

Promoter-driven splicing regulation in fission yeast

Alberto Moldón¹, Jordi Malapeira¹, Natalia Gabrielli¹, Madelaine Gogol², Blanca Gómez-Escoda¹, Tsvetomira Ivanova¹, Chris Seidel² & José Ayté¹

The meiotic cell cycle is modified from the mitotic cell cycle by having a pre-meiotic S phase that leads to high levels of recombination, two rounds of nuclear division with no intervening DNA synthesis and a reductional pattern of chromosome segregation. Rem1 is a cyclin that is only expressed during meiosis in the fission yeast *Schizosaccharomyces pombe*. Cells in which *rem1* has been deleted show decreased intragenic meiotic recombination and a delay at the onset of meiosis I (ref. 1). When ectopically expressed in mitotically growing cells, Rem1 induces a G1 arrest followed by severe mitotic catastrophes. Here we show that *rem1* expression is regulated at the level of both transcription and splicing, encoding two proteins with different functions depending on the intron retention. We have determined that the regulation of *rem1* splicing is not dependent on any transcribed region of the gene. Furthermore, when the *rem1* promoter is fused to other intron-containing genes, the chimaeras show a meiotic-specific regulation of splicing, exactly the same as endogenous *rem1*. This regulation is dependent on two transcription factors of the forkhead family, Mei4 (ref. 2) and Fkh2 (ref. 3). Whereas Mei4 induces both transcription and splicing of *rem1*, Fkh2 is responsible for the intron retention of the transcript during vegetative growth and the pre-meiotic S phase.

The switch from mitosis to meiosis is accompanied by a deep change in gene expression profiles⁴, as shown by genome-wide analyses in both *S. pombe*⁵ and *Saccharomyces cerevisiae*^{6,7}. One of the genes that is expressed and differentially spliced during meiosis is *rem1* (refs 1 and 8). $\Delta rem1$ cells complete meiosis and produce four-spore asci with a viability similar to wild-type cells (>90%). However, meiotic intragenic recombination is reduced twofold to threefold¹. Notably, *rem1* splicing takes place just after the recombination events, pointing to the possibility that full-length Rem1 might not be involved in the regulation of recombination. To determine whether this is the case, we measured recombination of a $\Delta rem1$ strain transformed with different plasmids and compared it with a wild-type strain. As shown in Fig. 1a, in the absence of *rem1* recombination efficiency was decreased twofold; it was restored with a plasmid containing the genomic region of *rem1* but not with an empty vector. Normal levels of recombination were not restored when the plasmid contained a mutation in the first codon of *rem1* (ATG-less), indicating that *rem1* messenger RNA is not directly involved in the regulation of recombination. However, a plasmid containing a mutation in the splicing donor site that produces an mRNA that is never spliced (NoS; Fig. 1b and Supplementary Fig. 1) and, as a consequence of the presence of a stop codon in the intron, renders a peptide encoded exclusively by the first exon, can restore wild-type levels of recombination. Thus, the 17 kDa peptide encoded by the first exon is sufficient to bring about normal levels of recombination, whereas the second exon (which contains the cyclin box) is dispensable for the recombination function of Rem1 (Fig. 1c). Thus, the timely regulation of splicing indicates that there are different functions of *rem1*.

To study this regulation, we analysed splicing of different chimaeras of *rem1* during meiosis (Fig. 2). Figure 2a shows the splicing profile of the wild-type *rem1*, compared to a mutant in which the 3'

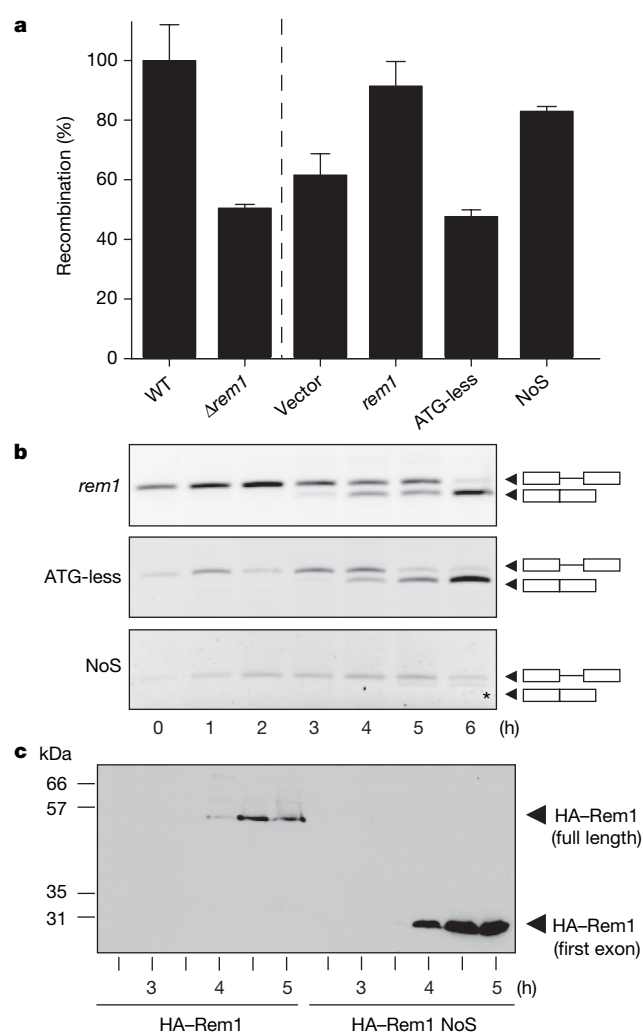


Figure 1 | Alternately spliced *rem1* restores normal recombination levels. **a**, Recombination was measured in wild-type (WT) and $\Delta rem1$ strains, and in $\Delta rem1$ strains transformed with different constructs (right of dashed line). Mean and s.d. are shown; $n = 5$. **b**, *rem1* splicing was monitored by RT-PCR in cells expressing wild-type *rem1* and the mutants ATG-less and NoS. The asterisk marks the splicing product from a cryptic 5' splicing. **c**, Protein extracts were prepared from meiotic cells expressing wild-type Rem1 (haemagglutinin (HA)-Rem1) or the NoS mutant (HA-Rem1 NoS) and proteins were detected using anti-HA antibodies. Molecular mass markers are indicated on the left.

¹Oxidative Stress and Cell Cycle Group, Universitat Pompeu Fabra, C/Doctor Aiguader 88, Barcelona 08003, Spain. ²Stowers Institute, Kansas City, Missouri 64110, USA.

untranslated region (UTR) and most of the second exon had been deleted. During synchronous meiosis (Supplementary Fig. 2), both constructs showed a wild-type profile of splicing with minor differences at the end of meiosis, probably due to the lack of a polyadenylation site in the mutant affecting its stability. This contrasted with the profile detected when the promoter was changed. As shown in Fig. 2b, when *rem1* expression was under the control of the inducible *nmt41* promoter (at the *rem1* locus), splicing was constitutive, either with or without the 5' UTR from *rem1*. Furthermore, when *rem1* was under the control of the *res2* promoter (another intron-containing gene), splicing was also constitutive (Fig. 2c). To ascertain fully whether the promoter was responsible for the regulation of the splicing, we proceeded with a reverse strategy. We fused the promoter of *rem1*, either with (data not shown) or without the *rem1* 5' UTR region, to different reporter genes such as the meiosis-specific *rec6* (data not shown) and the ubiquitous *cdc2*. Splicing of the different chimaeras as well as the endogenous genes was followed by polymerase chain reaction with reverse transcription (RT-PCR), as cells progressed into synchronous meiosis (Supplementary Fig. 3). As shown

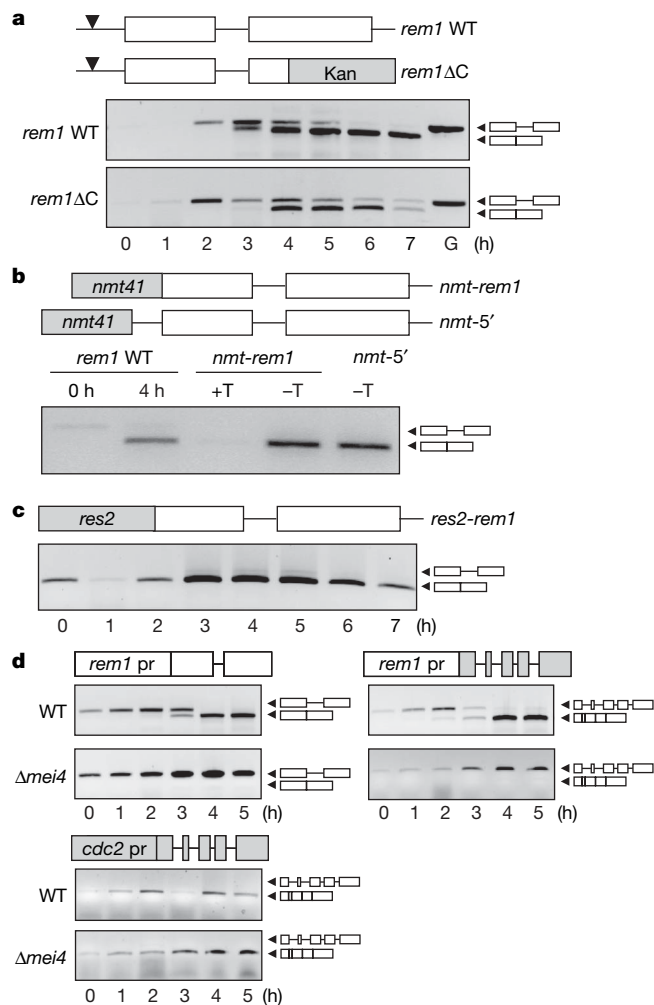


Figure 2 | The promoter of *rem1* controls splicing. **a**, *rem1* splicing analyses in a wild-type strain (*rem1* WT) and a strain deleted at the 3' end of *rem1* (*rem1*ΔC). The hours of meiosis are indicated; G, genomic DNA. Arrowheads indicate the transcription start site (-160 nucleotides). Kan, kanamycin cassette. **b**, *rem1* splicing analyses in cells carrying *nmt-rem1* and *nmt-5'* chimaeras (with or without *rem1* 5' UTR). Cells were grown with (promoter off) or without (promoter on) thiamine. Wild-type strains at 0 h and 4 h of meiosis were used as controls. **c**, *rem1* splicing analyses in cells carrying *res2-rem1* chimaera during meiosis. **d**, Splicing analyses of endogenous *rem1* and *cdc2* or *rem1-cdc2* chimaera in wild-type and Δ *mei4* strains. pr, promoter.

in Fig. 2d, the *rem1* promoter is sufficient to regulate intron retention during vegetative growth and the early stages of meiosis (top right panel). Furthermore, this splicing regulation is dependent on the presence of Mei4 (see later), because intron retention of the chimaeras can be detected in Δ *mei4* cells during the entire time course, as occurs with endogenous *rem1* (Fig. 2d; compare Δ *mei4* in top left and right panels). Conversely, endogenous *cdc2* (bottom panel) shows Mei4-independent constitutive splicing. Thus, the promoter of *rem1* is sufficient to direct intron retention in genes with up to four introns, such as *cdc2*.

The forkhead family is highly conserved in eukaryotes with roles in cell cycle control, cell death and differentiation^{9,10}. FLEX boxes are the *cis*-elements recognized by the forkhead transcription factors². There are two putative FLEX boxes in the *rem1* promoter, as indicated in Supplementary Fig. 4a. In fission yeast, four genes code for forkhead proteins: *sep1* (ref. 11), *fhl1* (ref. 12), *mei4* (ref. 2) and *fkh2* (refs 3 and 13). We have previously shown that Mei4 is required for proper regulation of *rem1* transcription¹. Mei4 overexpression in mitotically growing cells can induce splicing of *rem1* from a wild-type promoter but not in a FLEX box mutant background (Fig. 3a). Furthermore, *rem1* with mutations at the FLEX elements show induced transcription at early time points of meiosis, when there is no expression of *mei4* (Supplementary Fig. 4b, Δ FLEX2 panel). Together, these data indicate that other forkheads could also bind to these FLEX boxes and regulate transcription or splicing. Whereas deletion of *sep1* or *fhl1* had no effect, cells lacking Fkh2 showed *rem1* splicing during vegetative growth as well as meiosis (Fig. 3b, c and Supplementary Fig. 5). We determined that *rem1* splicing in Δ *fkh2* cells during vegetative growth was not due to the ectopic presence of Mei4 (data not shown). These findings prompted us to examine whether intron retention was a common feature of other genes during meiosis. We decided to use whole-genome microarray analysis to compare transcriptional profiles and intron retention of wild-type and Δ *mei4* strains. RNA was isolated from both strains at 0 h and 4 h after meiotic induction, and microarray data were collected mainly as described elsewhere¹⁴. Although the microarrays did not include probes for all *S. pombe* introns, the analyses showed that a set of genes presented intron retention in the absence of Mei4 (Supplementary Fig. 6a). Each one of these genes was analysed by RT-PCR, showing that they paralleled the pattern of *rem1* splicing during meiosis and vegetative growth (Supplementary Figs 6 and 7).

a Overexpression of Mei4 (lanes 2 and 4) in mitotically growing cells induces splicing of wild-type *rem1* (lane 2) but is unable to induce splicing when the two FLEX boxes are mutated (lane 4; F1/F2 mutant). Fkh2 and Mei4 regulate the splicing of *rem1*. The rate of splicing (**b**) and accumulation of *rem1* mRNA (**c**) was analysed by RT-PCR and northern blot in the indicated strains. The hours of meiosis are indicated at the bottom.

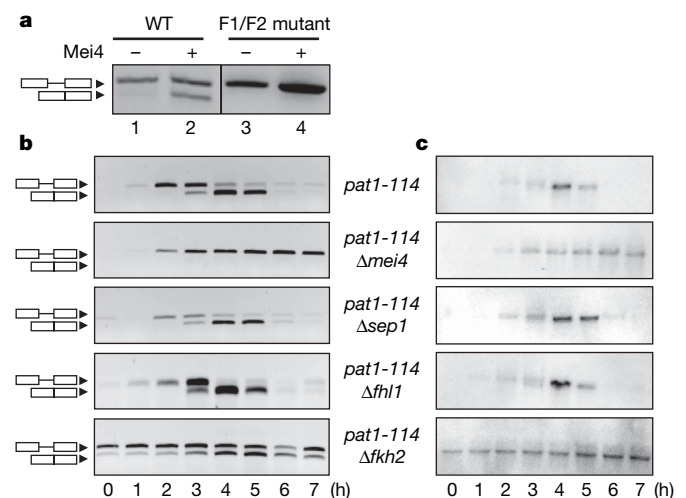


Figure 3 | *rem1* splicing is dependent on forkhead transcription factors. **a**, Overexpression of Mei4 (lanes 2 and 4) in mitotically growing cells induces splicing of wild-type *rem1* (lane 2) but is unable to induce splicing when the two FLEX boxes are mutated (lane 4; F1/F2 mutant). Fkh2 and Mei4 regulate the splicing of *rem1*. The rate of splicing (**b**) and accumulation of *rem1* mRNA (**c**) was analysed by RT-PCR and northern blot in the indicated strains. The hours of meiosis are indicated at the bottom.

To investigate further the role of the promoter in the regulation of splicing, we examined Mei4 and Fkh2 binding to specific promoters during meiosis and the timing of these interactions. Chromatin immunoprecipitation (ChIP) analysis showed that Mei4 and Fkh2 associated specifically with the *rem1* promoter (Fig. 4a). Notably, whereas the increased binding of Fkh2 was smoother during the time course—probably because Fkh2 was already bound to the promoter at the beginning of the meiosis (see the effect of *fkh2* deletion on splicing at early time points; Fig. 3b, lower panel)—there was an abrupt increase in binding of Mei4. Also, whereas Fkh2 was only detected in the promoter region, Mei4 was detected along the entire transcriptional unit. Next, we determined loading of RNA polymerase (Pol) II on *rem1*. ChIP analysis showed that Pol II is more efficiently loaded on the *rem1* promoter in the presence of Mei4 (Fig. 4b and Supplementary Fig. 8), although loading and transcription do occur to a certain extent in the absence of Mei4 at later time points of meiosis (see northern blots in Fig. 3c). We also noticed that in the absence of Mei4 there was an accumulation of the phospho-Ser 5 isoform of Pol II (concomitant with undetectable levels of the phospho-Ser 2 isoform), indicating that there might be a problem with Pol II elongation in Δ *mei4* cells that could have consequences on the coupling of transcription with pre-mRNA processing¹⁵. Next, we wanted to determine whether Mei4 and/or Fkh2 physically associated with proteins of the spliceosome to localize its function to specific genes. We tested the co-immunoprecipitation of Mei4 and Fkh2 with U1-70K (one of the 16 proteins of the U1 small nuclear ribonucleoprotein particles (snRNP) in fission yeast¹⁶), with Prp11 (the ATP-dependent helicase that bridges U1 and U2 snRNP, which is required for complex B formation during splicing¹⁷ and is known as PRP5 in other organisms) and with Cdc5 (a component of the nineteen complex (NTC), which is part of

the active form of the spliceosome¹⁸; the human homologue has been found associated with core Sm proteins¹⁹).

As shown in Fig. 4c and Supplementary Fig. 9, Mei4, but not Fkh2, was detected in the immunoprecipitates of U1-70K, Prp11 and Cdc5 at the time of *rem1* splicing. The interaction between Mei4 and the active spliceosome could explain why Mei4 is required for the regulated splicing of several meiotic genes. We next determined whether different splicing factors are recruited to specific genes in a Mei4-dependent manner. We could detect U1-70K, Prp11 and Cdc5 bound to the *rem1* open reading frame, with enrichment around the intron (Fig. 4d and Supplementary Fig. 10) and with maximum binding occurring concomitantly with the onset of meiosis I and *rem1* splicing. However, in the absence of Mei4, the binding of U1-70K, Prp11 or Cdc5 was abolished. Similar results were observed with two other genes, *spo4* and *mug137*, the splicing regulation of which paralleled the pattern of *rem1* (data not shown). Thus, loading of the active spliceosome onto several meiotic genes and its splicing depends on the presence of Mei4.

We report here that *rem1*, when it is not spliced, translates into a truncated protein that brings about normal levels of recombination. This could account for a sophisticated alternative splicing scheme in a unicellular organism which will generate a 17 kDa protein with a role in recombination when the mRNA is not processed (during the pre-meiotic S phase), and alternatively will produce a protein with a described function as a cyclin during meiosis I when the mRNA is processed (ref. 1 and Supplementary Fig. 11). This occurs as part of a regulatory mechanism that ensures the absence of cyclin in mitotically growing cells, because even very low levels of Rem1 are toxic in non-meiotic cells¹. Other mechanisms have been described to ensure the absence of meiotic-specific transcripts during vegetative growth²⁰. We propose here that the regulation of *rem1* splicing is exclusively under the control of its own promoter. In this model, the Mei4 transcription factor recruits the active spliceosome to specific genes, and the Fkh2 transcription factor interferes with coupling splicing to transcription. In budding yeast, it has been shown that two forkhead proteins, Fkh1 and Fkh2, have opposing functions in regulating Pol II elongation¹⁰. Thus, a switch from Fkh2 to Mei4 could explain the effect on splicing regulation. Other possibilities could be that Fkh2 and/or Mei4 act as chromatin-remodelling factors on specific promoters²¹, interfering with the coupling of splicing to transcription, or they could be affecting the processivity of Pol II, as in mammalian cells in which evidence for coupling between alternative splicing and promoter-specific transcription has been shown²².

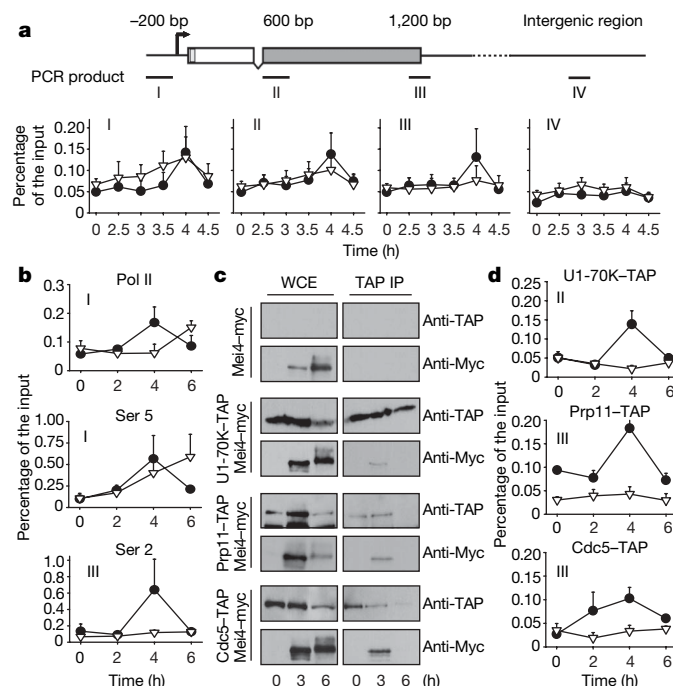


Figure 4 | Mei4 loads the spliceosome onto *rem1*. **a**, ChIPs of Mei4–13Myc (circles) and Fkh2–HA (triangles). *rem1* and the quantitative PCR products (I to IV) are shown at the top. **b**, ChIPs of Pol II, phosphor-Ser 5 or phosphor-Ser 2 from wild-type (circles) and Δ *mei4* (triangles) strains. **c**, Meiotic extracts from strains expressing Mei4–13Myc alone or co-expressing U1-70K–TAP, Prp11–TAP or Cdc5–TAP were IgG-sepharose-purified and proteins were detected by western blotting. TAP IP, tandem affinity purification; WCE, whole cell extracts. **d**, ChIPs of U1-70K–TAP, Prp11–TAP or Cdc5–TAP from wild-type (circles) and Δ *mei4* (triangles) strains. All the ChIPs were assayed for the presence of *rem1* at the indicated meiotic time points and the mean and s.e.m. were plotted ($n = 3$).

METHODS SUMMARY

Strains and media. All *S. pombe* strains used are isogenic to the wild-type 972h⁻ strain and are described fully in the Supplementary Information. Media was prepared as described²³. *S. pombe* cells were transformed using the lithium acetate method as described²³.

Synchronous meiosis. To obtain synchronous meiotic cultures in the *pat1-114* background, we used standard procedures described elsewhere^{1,24}. Synchronicity was assessed by flow cytometric analysis and 4',6'-diamidino-2-phenylindole (DAPI) staining as described previously²⁵.

Gene expression analysis. RNA was prepared by lysis in the presence of hot phenol, as described²⁶. For the RT-PCR reactions, 8 μ g of DNase-treated RNA was denatured at 65 °C for 10 min and then chilled on ice. Reverse transcription reactions were carried out with oligodT priming following manufacturer's guidelines (Promega), in the presence or absence of the enzyme. One microlitre of the newly synthesized complementary DNA was used in the PCR reactions with specific primers. Global expression analysis was carried out as described in the Supplementary Methods.

Protein extraction and immunoprecipitations. Extracts were prepared in NET-N buffer¹ and immunoprecipitations were performed with 30 μ l of immunoglobulin G (IgG) sepharose. Immunoprecipitates (8 mg) were performed in IPP buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), 5 μ g ml⁻¹ leupeptin and 5 μ g ml⁻¹ aprotinin), washed three times with the same buffer, resolved in 8% SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibody.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Microarray data are available at ArrayExpress (www.ebi.ac.uk/array-express/) under the accession number E-TABM-465. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.A. (jose.ayte@upf.edu).

METHODS

Growth and maintenance of *S. pombe* strains. All *S. pombe* strains used are isogenic to wild-type 972 *h*⁻ and are listed in Supplementary Table 1. Media were prepared as described previously²⁷ and *S. pombe* was transformed using the lithium acetate method²⁸. Flow cytometry was performed as described previously²⁹. For DAPI staining, 1 ml of cell culture (1×10^7 to 2×10^7 cells) was briefly centrifuged, fixed with 70% ethanol and stored. Fifty microlitres of fixed cells ($\sim 5 \times 10^5$ cells) were centrifuged and resuspended with 25 μ l of water, spotted onto slides and fixed by heating. Nuclei were stained with DAPI (5 μ g ml⁻¹). Recombination efficiency was determined as described³⁰. In brief, intragenic *ade6* recombination (gene conversion) was measured in wild-type and Δ *rem1* strains with the *ade6* alleles *ade6-M26* and *ade6-52*. Ade⁺ prototrophs per 10⁶ viable spores were measured and the efficiency relative to a wild-type strain (expressed as a percentage) was plotted.

Synchronous meiosis was performed as described previously³⁰ except for the experiments that involved co-immunoprecipitations (CoIPs), which were performed as follows: single colonies were grown in YE5S (ref. 27) to early stationary phase. The cells were diluted in minimal medium supplemented with 100 μ g ml⁻¹ leucine and grown at 25 °C to 1×10^7 to 2×10^7 cells ml⁻¹. The culture was then shifted to 35.5 °C in a water shaker (INFORS HT) to induce meiosis.

Gene expression analysis. Each cell pellet was resuspended in 0.4 ml of AE buffer (50 mM sodium acetate, pH 5.3, 10 mM EDTA, pH 8.0). SDS was then added to a final concentration of 1%, and proteins and DNA were extracted by adding 0.6 ml of 1:1 acidic phenol:chloroform and incubation at 65 °C for 1 h. The aqueous phase was separated by centrifugation at 10,000g for 2 min at 4 °C. The sample was washed twice with 0.2 ml of 1:1 acidic phenol:chloroform. After chloroform extraction, RNA was precipitated with ethanol. RNA concentration was determined by absorbance at 260 nm, and equal amounts (5 μ g) were loaded in formaldehyde-agarose gels³⁰ containing ethidium bromide to confirm equal loading by visualizing ribosomal RNA. RNA was then transferred to GeneScreen Plus nylon membranes (PerkinElmer Life Sciences). Hybridization and washes were performed as recommended by the manufacturer.

Splicing was analysed by RT-PCR. RNA was digested with DNase I for 30 min at 37 °C, phenol extracted and precipitated. Eight micrograms of total RNA was denatured at 65 °C for 10 min and then chilled on ice. Reverse transcriptase reactions were carried out (60 min at 42 °C, 30 min at 52 °C and 3 min at 94 °C) following the manufacturer's guidelines (Promega) in the presence or absence of the enzyme. One microlitre of the cDNA was used in the PCR reactions with the primers listed in Supplementary Table 3. Genomic DNA was used in Fig. 2a as a control of unspliced *rem1*.

Microarray experiments and data evaluation. Global expression analysis used custom designed *S. pombe* microarrays. Array construction, sample labelling and hybridization were carried out as described previously³¹. The arrays consisted of 8,785 70-mer oligonucleotides representing 6,918 exons, 1,521 introns and 333 3' UTRs (http://research.stowers-institute.org/microarray/S_pombe/). In brief, total RNA was prepared from wild-type and Δ *mei4* strains, at 0 h and 4 h from meiosis induction. Polyadenylated RNA was extracted from total RNA by purification with an oligo(dT) cellulose column. RNA quality was assessed on a Bioanalyser 2100 machine (Agilent). RNA (2 μ g per sample) was converted to cDNA by priming with oligo-dT18 and poly-dN9 in the presence of aminoallyl-dUTP (Ambion), followed by conjugation to Cy5 or Cy3 fluorescent dyes. Samples were dye-swapped for further technical replication. Labelled samples were mixed for comparative hybridization on poly-L-lysine-coated microarrays, which were printed with 70-base oligomers representing all known *S. pombe* reading frames and introns greater than 70 bases in length. The microarrays were scanned with a GenePix 4000B scanner and the images were analysed using GenePix Pro 6.0 software (Molecular Devices). Data analysis was performed with the R programming language. Differential expression was assessed using the Limma package³². Eight microarrays were used, with two experimental

factors: time points 0 h and 4 h from meiotic induction and wild-type and Δ *mei4* strains. Data within arrays were normalized using the print-tip loess method. Data between arrays were normalized using Aquantile normalization. Linear models were fit with coefficients estimated for wild type 0/*mei4* 0, wild type 4/*mei4* 4, wild type 0/*mei4* 4 and *mei4* 0/*mei4* 4. Microarray data are available at ArrayExpress (<http://www.ebi.ac.uk/array-express/>) under the accession number E-TABM-465.

After defining the genes that parallel the pattern of *rem1*, we have aligned the sequences of their promoters and searched for similar patterns in the direct and reverse strands. We have obtained the frequency of every nucleotide at each position (Supplementary Fig. 6b). The frequency of the nonamer FLEX box GTAAACAAA in the *S. pombe* genome is: 214 genes have 1 GTAAACAAA sequence, of which 9 of the genes have 2 GTAAACAAA sequences; 332 genes have 1 TTTGTTTAC sequence, of which 24 of the genes have 2 TTTGTTTAC sequences and 1 gene has 3 TTTGTTTAC sequences. The data were obtained by searching for FLEX boxes in both strands of the promoters of *S. pombe*.

Chromatin immunoprecipitation. Ninety millilitres of cells at an A_{600} of 0.5 (or equivalent) were crosslinked using 1% formaldehyde (final concentration) for 10 min at 25 °C. The pellet was washed twice with PBS. Cells were resuspended in breaking buffer (0.1 M Tris, pH 8.0, 20% glycerol and 1 mM PMSF) and broken in a Minibeat-beater three times at medium intensity. The pellet was washed twice with lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 1 mM PMSF), resuspended in 0.25 ml of lysis buffer and sonicated to a mean chromatin fragment size of 500 bp on a Diagenode water sonicator. The chromatin was centrifuged for 15 min at 16,000g and the pellet was discarded. Immunoprecipitations were performed overnight with the same amount of protein (measured by Bradford) with 15 μ l IgG-sepharose (Amersham) or a specific antibody (9E10 for Myc-tagged proteins, 12CA5 for HA-tagged proteins, anti RNA Pol II CTD monoclonal antibody (8WG16, Abcam), anti-RNA Pol II CTD (phospho-Ser5) polyclonal antibody (Abcam) or anti RNA Pol II CTD (phospho-Ser2) polyclonal antibody (Abcam)). Immunoprecipitated extracts were incubated with 15 μ l of protein G sepharose for 5 h. Beads were washed once with lysis buffer, twice with lysis buffer containing 500 mM NaCl, twice with wash buffer (10 mM Tris, pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA and 1 mM PMSF) and once with TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The beads were resuspended in 0.1 ml of elution buffer (50 mM Tris, pH 8, 10 mM EDTA, 1% SDS) and incubated at 65 °C for 20 min. The beads were then washed with 0.15 ml of TE buffer with 0.67% SDS and the crosslinking was reversed overnight at 65 °C. To clean up the DNA, 0.25 ml of TE buffer was added, 1 μ l of 20 mg ml⁻¹ glycogen and 7.5 μ l of 20 mg ml⁻¹ Proteinase K and incubated for 2 h at 37 °C. DNA was extracted with phenol:chloroform and precipitated with ethanol and sodium acetate for 30 min at -20 °C. DNA was pelleted for 30 min at 16,000g at 4 °C. The pellet was air-dried and resuspended in 100 μ l of TE buffer. To perform the PCR, a SYBR green kit was used and quantitative real time PCR was performed in an Applied Biosystems Cyler. Data were expressed as a percentage of the input.

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