamedi *et al*, 2004). Briefly, cells grown to an OD<sub>600</sub> of 1.3 (approximately 2 × 10<sup>7</sup> cells/ml) were crosslinked with 1% formaldehyde for 15 min. Whole cell extracts were treated with 700 units of DNase I (Sigma) for 1 h at 30 °C in a 50 mM HEPES-NaOH pH7.5, 150 mM NaCl, 1 mM EDTA pH8, 1% Triton X100, 0.1% Na-deoxycholate, 5 mM DTT buffer supplemented with 25 mM MgCl<sub>2</sub> and 5 mM CaCl<sub>2</sub>. IP was performed as described in ChIP section. Anti-Mmi1 IP was carried out using 10 μl of rabbit antibody. Immunoprecipitated RNA was isolated by phenol/chloroform extraction and reverse transcribed according to the manufacturer instructions (Superscript II, Invitrogen). Quantitative analysis was

performed using real-time PCR. For RNAs present in low abundance a quantitative preamplification step was made as described (Lao *et al*, 2006; Smith *et al*, 2011). 4  $\mu$ l of a 1/400 final dilution of the preamplification reaction was used for the second quantitative PCR. Primers are listed in Supplementary Table V.

#### Quantitative PCR (qPCR)

qPCR was performed on the LightCycler480 machine (Roche) using MESA Green qPCR mix (Eurogentec) and the following programme: 15 min incubation at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 70 °C for 30 s. Quantitative preamplification was done under the same conditions but with 14 cycles instead of 40.

#### Prediction of Mmi1 RNA-binding motif

Search for motif enrichment in *mei4*, *ssm4*, *rec8* and *spo5* DSR sequences was first performed with oligo-analysis programme from RSAT (<http://rsat.scmbb.ulb.ac.be/rsat/>), which uses an enumerative word discovery approach to detect statistically overrepresented motifs in a set of sequences (van Helden *et al*, 1998). Default settings and single strand as the oligomer counting mode were used. In addition, based on the detailed analysis of rat Yth521-B protein, the founding member of the YTH family, which demonstrated that its YTH RNA-binding module recognizes a hexameric sequence motif (Zhang *et al*, 2010), we searched for enrichment of hexameric sequences. The UUAAC hexameric motif was found overrepresented in the DSR sequences with an E-value for occurrences of 2.4E-3. A second analysis with the prediction algorithms Consensus, MEME, Gibbs and MDscan from melina II (<http://melina2.hgc.jp/public/index.html>) (Okumura *et al*, 2007) further validated the overrepresentation of the UUAAC motif in the four DSR sequences. This analysis also indicated that the UGAAAC, UCAAAC and UAAAAC motifs were potentially overrepresented in these sequences. Using a non-parametric and asymmetric Mann-Whitney-Wilcoxon test, UAAAAC motif was found to be statistically overrepresented with a P-value = 3.33E-4 in mRNA sequences of *mei4*, *ssm4*, *rec8* and *spo5*, compared to the control set formed by the 7039 annotated genomic regions in *S. pombe*.

#### Transcriptome analysis

Cultures of 25 ml YEA *S. pombe* cells were grown to approximately 7.10<sup>6</sup> cells/ml. Total RNA was extracted using hot phenol method. 100  $\mu$ g total RNA was further purified using RNeasy Qiagen columns, incubated with DNase I (Roche) digestion for 30 min at 37 °C, and extracted by classical phenol extraction. Microarray hybridization, processing and analysis were performed on the ProfileXpert core facility (Bron, France) following instructions of the manufacturer (Affymetrix, Santa Clara, CA, USA) and using a high-density oligonucleotide array (GeneChip *S. pombe* Tiling 1.0FR Array). Microarrays were read with a confocal laser (Genechip scanner 3000, Affymetrix). CEL files were generated using the Affymetrix GeneChip Command Console (AGCC) software 3.0. Probe sequences (from Affymetrix BPPMAP file) were aligned to the genome sequence of *S. pombe* from the *S. pombe* Genome project, using the R/Bioconductor package *Starr*. Probe sequences were mapped to unique positions on the genome and a new BPPMAP file was built (available upon request). RMA background correction

and quantile normalization were applied to the data using Robust Multiarray Average (RMA) statistical algorithm (Partek Genomic Suite software 6.5, St. Louis, MO, US). Transcriptome analysis of annotated regions was conducted performing a median summarization of probes covering each gene from Ensembl Fungi Database (EF1). Transcriptome analyses were done in duplicates, each duplicate corresponding to an independent biological replicate. A total of four pair-wise comparisons was carried out, where each annotated region from one duplicate was compared with the corresponding annotated region from the other duplicate. Only annotated genomic regions showing a variation greater than 1.5 fold in all four pair-wise comparisons were retained. A selected region was considered differentially expressed only if the ratio of the fold-changes (FC) [*mmi1* $\Delta$ /*wt* over *mei4-828/wt*] was above 1.5 fold. Transcriptome analysis was visualized using Integrated Genome Browser (IGB). The complete set of CEL files is available at the GEO database under accession number GSE31119.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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**Author contributions:** EH, AV, LTT and AV designed the experiments; EH, AV and LTT performed most of the experiments; EH, AV, LTT, DP and AV wrote the paper; BG, EL, NN, JL and DP contributed to the transcriptome analyses; CB and JP made specific contributions to the characterisation of *Mmi1* and *sme2*; AY, YS and MY provided plasmid constructions and contributed to the sexual differentiation rescue experiments; All authors discussed the results and the manuscript.

#### Conflict of interest

The authors declare that they have no conflict of interest.

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