

PROMOTER-DRIVEN SPLICING REGULATION IN FISSION YEAST

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PROMOTER-DRIVEN SPLICING REGULATION IN FISSION YEAST

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con todo el afecto del mundo*

Si es que cuando te pones
hasta sacas resultados bonitos
Alba Diz

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INTRODUCTION

Schizosaccharomyces pombe

The yeast known as *Schizosaccharomyces pombe* was first described by P. Lindner in 1893 after isolating it from an East Africa beer. The name derives from the Swahili word for beer, *pombe*. *S. pombe* forms a rod-shaped non-motile cell that grows by elongation and divides by medial fission with a doubling time of 2-4 hours in simple culture conditions (Forsburg). It was originally classified as a *fungus* from the family of ascomycete due to its capacity to produce *asci*. Recently, it has been shown by DNA and RNA sequence analysis to differ phylogenetically from the other known budding yeast *Saccaromyces cerevisiae* as much as from humans (2004). The complete taxonomic classification is:

Cellular organism
Eukaryota
Fungi/Metazoa group
Fungi
Dikarya
Ascomycota
Taphrinomycotina
Schizosaccharomycetes
Schizosaccharomycetales
Schizosaccharomycetaceae
Schizosaccharomyces
Schizosaccharomyces pombe

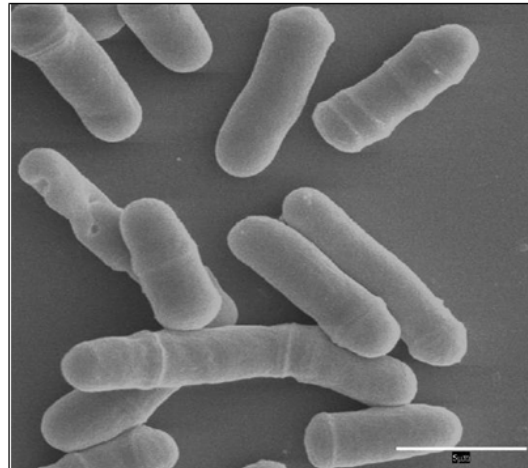
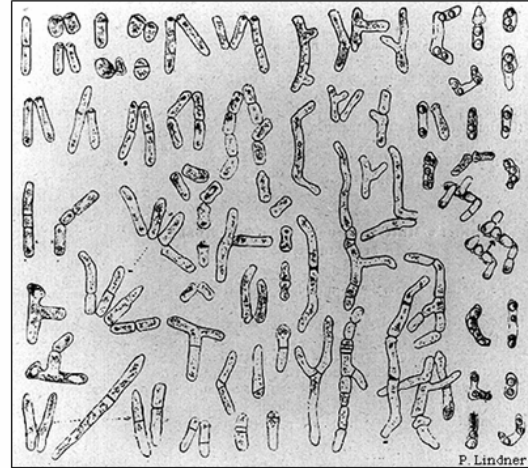


Figure 1. The fission yeast *Schizosaccharomyces pombe* has become today one of the most important model organisms.

Upper pannel, Lidner's draw of the isolated strain. Lower pannel, scanning electron microscopy image of some *S. pombe* cells.

The genetic material of *S. pombe* is organised in three chromosomes. The entire genome is completely sequenced containing 4824 genes in about 14 Mb of total DNA, making it the smallest free-living eukaryote (Wood et al., 2002). The *S. pombe* genome does not have many duplicated genes, making functional genetics very approachable.

Significantly, several groups of genes are conserved in *S. pombe* and humans, but missing in *S. cerevisiae*, including genes involved in heterochromatin and telomere function, some splicing genes, and components of the RNAi machinery (2004).

With simple culture conditions, *S. pombe* is easily adapted to any molecular biology laboratory. At present, *S. pombe* is one of the most important model organisms to study multiple cellular processes, such as cell cycle regulation, metabolism, cell signaling, transcription, splicing... The main reasons for this are its easy growing, maintainance and manipulation in the laboratory and its great molecular similarity to higher eukaryote cells. In the 50s, Murdoch Mitchison started his studies in cell cycle, cell division and growth; in the 70s this studies were linked with Paul Nurse's genetic approach and this led up to well-earned recognition by the award of the 2001 Nobel Prize (2004).

S. pombe is also known as fission yeast because its division is by bipartition. The study of the cell cycle is based basically in this type of division: microscopically it is possible to know in which cell cycle phase a cell is or in which moment of the cell cycle the cell is malfunctioning.

Life Cycle of fission yeast

During normal growing conditions, *S. pombe* can proliferate through mitotic cell cycles as a haploid cell, but this is just one of the three alternative fates that *S. pombe* may choose depending on the environmental conditions. When nutrient availability is reduced, fission yeast can undergo quiescent stationary phase (glucose deprivation) or conjugate with a cell of the opposite mating type and undergo meiosis (nitrogen deprivation). After conjugation, cells can grow as diploids if the media conditions change and enter in a diploid mitotic cell cycle, although they are very unstable and tend to haploidize. Meiotic progression finishes with the formation of spores that are specialized cells prepared to resist unfavourable environmental conditions. When the conditions improve, spores can germinate and re-enter mitotic cell cycle as haploid cells.

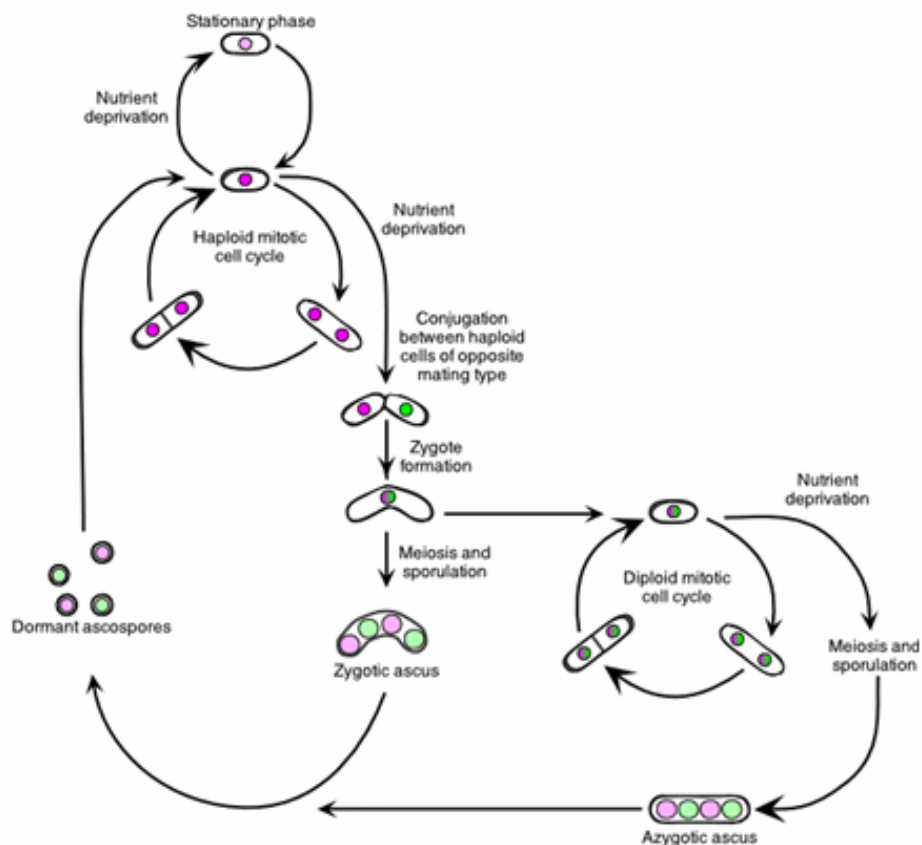


Figure 2. Complete cell cycle of *Schizosaccharomyces pombe*.

Vegetative growth

Schizosaccharomyces pombe has a typical cell cycle, with an S phase when DNA replicates and an M phase when the cell segregates the chromosomes into two different nuclei. These two phases (S and M) alternate with cell growing gaps, known as G1 and G2. Noticeably, the cytokinesis takes place just before next cycle S phase, so daughter cells remain together during nearly all G1 phase (2004).

During the vegetative growth, the onset of S and M phases is under tight regulation to ensure that they alternate and that they are coupled in the right order (Moser and Russell, 2000). There are more than 100 protein kinases in *S. pombe* that have a function in cell cycle progression but none of them is as important as Cdc2 (*Homo sapiens* Cdk1). Cdc2 is the unique cyclin-dependent kinase (CDK) in *S. pombe* and is required for both DNA replication and mitosis (Nurse and Thuriaux, 1980). Fission yeast cells require Cdc2 for passage through Start (restriction point in higher eukariots), at which cells commit to progress through the mitotic cell cycle. Cdc2 is also essential for the G2-M transition, at which cells initiate the processes of chromosome segregation and cytokinesis (Nurse and Bissett, 1981).

Though Cdc2 abundance is invariant during the cell cycle, its activity fluctuates, being low during G1 phase, moderate during S and G2 phases and high during M phase. This activity is regulated by phosphorylation, inhibitor proteins (CKIs) and mainly by its associated cyclins (Alfa et al., 1989).

Regulation of Cdc2 by phosphorylation

The specific phosphorylation status of the cyclin/Cdk complexes is very important for their function in any living organism. While Cdc2 phosphorylation in Threonine 167 (T167) of the T-loop is necessary for its activity (Gould et al., 1998), the phosphorylated form of Tyrosine 15 in the ATP-binding domain inhibits its function (Gould and Nurse, 1989).

Cdc2 Y15 dephosphorylation is essential to trigger mitotic entry. This is a key target for checkpoints in case of unreplicated or damaged DNA. This Y15 phosphorylation is carried out by the kinases Wee1 and Mik1. In the absence of these

kinases Cdc2 activity cannot be inhibited and cells undergo mitotic-catastrophe because of its uncontrolled premature entry into mitosis (Russell and Nurse, 1987). At the same time there are other proteins controlling the activity of Wee1 and Mik1. Wee1 is inhibited by Cdr1 as a response to nutrients limitation (Belenguer et al., 1995) and Mik1 activity is regulated in response to S-phase checkpoints.

On the other hand, Cdc25 is the most important phosphatase that dephosphorylates this residue. Cdc25 is also a target of checkpoint kinases that inhibit its function. Cdc25 accumulates just before M phase and its levels decrease after mitosis (Gautier et al., 1991).

Regulation of Cdc2 by Cyclins

The only essential cyclin in *S. pombe* is Cdc13, a member of the B-type cyclin family. Cdc13 abundance is low in G1 and increases during G2 until Anaphase, when its ubiquitin-directed degradation is necessary to separate sister chromatids. Cdc13 degradation is directed by proteins from the APC complex, which recognize the Destruction Box (DB) present in some cyclins. Three known APC-complex proteins in *S. pombe* are Slp1, in mitosis, Ste9, in G1 and Mfr1/Fzr1 present during meiosis and sporulation (Asakawa et al., 2001; Blanco et al., 2001; Kitamura et al., 1998; Matsumoto, 1997; Yamada et al., 2000; Yamaguchi et al., 1997). Cdc13 main function is to promote the events of mitosis and avoid re-initiation of S phase (endoreduplication).

S. pombe has two other B-type cyclins, Cig1 and Cig2. They are important in G1/S transition, when the kinase activity of the Cdc2/Cig2 complex peaks. Cig1 also participates in G1 although is a minor cyclin whose function is still not very well understood (Fisher and Nurse, 1996; Martin-Castellanos et al., 1996; Mondesert et al., 1996). Puc1 is another *S. pombe* G1 cyclin and it is supposed to couple initiation of S phase to the achievement of a critical size although its role is covered, under physiological conditions, by the function of Cig1 and Cig2 (Martin-Castellanos et al., 2000).

Importantly, Cdc13 can substitute all of the other cyclins. A *cig1-cig2* double deletion or a *cig1-cig2-puc1* triple deletion only causes a small delay at the G1/S

transition while cells with a *cdc13-cig1-cig2* triple deletion cannot perform DNA synthesis.

Regulation of Cdc2 by other proteins

Cdk proteins can also be regulated by inhibitors (CKIs). Rum1 inactivates Cdc2/Cdc13 and Cdc2/Cig2 complexes in G1. Its function is necessary for cells to arrest in G1 in response to mating factors (pheromones) and conjugate (Labib and Moreno, 1996; Martin-Castellanos and Moreno, 1996; Moreno and Nurse, 1994). Cdk-activating kinases (CAKs) phosphorylate T167, activating the Cdk. This phosphorylation increases the interaction between Cdc2 and the different cyclins. In *S. pombe*, the Mcs6/Mcs2 complex is able to phosphorylate (at least partially) Cdc2. In fission yeast, Csk1 is also considered a CAK protein because phosphorylates Cdc2, although some authors consider Csk1 as a CAKAK (CAK Activating Kinase) because can phosphorylate and activate Mcs6, which has a Cdk protein structure.

Sexual Reproduction

Under conditions of nutritional deprivation, specially the nitrogen source, fission yeast differentiate into ascospores. Spores represent a dormant state which is more resistant to unfavorable conditions. In this process, haploid G1 cells of opposite mating types (M or h^- and P or h^+) first mate to form a diploid zygote which will undergo meiosis and sporulation. This process will give rise to four haploid spores that will germinate when the nutritional conditions improve. Meiosis drives a specialized cell cycle that consists of one round of DNA synthesis followed by two successive rounds of M phase and is the main exception to the otherways tight S/M alternancy.

Meiotic differentiation includes complex progressive events. It can be divided into two parts: 1) exit from the mitotic cell cycle and 2) induction of the alternative cell cycle program. The former has been characterized as a complex network of activator and inhibitor factors which are regulated at different levels of transcription, post-translational modifications and degradation (Yamamoto, 1996). This network also involves many feedback loops to intensify the commitment of meiosis.

The latter process is achieved by a highly coordinated transcription induction of numerous genes that are specifically required for meiosis (Mata et al., 2002). These genes codify products required (1) to modify the basic machinery that operates in the mitotic cell cycle to ensure the DNA synthesis is followed by the two successive nuclear divisions and (2) to reorganize the cell and nuclear morphology to give rise to an asci containing four haploid spores.

Exit from mitotic cell cycle

The initiation of meiosis is mediated by two signal transduction pathways, one responding to the nutritional deprivation and the other to the mating pheromones.

Initiation of meiosis

After Nitrogen starvation, Tor2 kinase appears to decrease its activity inducing the switch between cell growth and cell differentiation (Alvarez and Moreno, 2006;

Uritani et al., 2006; Weisman et al., 2007). Nitrogen starvation results in the G1 arrest and the transcriptional activation of *ste11* (Maeda et al., 1990; Mochizuki and Yamamoto, 1992; Sugimoto et al., 1991):

a) Starvation-induced G1 arrest requires the inactivation of the Cdc2-Cig2 complex, which promotes S phase and the Cdc2-Cdc13 complex, which can take over Cig2 function in its absence (Fisher and Nurse, 1996). Degradation of Cig2 delays G1/S transition, which allows the accumulation of Rum1. Rum1 will promote the degradation of both cyclins via APC/Cste9 (Correa-Bordes and Nurse, 1995).

Both PKA and Sty1 pathways are necessary to promote this degradation: A reduction in PKA activity relieves the inhibitory phosphorylation of APC/C facilitating the degradation of Cig2 and Cdc13. Starvation of nutrients activates the stress-responsive Sty1 MAPK pathway (Shiozaki and Russell, 1997). The mechanism by which the stress pathway induces the G1 arrest is not yet known. It has been recently proposed, based in the fact that nitrogen starvation speeds up mitosis, that rapid mitosis results in smaller newborn G1 cells.

These small newborn cells stay longer in G1 since it takes longer to reach the critical size to enter S phase. Thus, the length of G1 is increased, leading to the accumulation of Rum1 and the CDK inhibition.

b) *Ste11* is a transcription factor that activates genes required for pheromone communication, mating and meiosis. Both, PKA and stress pathways are responsible for the induction of *Ste11* expression: *Rst1*, a target of PKA is inactivated when is phosphorylated by PKA (Higuchi et al., 2002). The unphosphorylated form of *Rst1* stimulates transcription

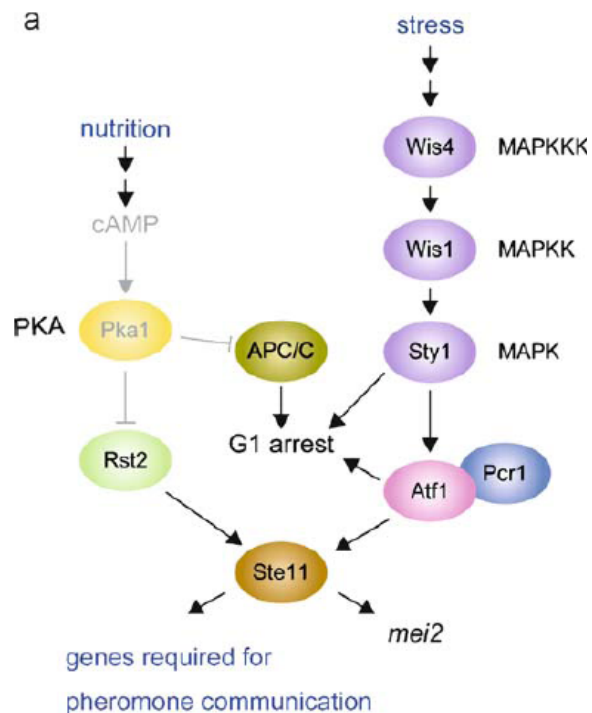


Figure 3. Two independent kinase pathways are needed to exit mitotic cell cycle. Pka pathway promotes Cig2 degradation and Sty1 pathway promotes Ste11 transcription, although both pathways form an interconnected network.

of *ste11*. Atf1, a substrate of the stress MAPK Sty1, forms a heterodimeric transcription factor with Pcr1, which also regulates *ste11* transcription.

The pheromone pathway

The pheromone system in fission yeast is based on the reciprocal recognition of specific proteins by the other mating type cell. *S. pombe* cells have two mating types; h^+ or h^- . This is genetically determined by the *mat1* locus (Egel, 1976b). In h^+ cells, two different genes are expressed from the *mat1* locus: *mat1-Pc*, which controls the sexual differentiation, and *mat1-Pm*, which controls the initiation of meiosis. Similarly, h^- cells express *mat1-Mc*, which also controls the sexual differentiation, and *mat1-Mm*, needed to enter meiosis (Kelly et al., 1988).

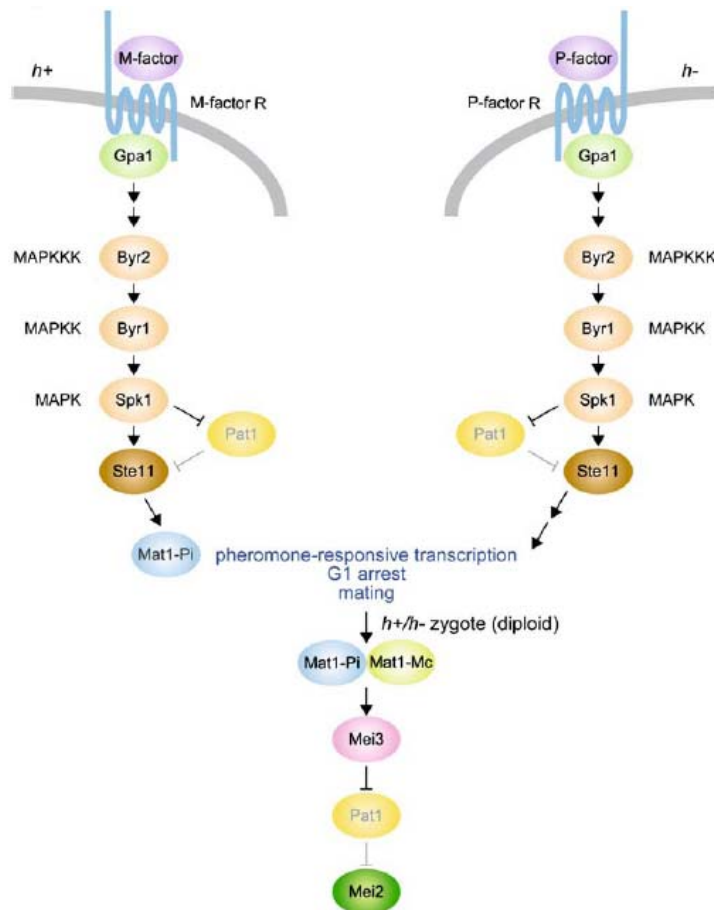


Figure 4. The pheromone pathways.

Both pheromone pathways activate when two opposite mating type cells conjugate. Binding of the opposite pheromone to the receptor triggers a MAPK cascade that triggers Ste11 transcription and inhibit the kinase Pat1. After the mating and the conjugation has taken place, Mat1-Pi and Mat1-Mc heterodimer promote Meiosis 3 transcription which will avoid the inhibitory phosphorylation of Pat1 to Meiosis 2..

The original isolated *S. pombe* strain was a homothallic h^0 strain (opposed to h^- or h^+ heterothallic strains), in which cells were switching from h^+ to h^- and viceversa. Changes in the *mat1* locus genetic information are controlled by the *mat2-P* and *mat3-M* genes. Transposing of the genetic material is the basis of this mechanism and it is

called “switching”. *mat2-P* and *mat3-M* are never transcribed because they are located in areas of silenced chromatin. These two genes are limited by homologue sequences also found in the boundaries of the *mat1* locus. These regions of homology will allow the recombination and the switch of information of *mat1* locus (Egel, 1976a). The switching is performed in the second generation of daughter cells. During DNA replication in S-phase, a single stranded break (SSB) is produced at the *mat1* locus and as a consequence one of the daughters will be switchable for changing its mating type (Arcangioli, 1998). Afterwards, this daughter cell will produce a double strand break in the *mat1* locus permitting the recombination with *mat2-P* or *mat3-P* and switching the mating type of the cell.

The reciprocal binding of the pheromones to their receptors leads to the activation of the Spk1 MAPK pathway which also induces *ste11* expression. Ste11 is the transcription factor of the master regulator of meiosis, Mei2. Mei2 is kept inhibited during vegetative growth by the kinase Pat1. After mating, in the zygote, Mat1-Pm (h⁺-specific) and Mat1-Mm (h⁻-specific) form a heterodimeric transcription factor that cooperatively induce the transcription of *mei3*. This cooperation is important to assure that meiosis will only be induced in heterozygous diploid cells. Mei3 binds and inhibits the Pat1 kinase (McLeod and Beach, 1988; Van Heeckeren et al., 1998) thereby liberating Mei2 from the inhibitory phosphorylation and triggering the initiation of meiosis. There is a mutant Pat1 protein commonly used in the laboratory to perform synchronous meiosis: the *pat1-114* mutant allele encodes a temperature-sensitive version of Pat1 that becomes inactive at high temperature inducing ectopic meiosis even in haploid cells (Iino and Yamamoto, 1985).

Meiotic cell cycle program

At the level of cyclin-cdks complexes, meiosis is regulated differentially depending on the meiotic step. As in the mitotic cell cycle, Cdc2 is essential to drive the meiotic G1/S transition and the

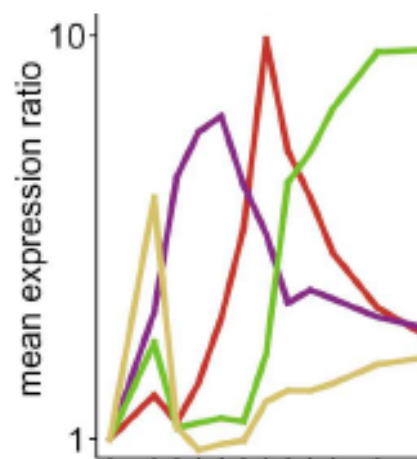


Figure 5. Different waves of transcription at meiosis. Yellow line represents early genes, purple line represents S-phase genes, red line represents meiosis I genes and green line represents meiosis II and sporulation genes (Mata et al., 2002).

second meiotic division and is not clear its role during the first meiotic division (Iino et al., 1995). Regarding its cyclin partners, Cdc2 is bound to Cig2 to promote pre-meiotic S phase (Borgne et al., 2002) and to Cdc13 in the two meiotic divisions. In spite of the cyclin-cdk complexes regulation, meiosis is strongly regulated by several waves of transcription factors.

Different waves of expression

The transcriptional program that drives sexual differentiation in *S. pombe* was studied extensively by the Bähler group using microarrays technology (Mata et al., 2002).

They found that the expression of hundreds of genes is modulated in successive waves during meiosis and sporulation and several known transcription factors are critical for these processes. All the genes that are upregulated during meiosis can be classified in four clusters, according to their time of transcription. These clusters represent successive waves of expression coinciding with the most important biological events of meiosis:

a) Starvation and pheromone-induced genes

This group is mainly regulated by the Ste11 transcription factor. These genes participate in the pheromone pathway and the first stages of meiosis.

b) Pre-meiotic S phase and recombination genes

During pre-meiotic S phase, two subclusters of genes are simultaneously expressed. (1) A group that is also expressed in mitotic G1 phase, required for DNA synthesis (*cdc18*, *cdt1*, *cdc22*...) and (2) a meiosis-specific group involved in recombination (*rec* genes) (Fox and Smith, 1998; Mata et al., 2002). Both groups are regulated by the MBF (MluI-binding factor) complex. This complex is formed by Cdc10, Res1, Res2 and the meiosis-specific Rep1 protein. This transcription factor regulates approximately 53% of the genes needed in this meiotic step.

c) Meiotic divisions and early steps of spore formation

The forkhead-family transcription factor Mei4 is essential for progression through the first meiotic division and has been reported to activate the expression of some middle genes. 55% of the middle genes are completely Mei4-dependent and this percentage increases until 92% if expression-reduced genes are considered (Mata et al., 2007). Cdc25 is under the regulation of Mei4 and its expression is necessary to progress through the meiotic divisions. $\Delta mei4$ strains are blocked just before meiosis I, but cells can undergo meiosis if Cdc25 is expressed ectopically (Murakami-Tonami et al., 2007).

d) Spore maturation

The late-genes cluster is regulated by two bZIP transcription factors, Atf21 and Atf31. They control the expression of approximately 55% of the sporulation genes. Atf21 and Atf31 seem to cooperate to induce this cluster of genes, probably by forming a heterodimer.

The alternation between transcriptional waves also requires that each wave is inactivated before the following wave is induced. It was found that the reduction in the levels of early genes that normally coincides with the induction of the middle genes did not occur in $\Delta mei4$ mutants. This effect has been previously observed for a small number of recombination genes. Similarly, Rep1, which is involved in the activation of a subset of early genes, is specifically required for the down-regulation of some of the genes induced in response to nitrogen starvation and pheromone signaling following entry into meiosis. These dependencies between transcription factors ensure the ordered, uni-directional succession of transcriptional waves.

Expression and selective elimination of meiosis-specific mRNAs in vegetative growth.

Until now, there have been described three mechanisms to control ectopic expression of meiotic-specific genes. The most important mechanism is based on the

absence/presence of specific transcription factors. For example Mei4, a general transcription factor of mid-meiosis genes is not present in mitotic cell cycle and is highly expressed on the onset of meiosis I.

It has recently been described that some meiotic-specific mRNAs, such as *mei4* and *rec8* mRNAs have a *cis*-acting region in the 3' end of the gene called DSR (determinant of selective removal) which is responsible for the block of the mRNA accumulation in vegetative growing cells (Harigaya et al., 2006). Mmi1 binds DSR-containing mRNAs promoting their degradation by the exosome. *mmi1* deletion is lethal which indicates that this system is not a mere safeguard mechanism to avoid accidental transcription of these genes, rather, cells should keep the system constantly active to curtail the expression of meiotic genes.

Another mechanism to avoid expression is based in the fact that some meiotic-specific genes contain introns. Genes such as *rem1* and *crs1* are only spliced in a very specific temporal window before meiosis I. Their transcription is mainly Mei4-dependent but in mitotic growing cells and in early stages of meiosis splicing seems to be actively repressed (Averbeck et al., 2005; Malapeira et al., 2005).

Forkhead transcription factors

The Forkhead Domain, also known as Winged Helix, is a B-DNA binding structure present in proteins of many different organisms, we can find them in eukaria, prokaria, and virus. Its structure was determined in 1993 (Clark et al., 1993) by X-ray crystallography. Forkhead transcription factors are part of the family of helix-turn-helix binding proteins (Gajiwala and Burley, 2000). Forkhead domain interacts with the DNA backbone through direct and water-mediated major and minor groove base contacts. The central alpha helix (H3) contacts the major groove of the DNA while the two “wings” (W1 and W2) (formed by alpha helixes, beta strands and random coiled aminoacids) contact the base pairs in the minor groove of the DNA.(Gajiwala and Burley, 2000)



Figure 6. Structure of the Forkhead domain. Helix 3 (H3) is shown binding to the major groove of the DNA, Wing 1 (W1) and Wing 2 (W2) contact the minor groove and recognize the DNA sequence.

Members of the forkhead-box family of transcription factors are present in almost all eukaryotes but not in plants (Mazet et al., 2003). More than 50 proteins that share homology in the winged-helix DNA-binding domain have been identified in higher eukaryotes (Shimada et al., 2007). This family of transcription factors is implicated in the regulation of a variety of cellular processes, including cell cycle, apoptosis, DNA repair, stress resistance, aging and metabolism.

In the human genome at least 100 genes encoding forkhead domains can be found, classified in many subgroups depending on the sequence (Mazet et al., 2003).

Due to the great range of functions of this family, the pathology associated to them is also really broad. FOXO subfamily, for example is implied in diabetes, obesity

and other metabolic failures, neurodegeneration and cancer (Partridge and Bruning, 2008).

In budding yeast there are 4 genes of this family. FKH1, FKH2 and HCM1 participate in the regulation of various cell cycle processes (Kumar et al., 2000), FKH1 and FKH2 coordinate in opposing ways elongation and concomitant phosphorylation of Ser2 of the CTD domain of RNApol II (Morillon et al., 2003). FHL1 and IFH1 are part of the rRNA processing machinery, these two members bound the DNA through an anchoring protein called RAP1 (Hermann-Le Denmat et al., 1994),(Rudra et al., 2007).

In fission yeast there are also 4 forkhead transcription factors. Fkh2, Fhl1, Sep1 and Mei4. The two former contain a Forkhead domain and a Forkhead associated domain (FHA) while the two latter contain just the Forkhead domain (Szilagyi et al., 2005).

Sep1 is a transcription factor that regulates cytokinesis and septation, it seems to be the link between nuclear and cell division (Grallert et al., 1998),(Ribar et al., 1997).

Fkh2 has many functions, sometimes opposite functions have been described. It is involved in septum formation, cytokinesis, nuclear structure and mitotic spindle function (Bulmer et al., 2004). It was also suggested that Fkh2 was implicated in the periodic gene expression of M and G1 phases (Buck et al., 2004), (when it is phosphorylated (Buck et al., 2004)) by negatively regulating the transcription of a cluster of genes (Rustici et al., 2004). The null mutant displays longer length, cold-sensitive, heat-sensitive and slow-growing phenotypes (Buck et al., 2004),(Bulmer et al., 2004). Regarding meiosis, a null mutant presents low levels of *ste11* mRNA, dephosphorylation at Fkh2 residues T314 and S462 is required for binding to *ste11* promoter region and induction of *ste11* transcription during nitrogen starvation (Shimada et al., 2007).

The function of Fhl1 is still not very well understood. The null mutant is more resistant to Methyl methanesulfonate treatment compared to wild type. The homologous protein in *S. cerevisiae* is involved in rRNA transcription and processing (Rudra et al., 2007).

Mei4 is the main transcription factor of mid-meiosis (Mata et al., 2002) and it is also a repressor of the previous transcriptional wave in meiosis (Mata et al., 2007). It is necessary to complete meiosis I because it controls the transcription of *Cdc25*, necessary for this progression (Murakami-Tonami et al., 2007). Its expression is completely avoided in vegetative growing cells by specifically degradation of meiotic mRNAs by the *mmi1* system (Harigaya et al., 2006). Mei4 has been reported to bind in vitro to the FLEX motif GTAAAYA (Horie et al., 1998).

Splicing

One of the most important conclusions of the post-genomic era is that in spite of the limited number of genes, a great protein complexity can be achieved. Mechanistically it is possible that one gene can increase its coding capacity to many protein products, pre-mRNAs have alternative coding (exons) and non-coding sequences (introns) that can be processed combinatorially to increase the protein output variability. An extreme example is the *Drosophila Dscam* gene, which codes for a cell surface protein involved in neuronal connectivity. Its pre-mRNA is alternatively spliced and can potentially generate 38,016 different protein isoforms, more than twice the number of genes in the entire *Drosophila* genome.

General mechanisms

Introduction

Most primary messenger RNA transcripts (pre-mRNAs) of RNA polymerase II in eukaryotes contain intervening sequences that must be precisely removed to generate a functional mRNA. The excision of the intervening sequences, the introns, from a pre-mRNA and the concomitant joining of the flanking sequences, the exons, is called pre-mRNA splicing. Pre-mRNA splicing takes place in large highly dynamic ribonucleo-protein (RNP) complexes, the spliceosome. It consists of five small nuclear RNAs (snRNAs) called U1, U2, U4, U5 and U6 and a great quantity of proteins (Kambach et al., 1999). RNA-RNA, RNA-protein and protein-protein interactions are involved in the recognition of the 5' splice site, 3' splice site, branch point, etc. and the intron removal from the pre-mRNA.

The splicing reaction can be functionally divided into several steps, including spliceosome assembly, activation, catalysis, and disassembly/recycling of the spliceosomal machinery. Many of these functional aspects have been investigated using *in vitro* splicing systems of mammalian cells and the budding yeast *Saccharomyces cerevisiae*. The picture emerging from these studies shows the spliceosome as a highly

dynamic machinery in which the snRNAs are rearranged multiple times concomitantly with a changing composition of the RNA and protein components (Bottner et al., 2005).

Molecular mechanism

This reaction takes place in the nucleus and can occur co- or post-transcriptionally. Afterwards, the mature mRNA is transported to cytoplasm to be

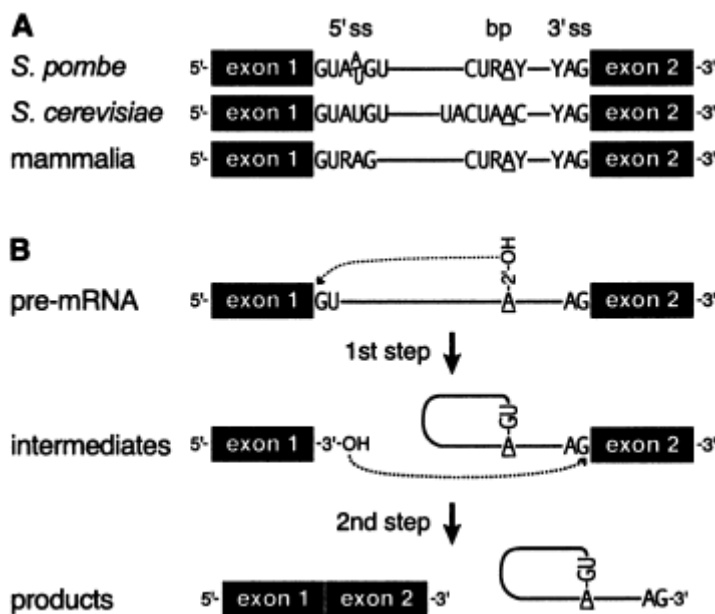


Figure 7. Schematic representation of introns and pre-mRNA splicing.

(A) 5' and 3' Splice Site (SS) and branch point consensus sequences in *S. pombe*, *S. cerevisiae* and mammalia.

(B) The two catalytic reactions in splicing and the products obtained. In the first step a nucleophilic attack of the branch site to the 5' SS produces a phosphodiester bond; in the second step, the 3' hydroxyl group of the 5' exon attacks the 3' splice site, resulting in the displacement of the lariat intron and the ligation of the exons (Kuhn and Kaufer, 2003).

translated.

In vitro studies demonstrated the mechanism by which introns are removed from the pre-mRNA. The process is based on two transesterification reactions: it is initiated by a nucleophilic attack of the 2' hydroxyl group of the adenosine at the branch site on the 3',5'-phosphodiester bond at the 5' splice site. Concomitant with cleavage, the 5' terminal guanosine of the intron is covalently attached to the branch site adenosine in a 2',5'-phosphodiester bond. The splicing intermediates thus formed are the cleaved-off 5' exon and the intron-3' exon intermediate in a branched circular, or lariat, configuration. The second step proceeds by attack of the 3' hydroxyl group of the 5' exon on the phosphodiester bond at the 3' splice site, resulting in the displacement of the lariat intron and the concurrent ligation of the exons.

The catalytic steps are preceded by the assembly of the pre-mRNA into a splicing-competent structure. The first specific stage in spliceosome assembly is the binding of U1 snRNP to the pre-mRNA, which occurs in an ATP- and temperature-independent fashion and involves base pairing of the 5' end of U1 snRNA to conserved sequences at the 5' splice site. U1 snRNP binding is essential for the subsequent binding of U2 snRNP and for the commitment of the pre-mRNA to the splicing pathway.

Pre-splicing complex A is formed upon binding of U2 snRNP to the branch site. This step, as well as all subsequent stages of the splicing reaction, is ATP-dependent. The U4/U6 and U5 snRNPs interact with complex A in the form of a U4/U6.U5 tri-snRNP complex to generate splicing complex B, which is converted into the catalytically active complex C after a conformational rearrangement. One of the consequences of this structural change is the destabilization of the extensive base pairing interaction between U4 and U6 snRNAs. The transesterification reactions are initiated during or subsequent to the structural reorganization of the spliceosome. The liberated intron remains associated with U2, U5, and U6 snRNAs, whereas the spliced mRNA is released from the spliceosome.

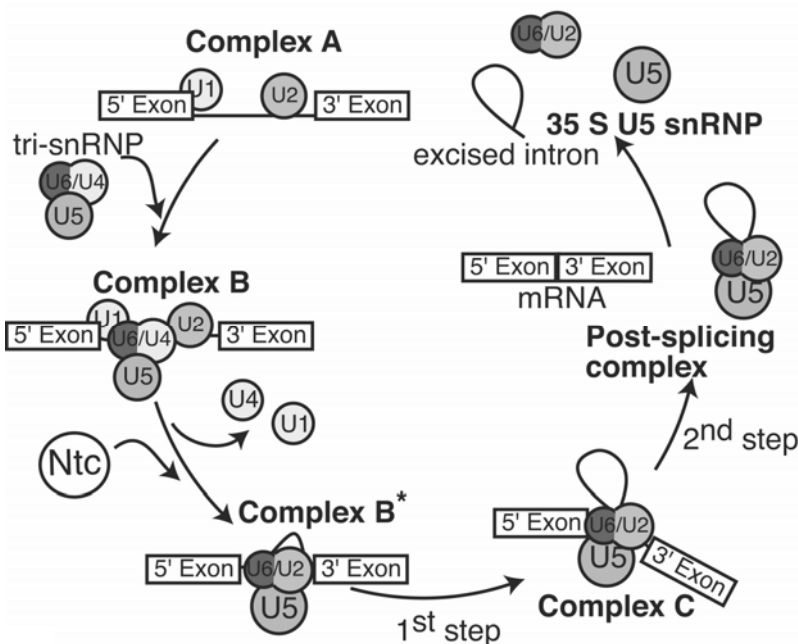


Figure 8. Dynamics of the spliceosome. Binding of the different snRNAs at the different complexes. The activated spliceosome complex is marked with an asterisk. The catalytic steps and the products are shown.

Spliceosome disassembly represents an active process that requires at least one protein factor and ATP. Subsequent to the release of the snRNPs, the intron lariat is

linearized by a debranching enzyme that specifically cleaves the 2',5'-phosphodiester bond at the branch site followed by degradation of the intron (Kramer, 1996).

Regulation of splicing

Splicing and/or alternative splicing has been shown to be regulated in multiple ways. An interplay of *cis*-acting sequences and *trans*-acting factors modulates splicing by the interaction of many complexes, proteins, DNA domains, etc.

A first degree of regulation is achieved by the 5' and 3' splice site sequences and the branch point. Specifically, splicing efficiency is dependent on their level of consensus with an optimal sequence. But there are other sequences in the pre-mRNA that can modulate the intron inclusion or exclusion.

- The Exonic Splicing Enhancers (ESEs) are motifs that promote the exclusion of the exon that contain the ESE.
- The Exonic Splicing Silencers have the opposite function of the ESEs.

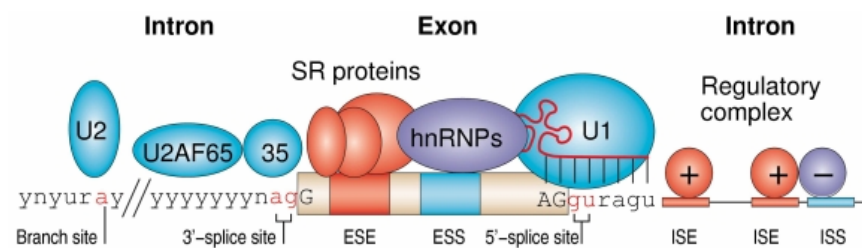


Figure 9. Splicing silencers and enhancers. Structure of the *cis* and *trans* elements in a gene affecting the splicing.

These motifs can also be found in the introns, so there are in a parallel way, Intronic Splicing Enhancers and Silencers. The *trans*-elements that participate in this regulation are mainly the SR family of proteins and the heterogeneous nuclear ribonucleoproteins (hnRNP).

The SR-proteins are highly conserved in eukaryotes, they are required for constitutive splicing and modulate alternative splicing. This family of proteins has one or two RNA-recognition motifs (RRM) and a Serine/Arginine rich domain (the SR domain) in the C-terminal region. There is a subtype of proteins called SR-related proteins (SRrps) which include, among others, the U1-70k protein and both subunits of

U2AF. The SR family and SR-related proteins function in the recognition of the ESEs leading to the activation of suboptimal adjacent 3' splice sites. The hnRNP represses splicing by ESSs binding. There are two hypothetical models to explain this mechanism, the “U2AF recruitment model”, where the SR proteins recruit the smaller subunit of the U2AF complex, U2AF³⁵, which will promote the binding of the U2snRNP to the adjacent intron promoting splicing or, the “inhibitor model”, where the inhibiting function of the hnRNPs over U2AF is inhibited at the same time by the SR proteins (Caceres and Kornblihtt, 2002; Cartegni et al., 2002).

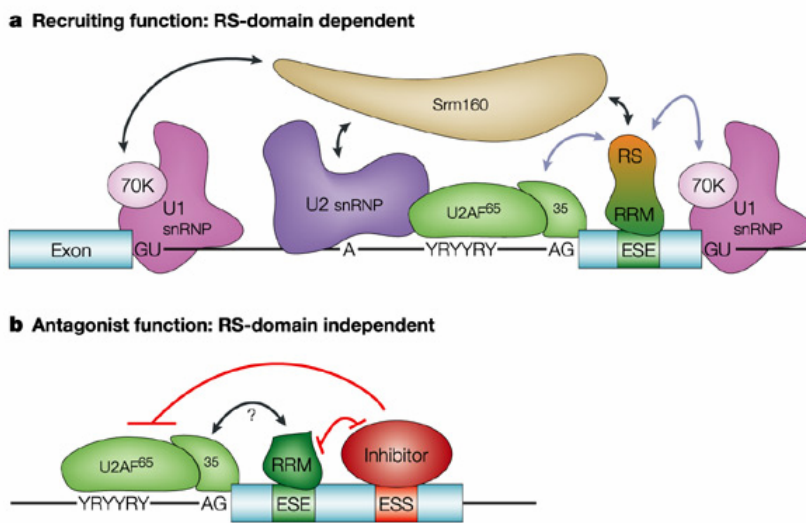


Figure 10. Function of the RS-domain-containing proteins. The recruiting (A) and the antagonist (B) function are shown. (Cartegni et al., 2002)

SR and hnRNP protein activities are modulated by phosphorylation, this way protein kinases related to splicing can have important roles in linking extracellular signalling to splicing regulation.

There are other groups of proteins that regulate splicing such as polypyrimidine tract proteins (PTB), the CELF protein family and other tissue-specific factors.

An important, although not very well known, mechanism to regulate splicing is through the RNAPolymerase II. RNAPol II transcribes mRNAs and some snRNAs. In the case of mRNAs, transcription is coordinated with other processes needed for the maturation of the mRNA. Capping at the 5' end minimizes degradation and permits its interaction with the ribosome, the 3' end polyadenylation increases the stability and the introns are removed. Transcription and all these processes are coordinated in time and space. RNAPol II is a multimeric protein, in which its largest subunit is characterized

by a C-terminal domain (CTD) composed by numerous repeats of the heptad consensus peptide YSPTSPS. The CTD and the phosphorylation of the Serines 2 and 5 mediate the recruitment of many proteins and complexes involved in this process and link the mRNA synthesis with the splicing machinery (McCracken et al., 1997). The phosphorylated form of CTD stimulates the early steps of spliceosome assembly while the hipophosphorylated form inhibits the formation of these complexes. The CTD is preferentially phosphorylated at Ser 5 when pol II is recruited at the promoter sites but becomes phosphorylated at Ser 2 when located at the coding region (Cho et al., 2001; O'Brien et al., 1994). This change of phosphorylation pattern might be relevant for the recruitment of splicing factors (Kim et al., 1997).

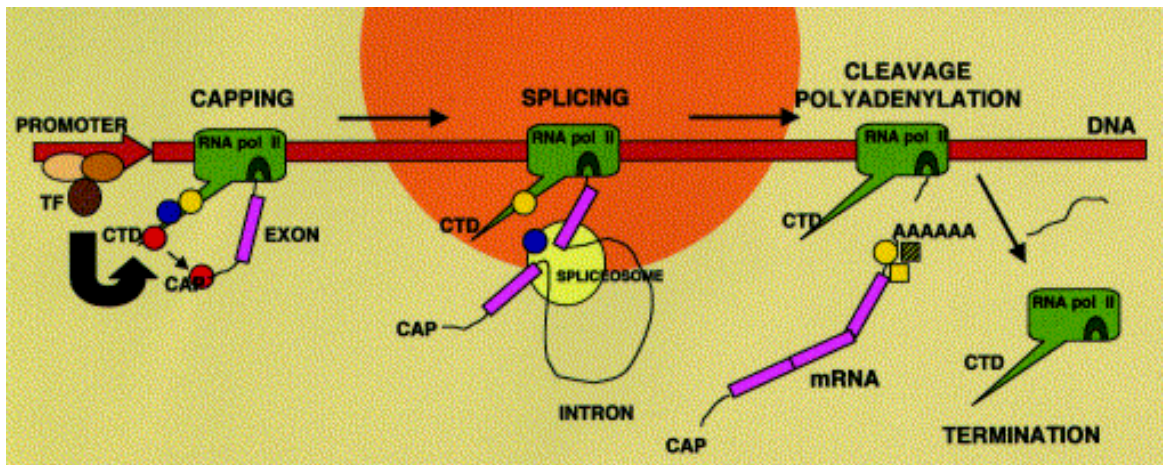


Figure 11. Involvement of RNA pol II in different steps of pre-mRNA processing. TF: transcription factors. Small circles represent proteins involved in pre-mRNA processing that interact with RNA pol II, participating in capping (red), splicing (blue) or cleavage/polyadenylation (yellow) mechanisms. Yellow squares represent factors involved in cleavage/polyadenylation which do not interact with RNA pol II (Cramer et al., 2001).

The promoter of any given gene, because it is responsible for the RNAPol II recruitment, is also supposed to have a paper in the coordination between transcription and splicing. That is to say, the structure of a promoter can affect alternative splicing of the gene. Moreover, since promoters are not modifiable elements, the only conceivable way by which promoters architecture could control splicing *in vivo* should be the differential occupation of promoters by transcription factors with different properties and/or functions (Cramer et al., 2001; Morillon et al., 2003).

Two alternative, but not exclusive, models that could explain the promoter control of splicing are:

- The promoter itself is responsible for recruiting splicing factors, such as SR proteins, to the site of transcription or the recruitment of factors that have a dual function in transcription and splicing. That is the case of the transcriptional activator of the human papillomavirus (Lai et al., 1999).
- The promoter mediates the recruitment of a slow or less processive RNAPol II (or internal pauses) that allows the preferential elimination of the upstream intron or the recruitment of a highly elongating RNAPol II (or the absence of internal pauses) which would favor the presentation of more than one intron to the splicing machinery (Caceres and Kornblihtt, 2002). That was the case of the model presented by Kadener et al in 2001 (Kadener et al., 2001).

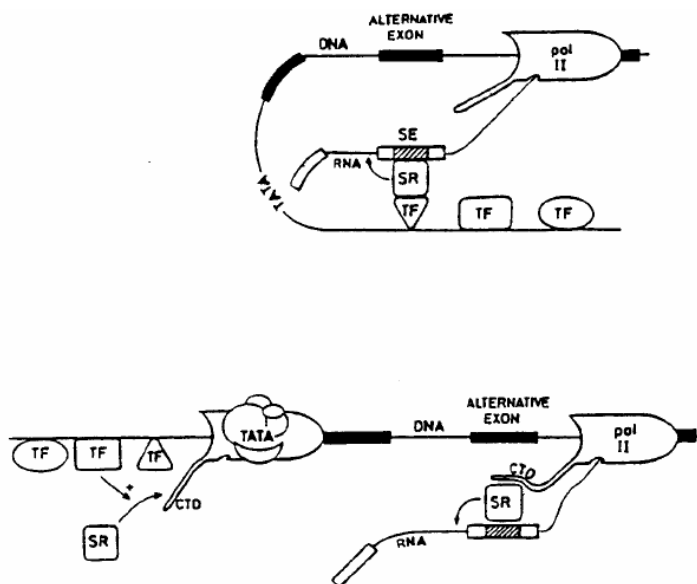


Figure 12. Two alternative models for putative interactions between SR proteins bound to an exon enhancer (SE, hatched box) with transcription proteins. (A) SR protein activity is modulated by interaction with a regulatory transcription factor (TF) positioned on its promoter site at the time splicing occurs. (B) Regulatory transcription factors interacting with the initiation complex promote loading of SR proteins (or other transcription factors) onto the moving RNA pol II complex (Cramer et al., 1997).

In any case, the difference between promoters is not due to its strength but due to its quality (Cramer et al., 1999).

Alternative splicing

Alternative splicing is the RNA splicing variation mechanism in which the exons of the pre-mRNA are separated and reconnected so as to produce alternative mature mRNAs. In this way, alternative splicing uses genetic expression to facilitate the synthesis of a greater variety of proteins.

There are four known modes of alternative splicing:

- Alternative selection of promoters: this is the only method of splicing which can produce an alternative N-terminus domain in proteins. In this case, different sets of promoters can be spliced with certain sets of other exons.
- Alternative selection of cleavage/polyadenylation sites: this is the only method of splicing which can produce an alternative C-terminus domain in proteins. In this case, different sets of polyadenylation sites can be spliced with the other exons.
- Intron retaining mode: in this case, instead of splicing out an intron, the intron is retained in the mRNA transcript. However, the intron must be properly encoding for aminoacids in frame, otherwise a stop codon or a shift in the reading frame will cause the protein to be non-functional.
- Exon cassette mode: in this case, certain exons are spliced out to alter the sequence of amino acids in the expressed protein.

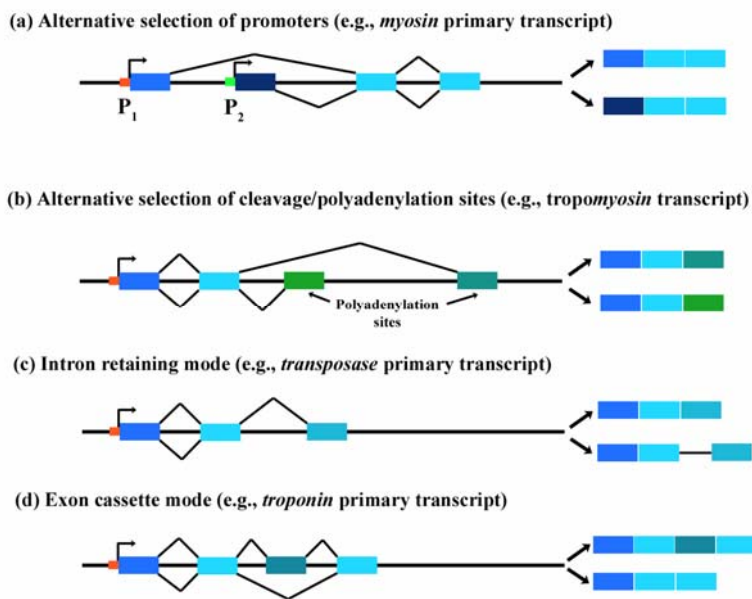


Figure 13. Representation of the four known models of alternative splicing.

Inter-specific splicing

Splicing can be found in species of all the kingdoms: There are four major classes of introns: self-splicing group I and group II introns, tRNA and/or archaeal introns, and spliceosomal introns.

Group I introns are found in bacterial and organellar genomes, and in the ribosomal RNAs (rRNAs) in the nucleus of protists and fungi.

Group II introns, less widespread than group I introns, are present in bacteria and organellar genomes of fungi, plants, and protists; in archaea they were identified only within the genus *Methanosarcina*, which illustrates probable horizontal transfers from bacteria; group II introns are absent from nuclear genomes and have never been observed in animal mitochondria.

Group I and group II introns fold into distinctive ribozymic structures essential for catalyzing their own excision.

tRNA introns are found in the nuclear tRNA genes of eukaryotes; introns are also found in archaeal mRNA, rRNA, and tRNA. These introns are enzymatically removed by a cut-and-rejoin mechanism through a pathway completely different from that of spliceosomal introns.

Spliceosomal introns are confined to the nuclear genome of all eukaryotes, and—except for the signals of splicing—evolve under selective regimes that do not impose a high degree of primary sequence definition.

Two types of spliceosomal introns have been described. Canonical introns start with GU and end with AG, and are spliced by the major, U2-dependent spliceosome, and the spliceosome is the multi-protein/RNA complex that catalyses the splicing of nuclear mRNA precursors in eukaryotes. Canonical introns account for the vast majority (>99.5%) of the spliceosomal introns in every species examined. The tiny minority remaining are noncanonical introns, which usually have AU-AC at their termini, and are spliced by the minor, U12-dependent spliceosome. U12-type introns are found in plants, insects, and vertebrates.

Taking into account the Spliceosomal canonical splicing, the function and components of this machinery appear to be highly conserved between organisms. Regarding yeasts, many distinct differences between budding and fission yeast have

been found, emphasizing their evolutionary distance. Most interestingly, fission yeast appears to reflect the more conservative evolutionary development regarding pre-mRNA splicing. Many spliceosomal components, including the five small nuclear RNAs, which most likely form the catalytic core of the spliceosome, show a higher degree of similarity with the components of the splicing machinery found in mammals. In addition, several regulatory components of the spliceosome detected in mammals are absent in *S. cerevisiae*, but present in *S. pombe* (Kuhn and Kaufer, 2003).

In mammals, introns usually comprise more than 95% of a pre-mRNA, they are usually larger than exons with a length of up to several kilobases long. In contrast, most fission yeast introns are only 40-75 nucleotides long. In humans, at least 70-80% of the genes have introns (Ben-Dov et al., 2008) and in *S. pombe* 43%, while in *S. cerevisiae* it is only about 5% (Kaufer and Potashkin, 2000).

Regarding the sequences defining an intron, both mammals and fission yeast introns have a very similar degenerated branchpoint consensus sequence, CURAY (R: purine; Y: pyrimidine) while in budding yeast is totally different (UACUAAC). The pyrimidine rich region between the branch point and the 3' splice site is very similar between mammals and *S. pombe* although the latter is considerably shorter (Prabhala et al., 1992). Regarding the proteins involved in pre-mRNA processing humans and fission yeast have also many similarities.

Importance of studying splicing

In humans, nearly 60% of the genes seem to be alternatively spliced and it is estimated that about 15% of all the mutations that cause genetic diseases lead to a failure in the correct splicing of the corresponding gene (Philips and Cooper, 2000). This estimate is only based on classical consensus sequences and does not consider other splicing motifs such as ESEs, so the real proportion may be closer to 50%. Most single nucleotide mutations that alter constitutive splicing generate aberrant transcripts as a result of exon skipping or the use of cryptic splice sites. In many cases these result in loss of expression through generation of isoforms that lose the open reading frame and are targeted for nonsense mediated decay.

Defects in splicing are nowadays being implicated in many diseases, such as growth hormone deficiency, Frasier syndrome, Parkinson's disease, cystic fibrosis,

retinitis pigmentosa, spinal muscular atrophy, myotonic dystrophy and a large number of cancers. Splicing defects can arise as a result of sequence mutations that disrupt splicing elements, mutations in splicing factors or through the unbalanced expression or normal alternative splice variants (Pettigrew and Brown, 2008).

A good model to study splicing at the molecular level is *S. pombe* because of its easy genetic manipulation and maintainance in the laboratory and because the similarity of the human machinery. The feasibility of using *S. pombe* in studying pre-mRNA splicing became clear when it was shown that the small T-antigen intron of the SV40 early region is properly spliced in fission yeast (Kaufer et al., 1985).

Pre-mRNA splicing in *Schizosaccharomyces pombe*

As explained before, pre-mRNA splicing is performed by the Spliceosome. In *S. pombe*, it consists of five small nuclear RNAs (snRNAs) called U1, U2, U4, U5 and U6 and more than 80 proteins (Kuhn et al. 2003). Regarding the proteins involved in pre-mRNA processing, at least 20 splicing factors have been functionally identified in fission yeast, genetically and/or using a yeast two-hybrid screen with Prp2 as a bait. Some of the most important ones are:

prp1 is an essential gene that encodes a protein with 19 consecutive tetracopeptide motifs (Urushiyama et al., 1997). Prp1 is a physiological substrate of Prp4 kinase *in vivo* (Schwelnus et al., 2001). This interaction seems to be evolutionary conserved because the human homologues also interact *in vitro* and *in vivo* (Dellaire et al., 2002). Biochemical studies of the human homologue of Prp1 indicated that it might be a bridge between the U5 and the U4/U6 snRNPs in the U4/U6·U5 tri-snRNP (Makarova et al., 2002).

prp2 (U2AF⁵⁹) encodes the counterpart of the large subunit of mammalian U2 auxiliary factor (U2AF⁶⁵). While in fission yeast we can find the homologue of the mammalian small subunit U2AF³⁵, called U2AF²⁵, no homologue for this protein can be found in budding yeast by database analysis. The U2AF complex is involved in the recognition of the 3' splice site. U2AF⁶⁵ binds to the polypyrimidine tract facilitating

the interaction of the U2 snRNP with the branchpoint region (Zamore and Green, 1989). Bbp1 interacts with both subunits and its is necessary for the spliceosome formation (Huang et al., 2002).

prp4 encodes the first protein kinase described to be involved in pre-mRNA splicing. There are Prp4 homologues in many organisms. In general, this protein has an N-terminal domain characterized by containing Arginine/Serine rich domains, typically found in splicing regulators (SR proteins), but in Prp4 this domain is missing. Although genetic interactions were described with five splicing factors (Cdc5, Prp1, Prp5, Prp10 and Rnps1), Prp4 kinase appears not to be associated with any of the spliceosomal snRNPs.

Prp5 in a 40S complex which also contains the U2, U5 and U6 RNAs (McDonald et al., 1999). Prp5 consists of seven WD domains.

prp8/cdc28 is an RNA-dependent ATPase. The *cdc28* mutant produces elongated cells blocked in G2 but its role in cell cycle and splicing is still unknown. The orthologue in *S. cerevisiae* (PRP2) is necessary just before or during the first transesterification reaction (Kim and Lin, 1993).

prp10 also shows functional interaction with *prp4* (Richert et al., 2002). This protein contains consensus HEAT repeats found in regulatory subunits of the phosphatase PP2A (Habara et al., 1998). The mammalian homologue, SF3b155/SAP155 is part of the SF3b complex, part of the U2 snRNP (Wang et al., 1998). SAP155 is localized in close proximity to both sites of the branchpoint region and stabilizes the binding of the U2 snRNP with the spliceosome (Gozani et al., 1998).

prp11 encodes for an ATP-dependent RNA helicase homologue to *S. cerevisiae* PRP5. It is necessary for pre-spliceosome assembly *in vitro*, and interacts with U2snRNA in the early steps of spliceosomal assembly.

prp12 encodes a protein with high similarity to the human splicing factor SF3b130 (Habara et al., 2001). SF3b130 is part of the SF3b complex, a highly stable

subcomplex of the U2snRNP. It is required to form a functional U2snRNP although the function of its different components is not well known.

prp31/spp13 is closely related to its human and *S. cerevisiae* homologues. Studies in these organisms showed that are important for tri-snRNP formation and spliceosome activation (Kuhn et al., 2002; Makarova et al., 2002; Weidenhammer et al., 1997).

cdc5 was first identified as being necessary for G2/M transition. It is also part of the complex containing the U2, U5 and U6 RNAs. It seems very likely that the 40S U2·U5·U6 complex represents a post-catalytic complex prior to the recycling of the individual snRNPs (Huang et al., 2002; McDonald et al., 1999).

U1-70k is a SR-related protein that binds to U1snRNP and participates in the regulation of the pre.mRNA processing by Exonic Splicing Enhancers and Inhibitors.

Uap2 was the second *U2AF* associated protein identified (McKinney et al., 1997). It is most similar to *S. cerevisiae* CUS2, which is associated with U2snRNA and is probably playing a role in the proper folding of this RNA (Yan et al., 1998). Uap2 is postulated to have a function mediating the U2AF-dependent association of the U2snRNP with the intron.

spp42 was identified by suppression of the temperature sensitivity of a *prp4-73* mutation. Its homologue in *S. cerevisiae* (PRP8) is part of the U5 snRNP, it is a highly conserved spliceosomal protein which plays a pivotal role right at the active center of the spliceosome. Spp42 human homologue (called U5-220kK/hPrp8) coordinates multiple RNA-rearranging activities during assembly and activation of the spliceosome (Kuhn et al., 2002).

rnps1 is synthetically lethal with *prp4-73* mutation, it was first identified in the mammalian system, called RNPS1, as a general activator of splicing (Mayeda et al., 1999). It was also shown that RNPS1 remains on the mature mRNA at the position of an exon-exon junction (Le Hir et al., 2000). This complex seems to be important for the

transport of the mRNA to the cytoplasm and also seems to have a function in differentiating premature and correct stop codons (Lykke-Andersen et al., 2001).

Rem1

Rem1 was previously found by our group while searching for other cyclins involved in the G1/S transition. The fact that the deletion of all the G1/S cyclins (Cig1, Cig2 and Puc1) does not avoid the DNA replication might mean that some other cyclins may be doing this function. This way *rem1* (SPBC16E9.17c) was found by sequence homology and cloned. Its characterization was published by Malapeira et al, 2005.

Rem1 (Required for Entry into Meiosis) is a B-type cyclin with high homology to other cyclins of *S. pombe*. *rem1* is composed by 2 exons separated by an intron of 80 bp. The first exon, which represents 1/3 of the protein has no significant homology with any other protein, except for the Destruction Box (DB) located in the N-terminal

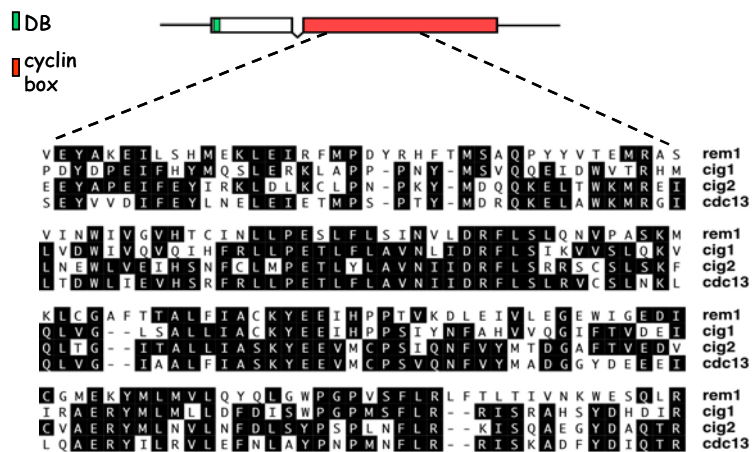


Figure 14. Alignment of the cyclin domain of *rem1* and three other B-type cyclins of *S. pombe*.

region, responsible of the degradation of the cyclin by the proteasome. The second exon encodes the cyclin domain. The intron contains a stop codon in frame, so to obtain the mature (full length) protein of Rem1 its mRNA has to be processed.

rem1 mRNA is nearly undetectable during vegetative growth. In fact, the only moment when *rem1* is highly transcribed is in meiosis I. The mRNA can be detected some time before meiosis I but it is not spliced until the onset of meiosis I, when the protein is expressed.

If the kinase activity of Rem1 is assayed (using Histone H1 as a substrate) during the progression of meiosis, we can observe that the kinase activity coincides with the protein expression. Concerning the regulation of Rem1, $\Delta mei4$ strains are blocked

before meiosis I, *rem1* mRNA splicing was not performed and *rem1* levels were decreased so Mei4 transcription factor appeared to be the main regulator of *rem1*, either directly or indirectly.

There were also some hints about the function of Rem1: the entrance in meiosis I was delayed 30 min in a *rem1* mutant strains and the levels of intragenic recombination were reduced while the levels of intergenic recombination were normal. It was shown that Rem1 has a redundant function with Cig2 cyclin. Strains deleted for *rem1* or *cig2* were able to complete meiosis while the double *rem1/cig2* mutant was blocked before finishing pre-meiotic S phase.

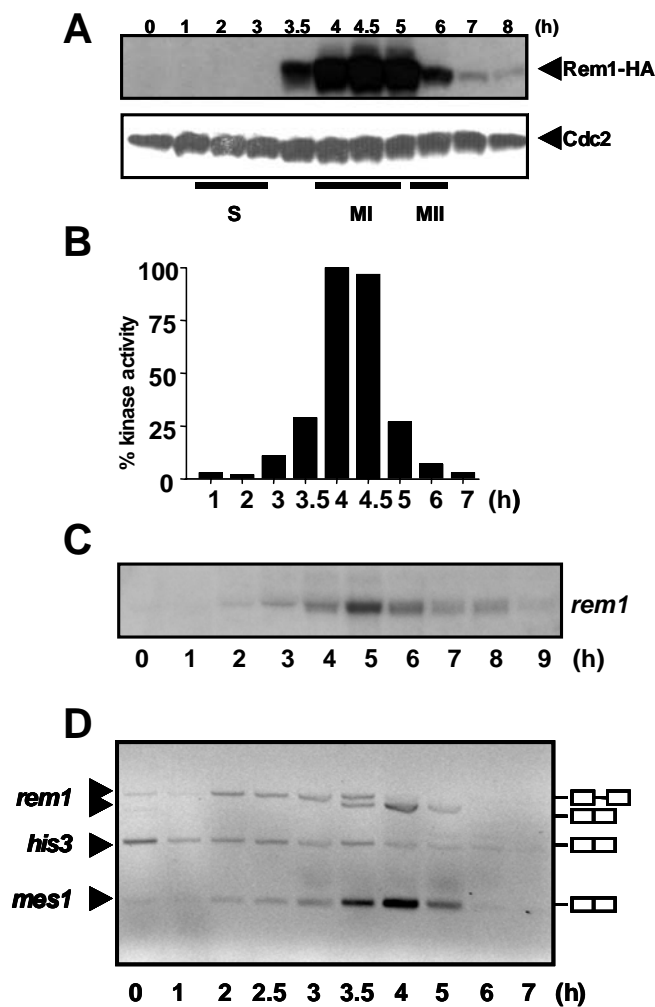


Figure 15. Characterization of Rem1.

(A) Western blot indicating Rem1 protein expression during meiosis, Cdc2 was used as a loading control. Hours at meiosis are indicated. (B) Kinase activity of immunoprecipitated Rem1 was assayed over H1 Histone with radiolabelled phosphate, detected by radiography and quantified. Hours at meiosis are indicated. (C) *rem1* northern blot during meiosis. A probe for the complete ORF was used. Hours at meiosis are indicated. (D) RNA from cells at different meiotic time points were harvested. Reverse transcriptase assay with specific oligos flanking *rem1* intron were used. Both processed and non-processed products are indicated on the left. Hours at meiosis are indicated.

OBJECTIVES

The main objective of this project was in the beginning the characterization of the meiosis-specific cyclin Rem1 in *Schizosaccharomyces pombe*. While advancing in the research the study became broader and the main objective change to understand the transcription and splicing regulation of a subset of genes expressed specifically in the meiotic differentiation.

The specific objectives were:

Understand regulation of *rem1* transcription and splicing

Determine the regions important for this regulation

Study the promoter and the *cis* elements responsible of this regulation

Search for transcription factors regulating *rem1*

Extend the regulation model of *rem1* to other meiotic intron-containing genes.

Characterization of the function of these regulators

Spatio-temporal localization in the gene

Their relationship with the RNA polymerase II

Interaction with the spliceosome

Interaction between them

Post-translational modifications of the factors.

Study Rem1 function and relate it to its regulation

RESULTS

rem1 regulation

Rem1 is regulated at different levels. *rem1* expression is at least regulated at the level of transcription, splicing and protein stability. This project has been focused mainly in the two former although I also will talk briefly about the last one.

Transcription and splicing of *rem1*

rem1 is only expressed during meiosis, with its transcription induced early in the moment of meiotic recombination and then highly induced at the onset of meiosis I. After meiosis II *rem1* mRNA levels decrease during the rest of the meiotic process.

At the level of splicing, *rem1* is only spliced at the onset of meiosis I, concomitantly to the high induction of its expression.

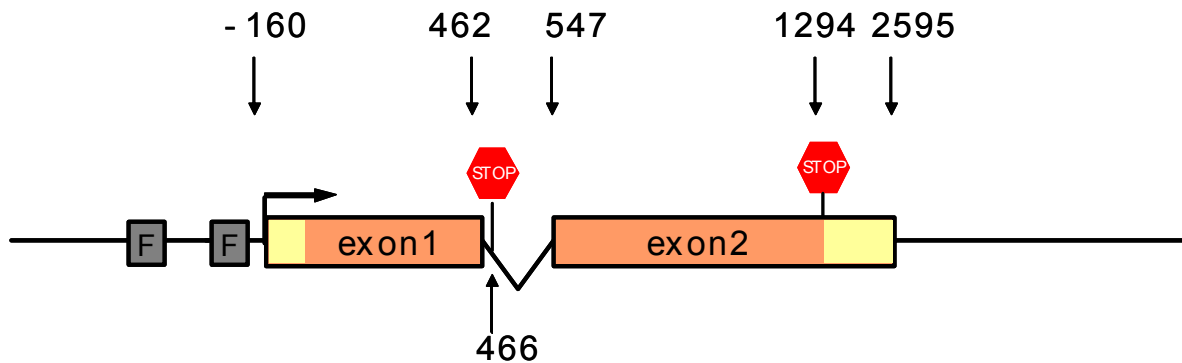


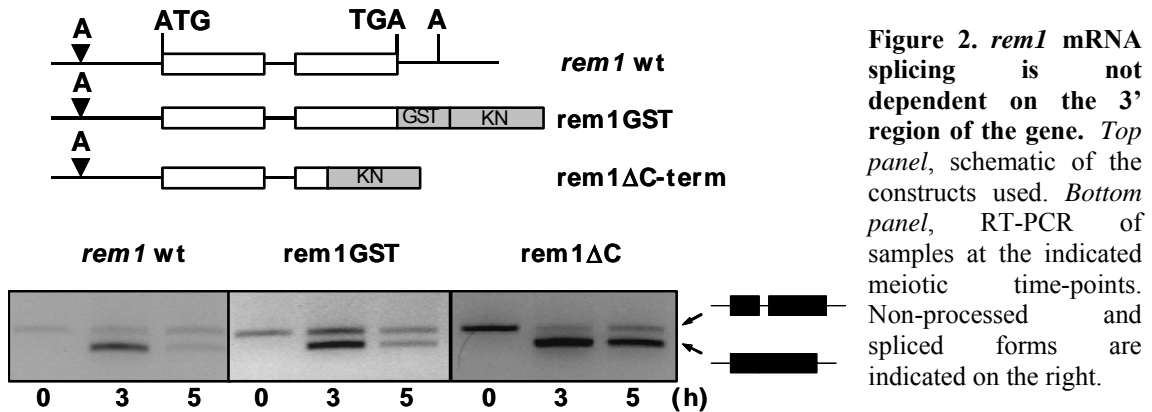
Figure 1. Gene structure of *rem1*. *rem1* ORF is represented by brown boxes (exon1 and exon2), the Untranslated Regions (UTR) are shown as yellow boxes. F stands by the FLEX boxes and the STOP signals indicate the position of the in frame STOP codons.

The transcriptional start point (tsp) of *rem1* was determined to be located at 162 bp upstream the first Methionine codon, so *rem1* has a 5' Untranslated Region (UTR) of 162 bp. *rem1* belongs to the family of intron-containing genes, its first exon has 462 bp and the second one 747 bp. Both are intervened by an intron of 85 bp. Downstream, the 3'UTR is about 88 bp (Malapeira et al., 2005).

To study the regulation of transcription and splicing we decided to analyse all these regions.

3' UnTranslated Region (UTR)

To analyse the paper of the 3'UTR in splicing we did modifications in the gene by substituting the 3'UTR and most of the second exon for the kannamycin cassette or adding a tag in the C-terminus to modify the region. We checked *rem1* intron retention and, as shown in figure 2, wt, *rem1*-GST and *rem1*ΔC652 mutant strains behave in a similar manner with some minimal differences at late time points. This could be indicative that the 3'UTR region does not have any paper in the splicing regulation, although it probably has a paper in the stabilization of the mRNA.



5' Splice site

The 5' splice site of *rem1* intron (GUAUGA) is similar to the consensus sequence for *S. pombe* introns (GUAAGU). Even though, we wanted to know if by changing the two main nucleotides of the sequence (GU → AC) we could detect any difference in the intron retention. The experiment shown in figure 3 is a synchronous time course of cells entering in meiosis, both wild type strain and the mutant (GU → AC) strain (NoS) were analysed by RT-PCR for intron retention. The NoS mutant was unable to perform physiological splicing and to process *rem1* mRNA. Even though, we could detect a band (Fig. 3, *) with an intermediate mobility between the unprocessed and the spliced form of *rem1*. This band was isolated, re-amplified and sequenced and

the result was an mRNA spliced from a cryptic 5' splice site in which the first exon was extended 26 bp (Fig. 3). This splicing product still contains the stop codon in frame and

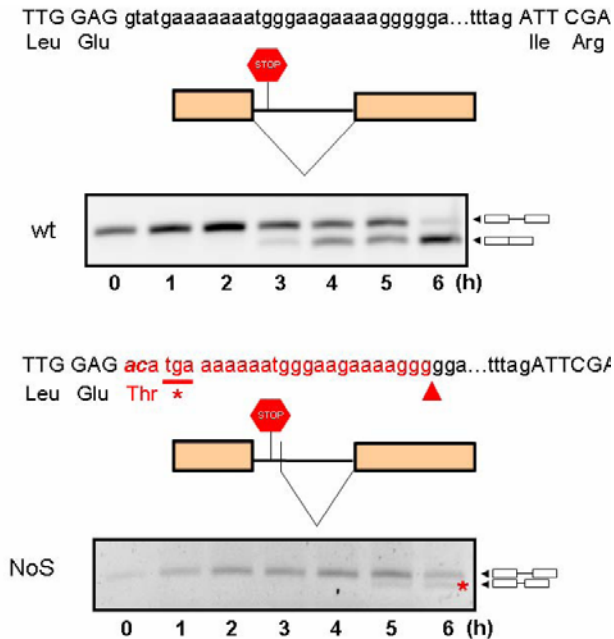


Figure 3. Analysis of the 5' Splice Site (SS). *Top panel*, RT-PCR of a wild type strain at the indicated meiotic time points. *Bottom panel*, RT-PCR of a mutant strain, carrying a GT→AC mutation in the 5'SS (NoS), at the indicated meiotic time points. In the NoS mutant there is some splicing (although very inefficient) from a cryptic 5' splice site (indicated by the red arrowhead) in which the first exon is extended 26 nucleotides (in red). This splicing product contains the Stop codon (tga) and renders a truncated protein that corresponds to the first exon. The band marked with an asterisk in the NoS mutant panel (which has an intermediate mobility between spliced and unspliced bands), was excised from the gel, reamplified with primers JA71 and JA174 and sequenced.

is unable to produce a mature full-length protein. The phenotype observed in this mutant is probably not due to a paper in the regulation of *rem1* splicing but a lack of operability of the splicing machinery. So, the 5' splice site of *rem1* doesn't have any role in splicing-specific regulation of *rem1*.

Regulation by the same product

Another way of regulating the splicing is by the own protein encoded by the mRNA as the case described by Macías *et al* (Macias *et al.*, 2008). To study this possibility, we mutated the ATG start codon to a TAG codon disabling the possibility to translate Rem1

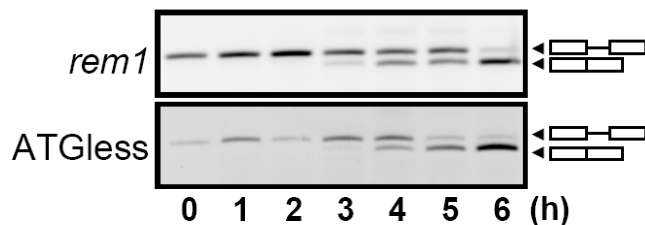


Figure 4. Regulation of the splicing by Rem1 protein. RT-PCR of a wild-type (*rem1*) or a mutant *rem1* lacking the initiation codon (ATG→TAG) (ATGless) were performed at the indicated meiotic time points.

(ATGless mutant). Both wild type and ATGless mutant strains were induced to enter synchronous meiosis and were analysed by RT-PCR with no differences in the splicing of the pre-mRNA (Fig. 4).

Promoter and 5' UTR

Both the promoter, mediating the binding of transcription or splicing factors, and the 5' untranslated region, by a specific spatial conformation, have been described for some genes as essential or important for the regulation of splicing (Shimoseki and Shimoda, 2001),(Cramer et al., 1997). We wanted to elucidate if it was the case of *rem1*.

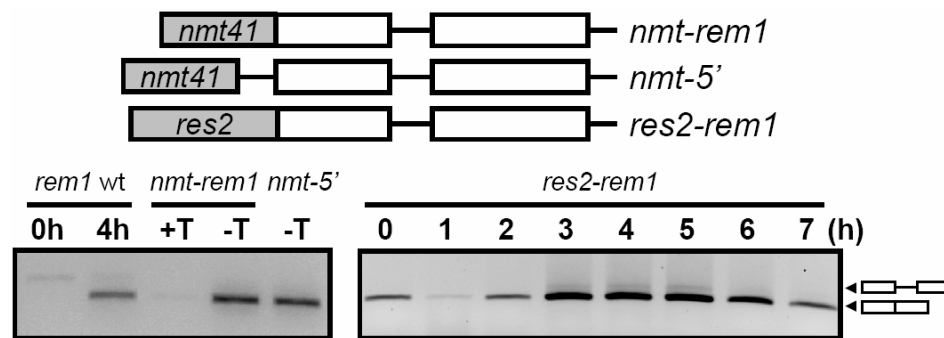


Figure 5. Analysis of the 5' region of *rem1* gene. *Top panel*, schematic of the constructs used: the promoter was replaced by (i) the inducible *nmt41* promoter with (*nmt-5'*) or without (*nmt-rem1*) the 5'UTR of *rem1* (shown on the diagram) and (ii) *res2* promoter. *Bottom panel*, *rem1* splicing in strains carrying the replacements described above. RNA was isolated from meiotic wild-type cells as a control (*rem1* wt; 0 and 4 hours into meiosis), from cultures vegetatively growing in the presence (+T, promoter OFF) or absence (-T, promoter ON) of thiamine (*left panel*), or from meiotic cells carrying a *res2-rem1* chimaera (*right panel*).

Thus, we analysed intron retention of different chimaeras of *rem1*. First of all we studied the behaviour of *rem1* under the control of the *nmt41* inducible promoter. As shown in figure 4 (*left panel*), when the *nmt41* promoter is active all the transcribed mRNA is spliced, independently of the presence or absence of the 5'UTR. Since the endogenous *nmt1* gene has no introns and maybe the high levels of transcription could lead to higher splicing efficiency we decided to study the effect of a weak promoter in similar conditions as the ones suffered by *rem1*: meiotic progression. We fused *res2*

promoter to *rem1* ORF and induced synchronous meiosis (Fig. 4, right panel). Surprisingly, we could detect constitutive splicing of *rem1* mRNA, even in early time points of meiosis. This result was pinpointing the possibility that the regulation of *rem1* mRNA splicing at early meiosis was based in a negative splicing factor. This factor would avoid *rem1* mRNA processing until meiosis I.

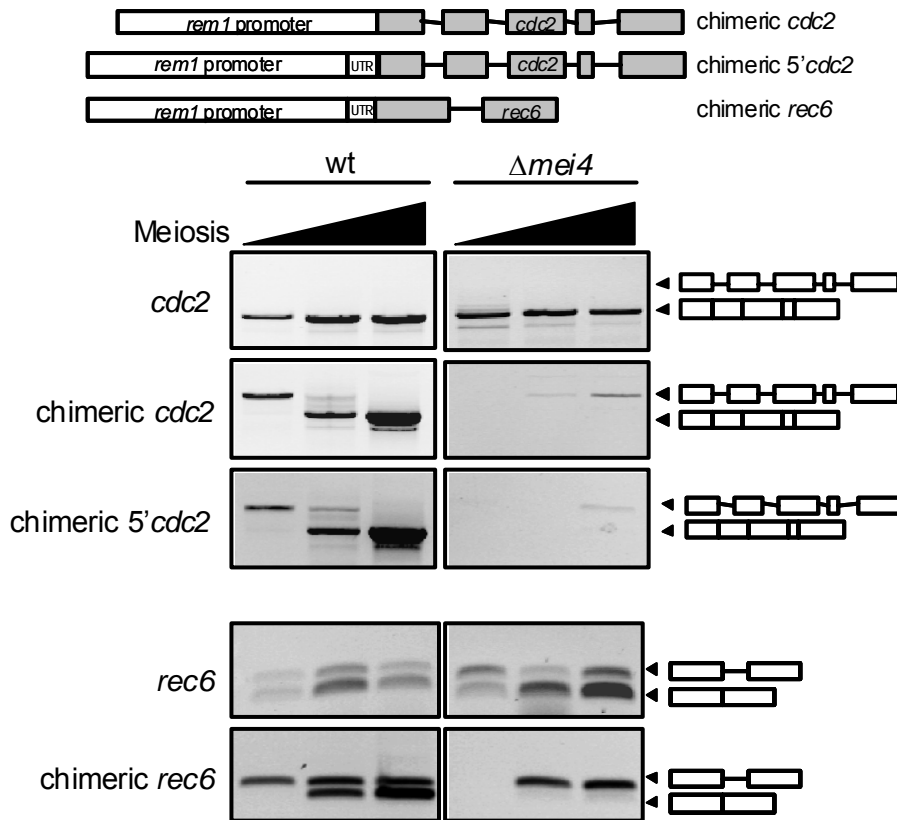


Figure 6. *rem1* promoter controls other genes splicing. *Top pannel*, schematic of the constructs used: the ORF of *rem1* was replaced by the *cdc2* ORF either (i) without (chimeric *cdc2*) or (ii) with (chimeric 5' *cdc2*) the 5'UTR of *rem1* and (iii) replaced by *rec6* ORF. *Bottom pannel*, RT-PCR analysis of the endogenous genes and the quimeras described above. RNA was purified from cells at 0, 3 and 5 hours of meiosis.

To fully ascertain this possibility, we proceeded with a reverse strategy. We fused the promoter of *rem1* to different reporter genes, like the ubiquitous *cdc2* (Fig. 6 and 7, wt panels) or the meiosis-specific *rec6* (Fig. 6, wt panels). Fission yeast cells were transformed with the different chimaeras and were induced to enter into synchronous meiosis. Splicing of the different chimaeras as well as the endogenous genes was followed by RT-PCR. As shown in Figures 6 and 7, *rem1* promoter is

sufficient to promote intron retention during vegetative growth and early stages of meiosis and can control the splicing of genes placed under its control. Furthermore, this regulation is dependent on *mei4*, directly or indirectly (Figure 6 and 7, $\Delta mei4$ panels). We can also conclude from these experiments that the 5'UTR does not affect the regulation of *rem1* splicing (Fig. 6).

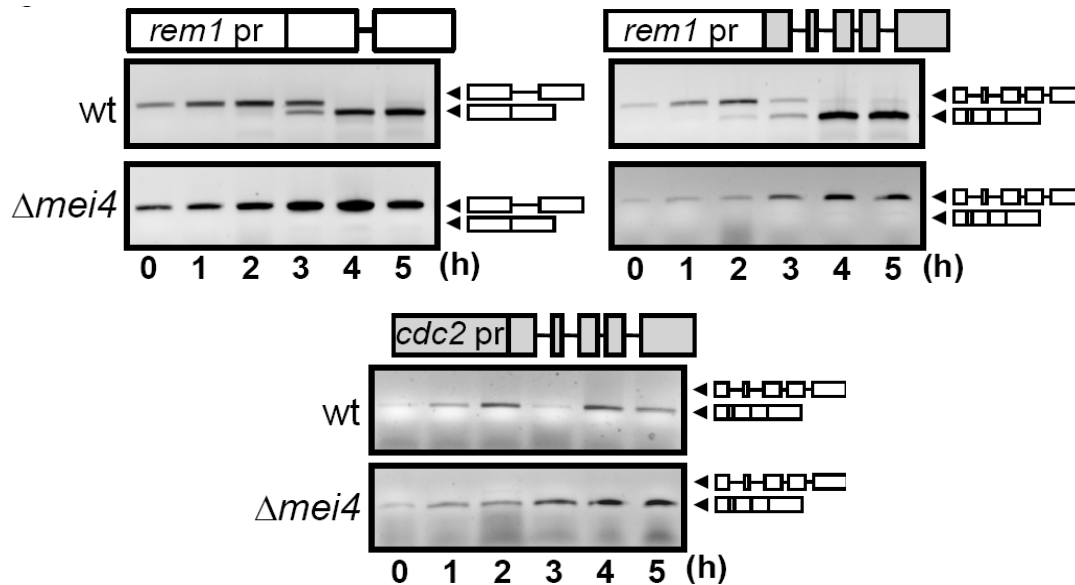


Figure 7. *rem1* promoter controls splicing. RNA was isolated from wild type (wt) or $\Delta mei4$ cells progressing into meiosis and *rem1* (top left panel) *cdc2* (bottom panel) or *rem1:cdc2* chimera (upper right panel) and splicing was monitored by RT-PCR with specific primers. Hours of meiosis are indicated at the bottom.

Analysis of the promoter function

As previously shown, we have determined that the promoter was the main regulator of *rem1* mRNA splicing. In this section I am going to focus on the *cis* and *trans* elements of the promoter found to regulate *rem1*.

Cis elements

The promoter of *rem1* contains two putative FLEX boxes (Fig. 1). These sequences are described to bind forkhead transcription factors *in vivo* (Horie et al., 1998). Since Mei4 is known to induce transcription and splicing of *rem1* (Malapeira et al., 2005), we decided to mutate these FLEX boxes and study this modifications at the level of transcription and splicing. The mutations performed consisted in the complete deletion of FLEX1 (GTAAACAAA), point mutations of FLEX2 (GTAAACA →

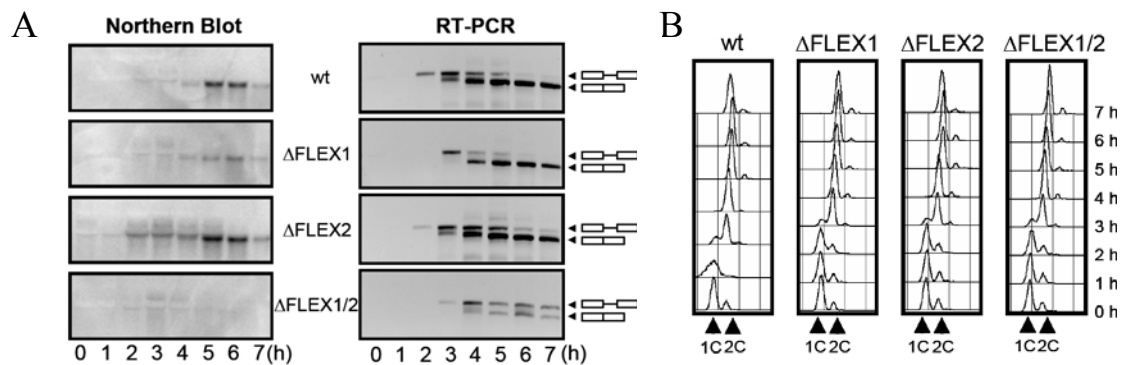


Figure 8. Mutations at the FLEX boxes alter transcription and splicing of *rem1*. There are two putative FLEX boxes in the *rem1* promoter as shown in Figure 1. A, Transcription (Northern Blot) and splicing (RT-PCR) of *rem1* was analysed in a wild-type (wt), single FLEX box mutant (Δ FLEX1 and Δ FLEX 2) or double FLEX box mutant (Δ FLEX1/2) strains as they progressed into meiosis. B, synchronicity of meiosis was assessed by flow cytometry. Time in hours after the culture was shifted to 35.5°C is indicated on the right.

CTAATGA) or both of them. The double deletion was not used because it would lead to a destructured promoter with no transcription (data not shown). Strains with these mutations were induced to enter meiosis synchronously and *rem1* mRNA was analysed by Northern blot and by RT-PCR. In a Δ FLEX1 mutant the levels of mRNA are lower than the wild type while in a FLEX2 mutation *rem1* seems to be expressed earlier, with increased basal levels. In the double mutant the transcription is nearly abolished (Fig. 8, A, Northern Blot). Concerning splicing, both of the single mutants seem to have not significant differences with the wild type while the double mutant has impaired splicing with only residual processing of the pre-mRNA (Fig. 8, A, RT-PCR). This experiment indicates that the most important elements of the promoters regulating transcription and splicing of *rem1* are the FLEX boxes. Even though, we cannot discard additional elements co-regulating or modulating the expression of *rem1*.

Trans elements

a) Forkhead transcription factors

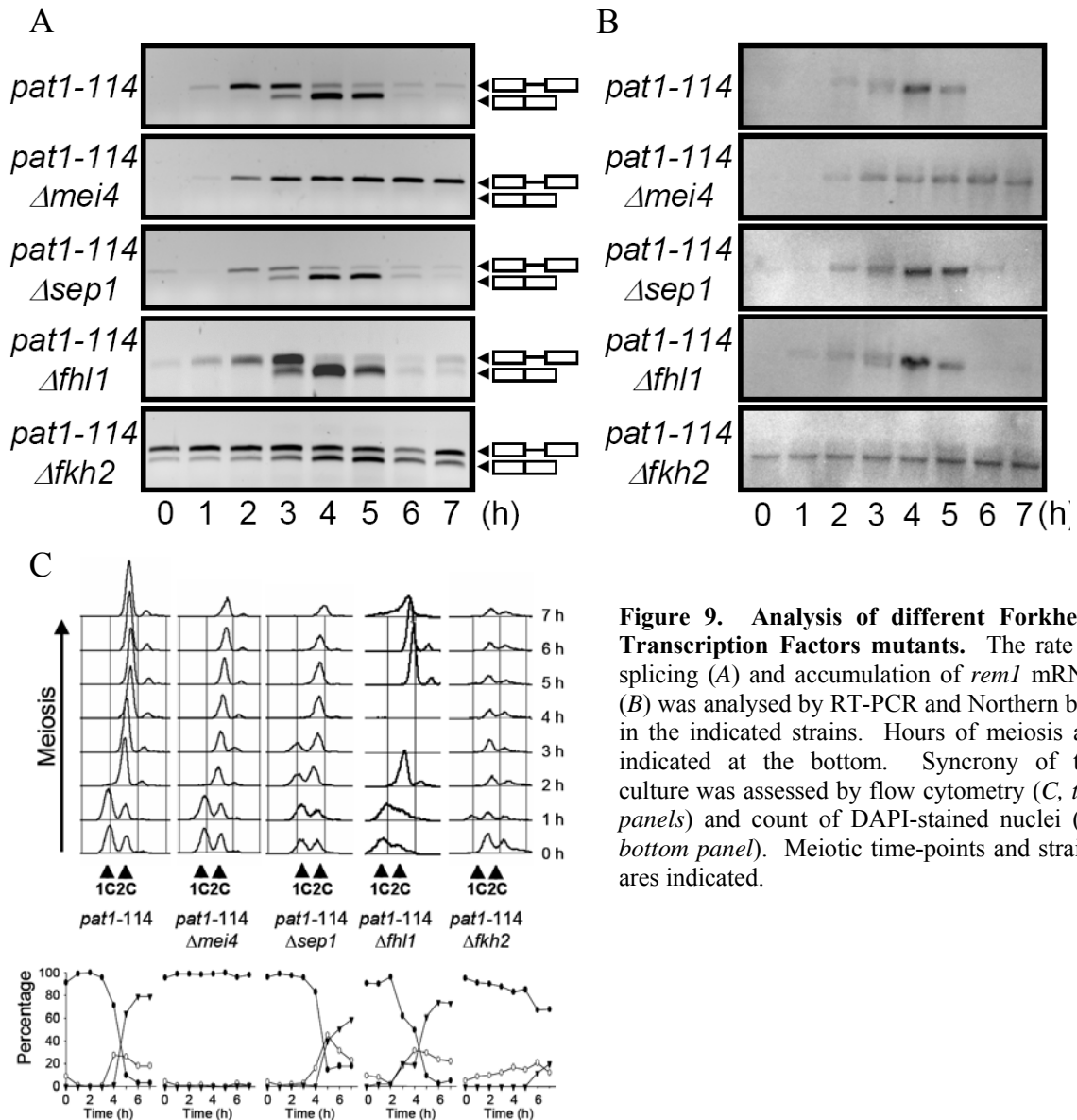


Figure 9. Analysis of different Forkhead Transcription Factors mutants. The rate of splicing (A) and accumulation of *rem1* mRNA (B) was analysed by RT-PCR and Northern blot in the indicated strains. Hours of meiosis are indicated at the bottom. Synchrony of the culture was assessed by flow cytometry (C, top panels) and count of DAPI-stained nuclei (C, bottom panel). Meiotic time-points and strains are indicated.

As explained before, FLEX boxes are the *cis* elements recognized by the forkhead family of transcription factors. We have observed that *rem1* expression is regulated mainly by the FLEX boxes (Fig. 8) and that *rem1* regulation is based on a two-component factor (Fig. 5, 6 and 7), negative in early meiosis and positive (Mei4, Figure 6 and 7 and (Malapeira et al., 2005)) in Meiosis I. To search the negative

regulator, we studied the winged-helix forkhead transcription factors present in *S. pombe*. In fission yeast, there are four genes coding for forkhead proteins: *sep1*, *fh11*, *mei4* and *fkh2*.

We have characterized the effect of the other forkhead transcription factors on transcription and splicing of *rem1* during meiosis (Fig. 9). While deletion of *sep1* and *fh11* had no effect neither in transcription nor in splicing regulation, cells without Fkh2 showed constitutive splicing, during vegetative growth as well as during early time points of meiosis (bottom panels of Fig. 9), pointing to a direct role of Fkh2 in the regulation of *rem1* splicing.

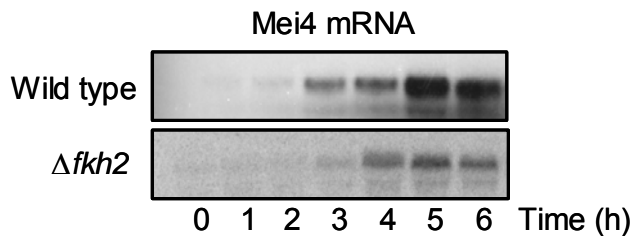


Figure 10. *mei4* expression in a $\Delta fkh2$ strain. Northern blot of wild type and $\Delta fkh2$ strains at the indicated meiotic time-points.

To discard the possibility that the constitutive pattern of splicing of *rem1* mRNA in a $\Delta fkh2$ strain was due to the ectopic presence of Mei4, we determined the levels of *mei4* mRNA by northern blot in $\Delta fkh2$ cells during meiosis. As shown in Figure 10, *mei4* mRNA behaves similarly in a wild type and a $\Delta fkh2$ strain, being absent in early meiotic time points.

After determining that Fkh2 and Mei4 are the main regulators of *rem1*, we wanted to search if there was any residual regulation after deleting both factors. Surprisingly, in the double mutant $\Delta fkh2\Delta mei4$, although we expected the same behaviour than in a $\Delta fkh2$ strain, *rem1* mRNA was spliced in early meiosis but the efficiency of splicing instead of be maintained as the the $\Delta fkh2$ single mutant, was decreasing along meiosis (Fig. 11).

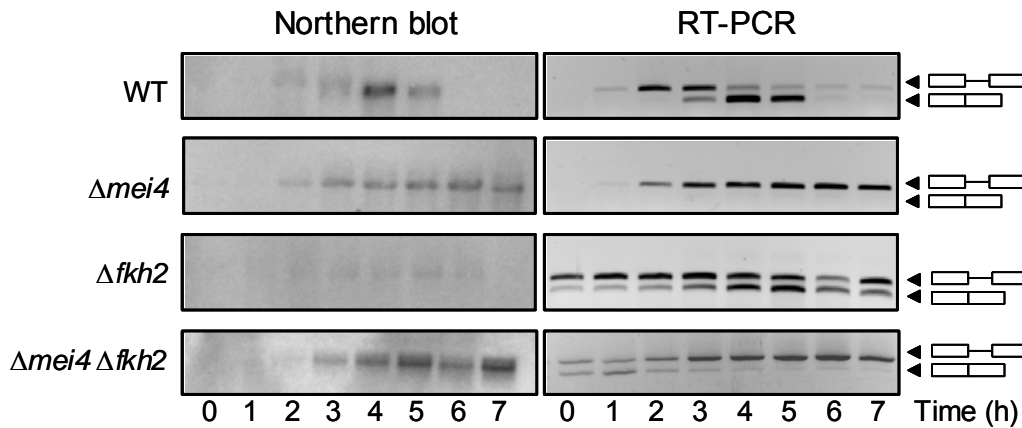


Figure 11. Genetic interaction between *mei4* and *kh2* in vivo. Northern blot (left panels) and RT-PCR (right panels) analysis of a wild type strain, single $\Delta mei4$ and $\Delta fkh2$ mutants and the double $\Delta mei4 \Delta fkh2$ mutant. Meiotic time-points are indicated.

To confirm the direct function of Mei4 in *rem1* promoter, *in vitro* experiments were performed. Binding of recombinant GST-Mei4 (DNA-binding domain) to a probe containing part of *rem1* promoter was studied by electrophoretic mobility shift assay (EMSA). We can observe a shift in the mobility of the probe in the presence of GST-Mei4 and a supershift if we add a specific antibody (Fig. 12, left panel), with similar

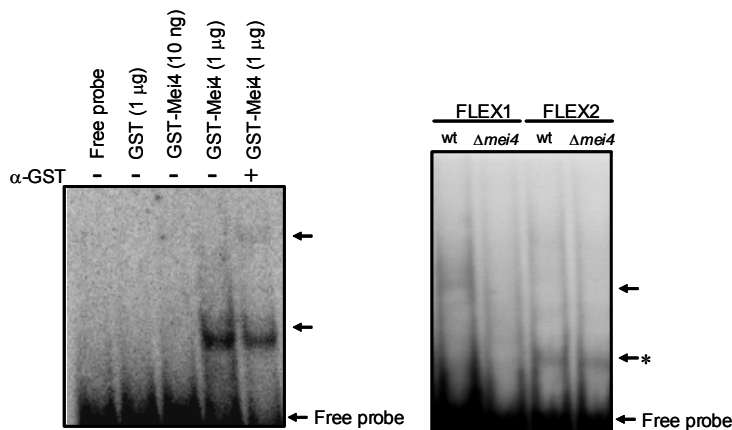


Figure 12. In vitro binding to the FLEX motifs. Left panel, electrophoretic mobility shift assay (EMSA) of recombinant GST-Mei4 with a radiolabelled probe containing FLEX1. Right panel, EMSA of meiotic extracts from wild type and $\Delta mei4$ strains with radiolabelled FLEX1 and FLEX2 probes. The asterisk indicates an unspecific band.

results to the ones obtained by C. Shimoda's group previously (Horie et al., 1998). A similar assay was performed with native extracts of wt and $\Delta mei4$ strains at 4 hours of meiosis (Fig. 12, right panel). We can observe a shift of the FLEX1 probe after adding wild type extracts but not with extracts from cells lacking *mei4*. For the FLEX2 probe, we observed no differences between both strains. We can observe an unspecific band

that can be Fkh2 but it has not been checked. These results indicate that Mei4 can bind directly to *rem1* promoter *in vitro* and gave us some hints of its binding *in vivo*.

To analyse the binding of Mei4 and Fkh2 to *rem1* *in vivo*, we applied the chromatin immunoprecipitation methodology (ChIP). We expressed tagged versions of Mei4 and Fkh2 on their own *loci* and performed synchronous meiosis. Cross-linked extracts were immunoprecipitated with specific antibodies and quantitative PCR reactions of different regions of *rem1* (Fig. 13 A) were performed. As shown in Figure 13 B, Fkh2 and Mei4 bind specifically to *rem1* promoter (compare Fig. 13, I and IV) in a time-specific manner. Concerning the timing, while Fkh2 increase its binding gradually along meiosis, Mei4 has a narrow window of interaction with the promoter, just at the onset of meiosis I. Interestingly, while Fkh2 seems to remain bound to the promoter, Mei4 has a more ubiquitous positioning along the entire gene, meaning that Mei4 is probably travelling with the RNAPolymerase (pol) II in the process of transcription.

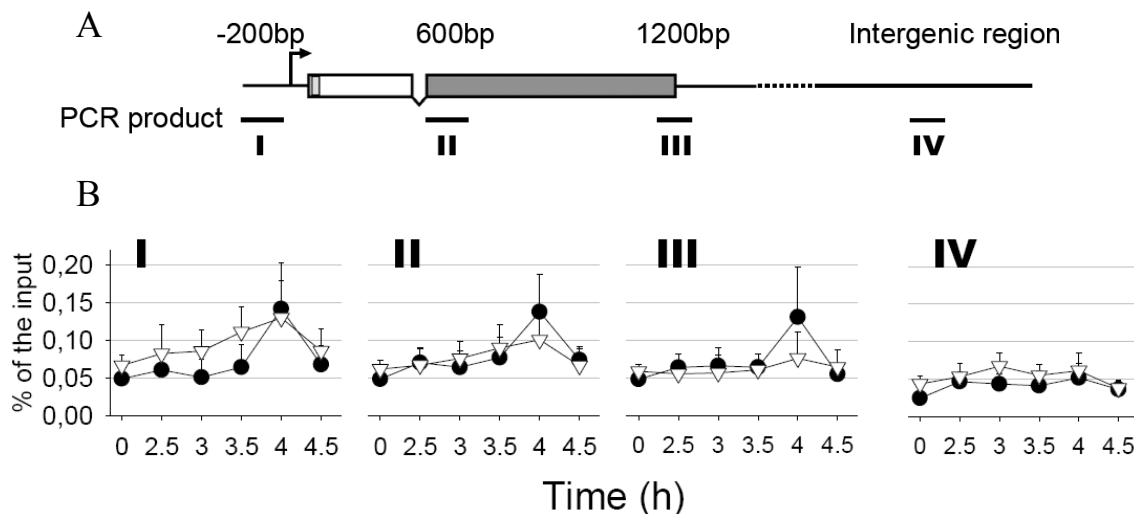


Figure 13. Mei4 and Fkh2 bind to *rem1* *in vivo*. (A) Schematic of *rem1* with the position of the quantitative PCR products (I to IV). (B) Recruitment of Mei4 and Fkh2 to *rem1* promoter and coding regions (I to III) during meiosis. Cross-linked cell extracts from a wild-type strain expressing chromosomal Mei4-13Myc (closed circles) or Fkh2-HA (open triangles) were immunoprecipitated, and assayed for the presence of *rem1*. Hours of meiosis are indicated at the bottom. Data represent the mean and standard error of the mean of three independent experiments.

b) RNAPolymerase II

To further investigate the relationship of the RNAPol II with Mei4, we performed ChIPs of the RNAPol II with different antibodies in wild type and $\Delta mei4$ strains. It has been described that the phosphorylation of the C-terminal domain (CTD)

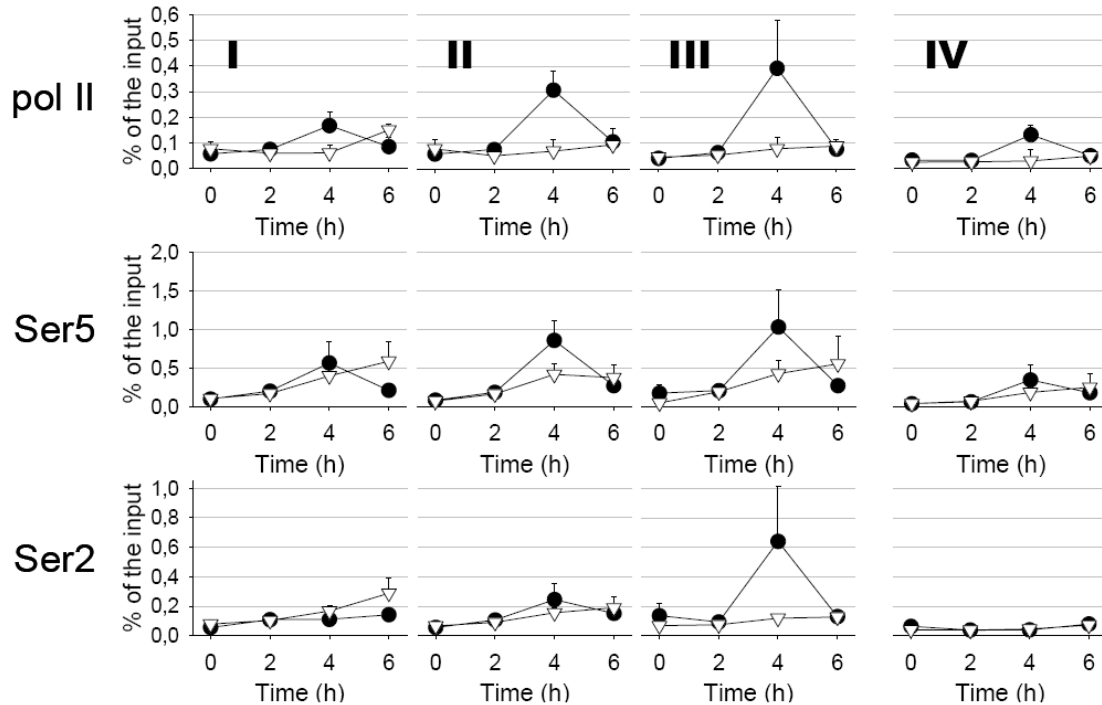


Figure 14. Recruitment of the RNAPolymerase II is *mei4*-dependent. Loading of RNAPol II during meiosis. Extracts from a wild type (closed circles) or $\Delta mei4$ (open triangles) strain were immunoprecipitated with antibodies against pol II (*top panels*), phosphor-Ser5 (*middle panels*) or phosphor-Ser2 (*bottom panels*), and assayed for the presence of different regions (shown in Figure 13,A) of *rem1*. Time in hours after meiosis was induced is indicated at the bottom. Data represent the mean and standard error of the mean of three independent experiments.

repeats (YSPTSPS) of the polymerase are indicative of the activity of the RNAPol II. (see Introduction).

Phosphorylation of Ser5 of the heptapeptide is indicative of the initiation of transcription, that is to say, the loading of the RNAPol II to the promoter and 5' end of genes and does not correlate with spliceosome assembly. On the other hand, phosphorylation of Ser2 of the heptapeptide is indicative of the active elongating form of the RNAPol II, the phosphorylation of this residue correlates with the recruitment of elongating factors and spliceosome (see Introduction). We have chromatin immunoprecipitated RNAPol II from extracts of wild type or $\Delta mei4$ meiotic progressing cells with an antibody recognizing total RNAPol II, specifically phosphoserine-5 and specifically phosphoserine-2. As shown in Figure 14, RNAPol II accumulates

specifically in *rem1* gene at 4 hours of meiosis in a *mei4*-dependent manner. For the phosphoserine-5 isoform of RNAPol II, we can also see differences between wild type and Δ *mei4* strains. In the Δ *mei4* mutant it can be loaded to some extent to the promoter and induce some transcription (see Figure 14, Ser5 panel,) but the accumulation in the promoter lead us to suspect there are some problems in the elongation. This unefficient loading fails to elongate in the mutant strain as confirmed in the ChIP for Ser2 (Figure 14, Ser2 panel). The problems of the RNAPol II to elongate at *rem1* gene in a Δ *mei4* strain could have consequences in coupling transcription to pre-mRNA processing.

c) Splicing Factors

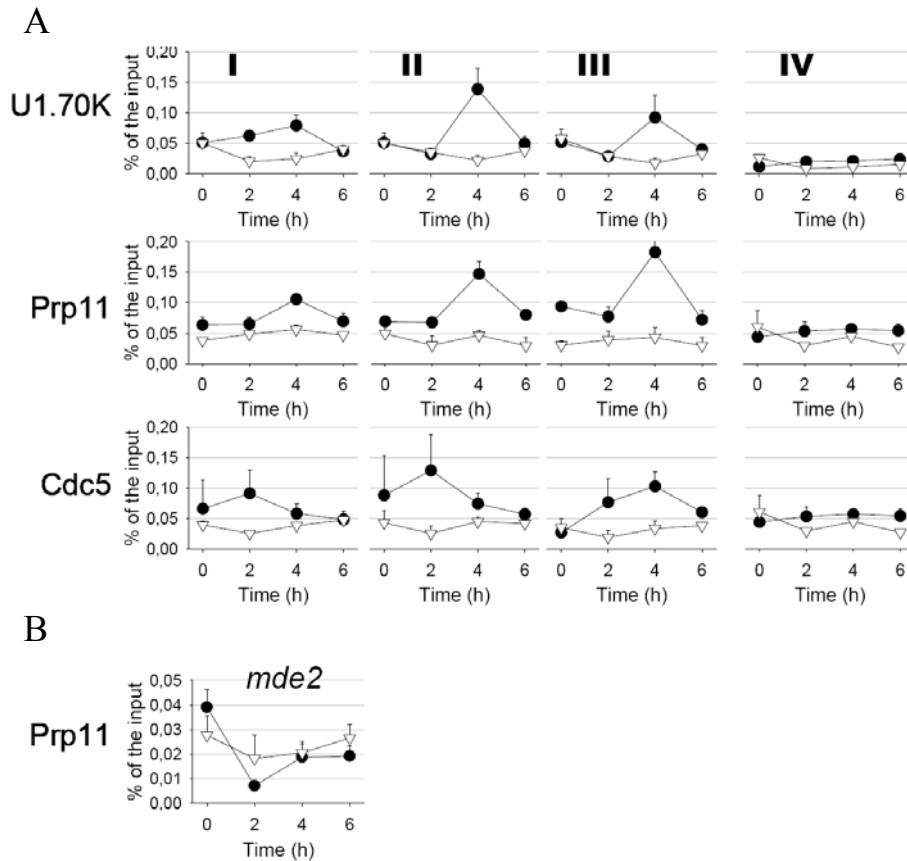


Figure 15. Loading of U1·70k, Prp11 and Cdc5 to *rem1* locus depends on Mei4. (A) Wild-type strains (closed circles) containing TAP tagged U1·70k, Prp11 or Cdc5 or their Δ *mei4* counterparts (open triangles) were induced into meiosis (time indicated at the bottom), and samples for ChIP assay were analysed for the presence of different regions (shown in Figure 13A) of *rem1* by real-time PCR. (B) Loading of Prp11-TAP on *mde2*, an intronless *mei4*-dependent gene. Closed circles correspond to wild-type strain and open triangles to Δ *mei4* strain. Note the different scale used compared to (A). Data represent the mean and standard error of the mean of at least 3 independent experiments.

We have determined that cells lacking Mei4 can not promote an efficient transcription of *rem1* because although RNAPol II can be recruited to the promoter, no efficient elongation can be achieved.

Regarding the splicing regulation, we wanted to know if Mei4 and/or Fkh2 can recruit the spliceosome to specific promoters. To answer this question we used the same approach as for the RNAPol II, we performed ChIPs of some components of the spliceosome. U1-70k, which is one of the 16 proteins of the U1 snRNP; Prp11, a complex B component, also known as PRP5 in other organisms; and Cdc5, a component of the nineteen complex (NTC) were tagged with TAP and chromatin immunoprecipitated from extracts of wild type or $\Delta mei4$ meiotic progressing cells. We could detect U1-70k, Prp11 and Cdc5 bound to the *rem1* ORF with an enrichment around the intron at 4 hours of meiosis in wild type cells. However, in the absence of Mei4, binding of U1-70k, Prp11 or Cdc5 was abolished (Figure 15 A).

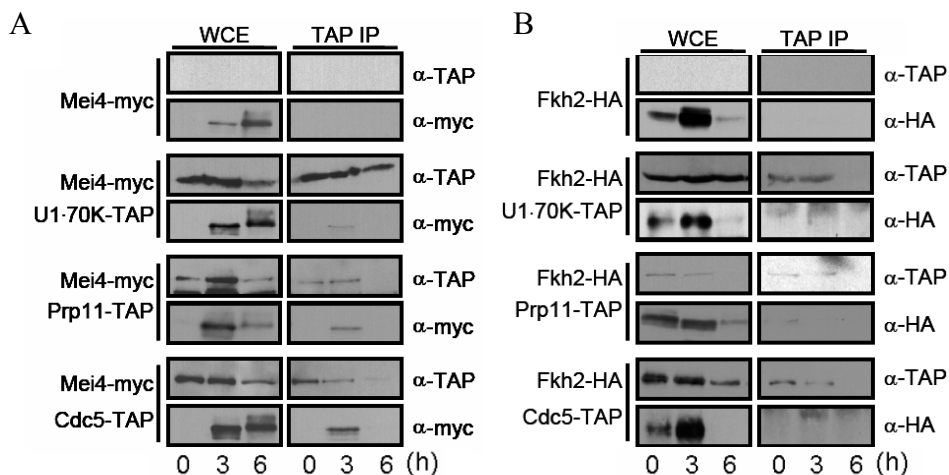


Figure 16. Mei4 coprecipitates with U1-70K, Prp11 and Cdc5. (A) Strains expressing Mei4-13Myc alone or co-expressing U1-70K-TAP, Prp11-TAP or Cdc5-TAP were induced into synchronous meiosis (time in hours is indicated at the bottom). (B) Strains expressing Fkh2-3HA alone or co-expressing U1-70K-TAP, Prp11-TAP or Cdc5-TAP were induced into synchronous meiosis (time in hours is indicated at the bottom). Extracts (8 mg) containing tagged U1-70K, Prp11 or Cdc5 were pulled down with IgGsepharose beads and Mei4 (A) or Fkh2 (B) were detected by western blotting using antibodies against Myc or HA, respectively (*right panels*, TAP IP). Expression of the different proteins was analysed on 50 μ g of total extracts (*left panels*, WCE).

Mei4, thus, is able to recruit RNAPol II and the splicing machinery to specific genes on the onset of meiosis I.

Furthermore, to check if Mei4 was able to recruit the splicing machinery constitutively or just on intron-containing genes, we performed ChIP of Prp11 and assayed the presence of the Mei4-dependent intron-lacking gene *mde2*. As shown in

figure 15 *B*, we cannot detect the presence of the spliceosome in *mde2* open reading frame, so Mei4 is only recruiting the spliceosome in intron-containing genes.

We have seen that Mei4 and the splicing machinery colocalize in *rem1* gene in meiosis. We wondered now if Mei4 could interact physically with the splicing machinery to recruit it. To determine this interaction, we performed Co-Immunoprecipitations (CoIPs) from native ^A extracts of cells progressing into meiosis. We pulled down U1-70k-TAP, Prp11-TAP and Cdc5-TAP and analysed the presence of Mei4 or Fkh2 in the immunoprecipitates. As shown in Figure 16, *A*, Mei4 can CoIP with the three members of the spliceosome studied, meaning the direct or indirect physical interaction between the transcription factor and the spliceosome. On the other hand, we can not detect at all Fkh2 in the immunoprecipitates of the spliceosome components (Figure 16, *B*).

Taking into account all these results, Mei4 seems to be promoting the transcription of *rem1* by recruitment of RNAPol II on the onset of Meiosis I, travelling with it and stabilizing the elongation through the gene. Mei4 also seems to recruit the spliceosome to *rem1* gene in mid-meiosis, promoting the processment of *rem1* pre-mRNA.

Protein stability

Rem1 is also regulated at the level of protein. As in mitosis, the APC complex is necessary to trigger the anaphase and separate the sister chromatides in the two new cells or spores. Mfr1 is a fizzy-related protein exclusively expressed in meiosis that activates the APC in meiosis II inducing the degradation of Cdc13 (Blanco et al., 2001).

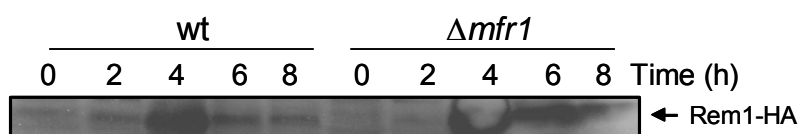


Figure 17. Rem1 degradation by Mfr1. Western blot of meiotic boiled extracts from wild-type and $\Delta mfr1$ strains carrying Rem1-HA.

We have shown that Mfr1 also regulates Rem1 stability since in a strain lacking *mfr1*, we can detect Rem1 for a longer time in meiosis (Figure 17). This indicates a usual cyclin-like regulation for Rem1. The other APC-related E3 ubiquitin ligases expressed in vegetative cycle are not able to degrade Rem1 (Hiro Yamano, personal communication), this could partially explain the toxicity presented in cells expressing ectopically full length Rem1.

Regulation of other meiotic intron-containing genes

We have determined that Fkh2 seems to avoid intron processing and keep *rem1* at low transcription levels at early meiosis and on the onset of meiosis I, Mei4 promotes high levels of transcription and splicing of the intron allowing the expression of the full length protein. To examine whether intron retention was a common feature of other meiosis-specific genes apart from *rem1*, we decided to use whole-genome microarray analysis to compare transcriptional profile and intron retention of wild-type and $\Delta mei4$ strains. These strains were induced to enter synchronous meiosis and cells were harvested at 0 and 4 hours after the temperature shift. RNA was isolated and sent to Dr. Chris Seidel's laboratory, at Stowers Institute (Kansas City). There, Madelaine Gogol, processed the samples and the microarray data was collected as described in Material and Methods. The microarray consisted of 8785 70-mer oligonucleotides representing 6918 exons, 1521 introns and 333 3' Untranslated Regions. Although the microarrays only included oligonucleotides for 31% of the total introns, the analysis showed that a set of genes presented intron retention in the absence of Mei4. First of all

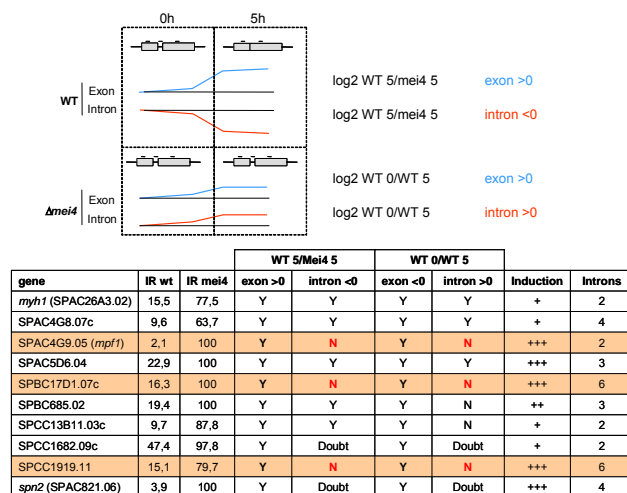
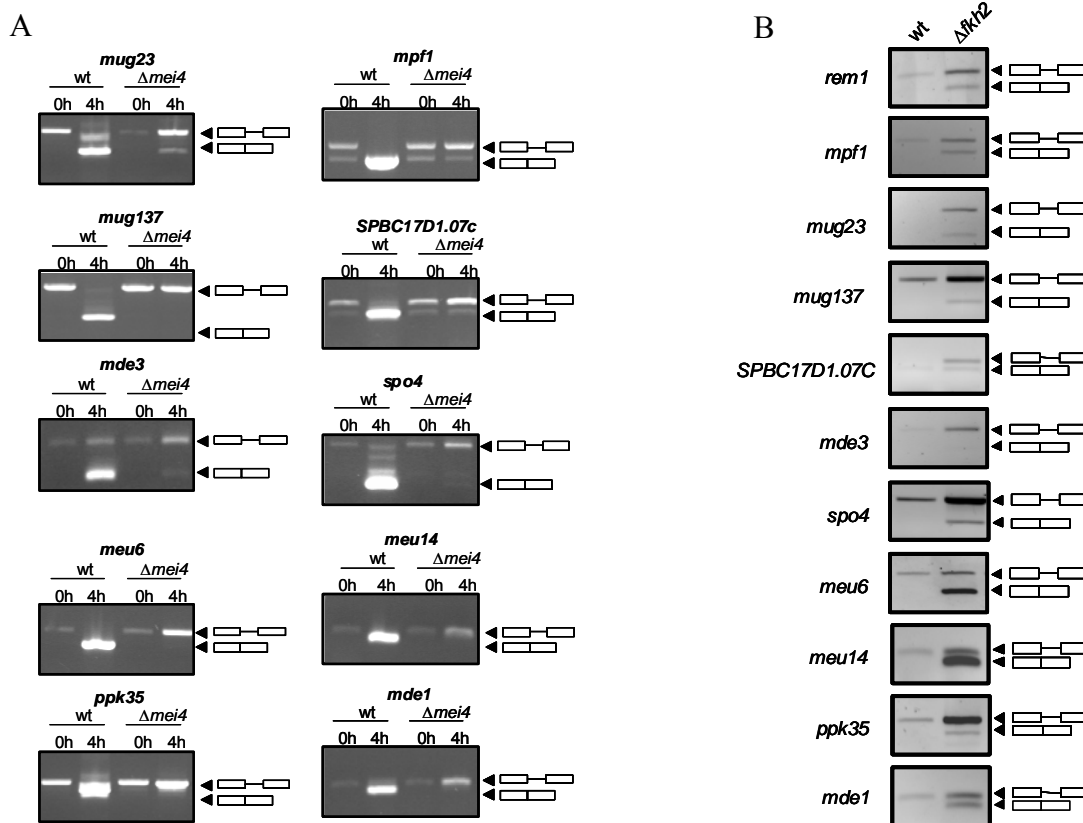


Figure 18. Microarray analysis. Mathematical criteria followed to search the candidate genes from the microarrays.

Considering Mei4 dependence, induction in Meiosis, the IR and genes matching the criteria presented in Figure 18, we could find some candidate genes. After checking all of them by RT-PCR, we noticed that not all of them behaved as *rem1*. Comparing the results back to the microarrays and considering new criteria derived from the positive ones, we restarted the search (we found that from the criteria at Fig. 18, the

we calculated the Intron Retention Rate (IR) of all the genes with plotted exons and introns with the data from the microarrays. We considered the signal from the exon as the total mRNA (processed and unprocessed mRNA) and the signal from the intron as the non-processed mRNA. So we had $IR = \text{intron} / \text{exon}$. Then, we compared the IR of the wt and the $\Delta mei4$ samples.

conditions 1 and 3 should be real). In the new search we looked for genes with “exon > 0” in the “log₂(wt 5h/mei4 5h)” values (meaning Mei4-dependence) and “exon > 0” in the “log₂(wt 0h/wt 5h)” values (meaning induction in meiosis I) and genes with a FLEX motif similar to the first 3 positive genes (GTAAAYAAAYAAA and reverse), all of them were afterwards checked by RT-PCR. At the end of the process we could define a list of 10 *rem1*-like genes, behaving similarly in a wt, a Δ *mei4* and a Δ *fhk2* strains (Figure 19).



Gene Name	IR wt	IR Δ <i>mei4</i>	FLEX box position
<i>mug23</i>	18.8	56.5	-269,-125
<i>mpf1</i>	2.1	100.0	-192,-173
<i>mug137</i>	15.1	79.7	-905,-184
<i>SPBC17D1.07c</i>	16.3	100.0	-166,-114
<i>mde3</i>	16.2	29.6	-251,-75
<i>spo4</i>	11.2	38.8	-413,-193,-97
<i>rem1</i>			-223,-182
<i>meu6</i>			-607,-135
<i>meu14</i>			-189,-12
<i>ppk35</i>			-294,-176
<i>mde1</i>			-440,-181

Figure 19. Validation of the candidates found. (A) RT-PCR of wild-type and Δ *mei4* strains at 0 and 4 hours of meiosis with specific oligonucleotides for each gene. (B) RT-PCR of wild-type and Δ *fhk2* strains vegetatively growing with specific oligonucleotides for each gene. (C) Table with the candidate genes, the intron retention rates from wild-type and Δ *mei4* strains and the FLEX boxes position relative to the starting codon.

These results indicate that the regulation studied here for *rem1* is not only an individual case but a general mechanism regulating gene expression in meiosis.

Even though, it remained unclear which was the difference between the group of genes found to behave as *rem1* and the one behaving differently. To analyse the possibility that the difference may be in the promoter region we mapped the FLEX motives of both groups. The results are shown in Figure 20, where it is shown a scheme of the promoters with the localization, sense and length of the FLEX motif. We could observe that nearly all the FLEX boxes were localized between -200 and -50 from the ATG with a great variability regarding the length and direction of the motif. Though, we could not find any difference between both groups.

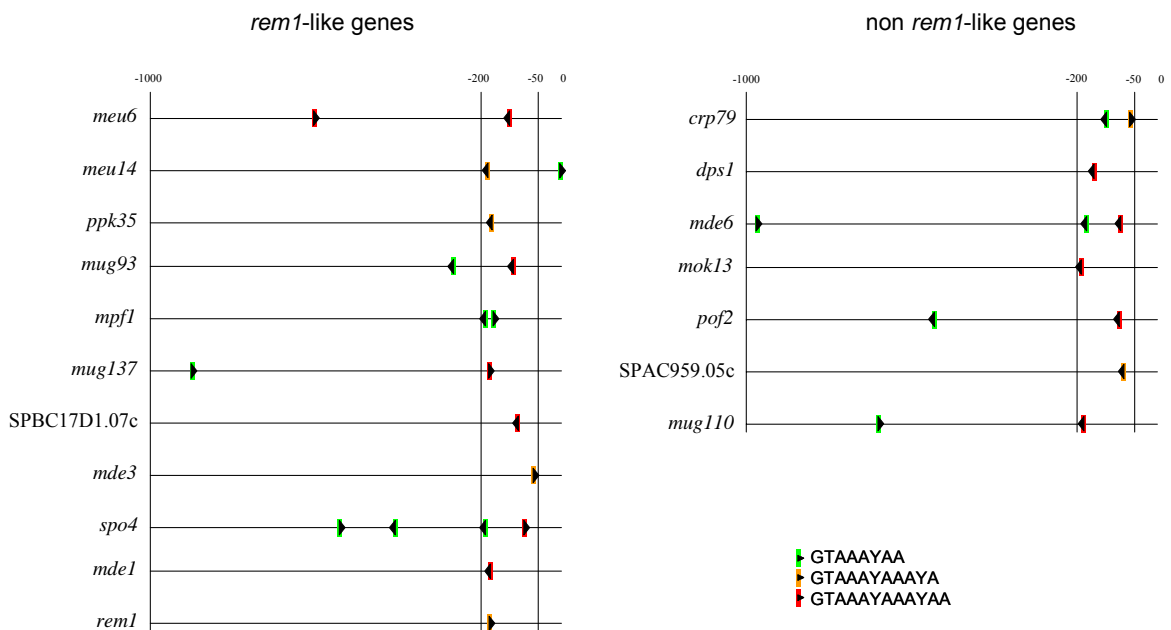


Figure 20. Relative position of FLEX boxes. Arrowheads indicate the orientation of the box and the colours indicate the length of the box. Positions are relative to the start codon

Although, previously a consensus FLEX box was described (Horie et al., 1998), we have used the list of 11 genes to align their FLEX sequences and define a new and longer weight matrix FLEX consensus box represented in Figure 21.

After defining this new FLEX box we decided to search the spreading among all the promoters of *S. pombe* with an algorithm designed for Microsoft Excel. We have found that 214 gene promoters have at least one copy of the nonamer GTAAACAAA, from which 9 have two copies of the FLEX box. Searching for the reverse sequence, we found that 332 gene promoters have at least one copy of the nonamer TTTGTTTAC, from which 23 gene promoters have 2 copies and 1 gene promoter has three FLEX boxes.

Taking into account all these results we can conclude that the regulation of splicing is a general mechanism to control gene expression in meiosis.



Figure 21. FLEX consensus box. The graph was obtained from the alignment of the FLEX boxes of the ten genes found. The software used was found at <http://weblogo.berkeley.edu>

Mei4 and Fkh2 regulation

To further study the mechanism of splicing regulation in meiosis, we decided to analyse the regulation of the two main factors of this process, Mei4 and Fkh2. We wanted to characterize them at different levels: mRNA and protein levels, postraductional modifications and ability to bind to different promoters.

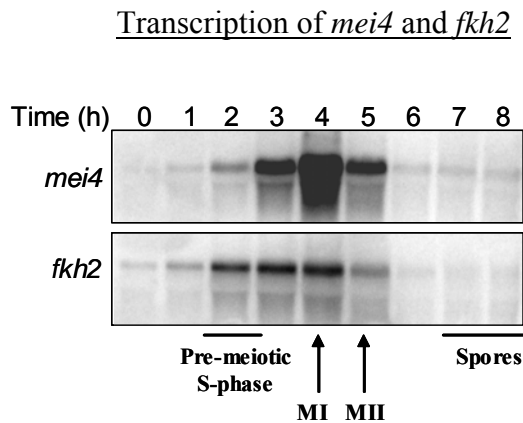


Figure 22. mRNA levels of *mei4* and *fkh2* during meiosis. Northern blot analysis of *mei4* and *fkh2* mRNAs with specific radiolabelled probes. Meiotic time-points are indicated.

Mei4 is only expressed in meiosis. mRNA levels in vegetative growing cells are nearly absent because the extremely low levels of mRNA transcribed are eliminated by the *mmi1* system (Harigaya et al., 2006). In meiosis, *mei4* mRNA is only detectable when pre-meiotic S-phase has finished and until Meiosis II. Concerning *fkh2* mRNA, we can detect it from nearly the beginning of meiosis, then it increases slowly until meiosis I and then decreases until being not detectable (Figure 22).

Protein expression of Mei4 and Fkh2

At the protein level, Mei4 follows a similar pattern as its mRNA, reaching the maximum levels at meiosis I. Fkh2 seems to be expressed constitutively, with a minor increase in the protein levels at mid-meiosis as seen on boiled extracts (Figure 23, A). In Mei4, a slow migrating band was observed at 4h and since Mei4 has 4 putative phosphorylatable sites we wanted to check if it was a mobility shift due to a phosphorylation. We treated the sample with alkaline phosphatase or lambda phosphatase and the result is shown in Figure 23, B. The alkaline phosphatase had no effect but the lambda phosphatase we could observe that the band disappeared. Then, an assay with phosphatase inhibitor was performed to confirm the result. We could

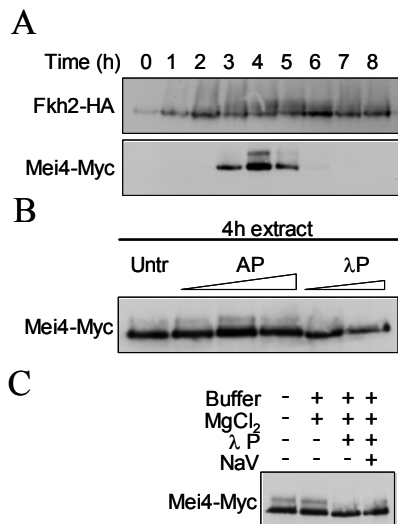


Figure 23. Mei4 and Fkh2 are phosphoproteins. (A) Western blot of boiled extracts at the indicated meiotic time points. (B) Alkaline and lambda phosphatases assay of Mei4-Myc at 4 hours of meiosis. (C) Phosphatase assay of Mei4-Myc at 4 hours of meiosis.

observe that the band disappeared and that the mobility of the second band migrated faster. The results on figure 23 indicated that Mei4 suffers at least two phosphorylations.

Regarding Fkh2, it has 18 putative phosphorylatable sites in its sequence. The western blot indicates that at mid-meiosis Fkh2 suffers a series of phosphorylation (Fig. 23). It has been reported that Cig2 phosphorylates Fkh2 during pre-meiotic S-phase (Shimada et al., 2007) but nothing is reported about the multiple phosphorylations at meiosis I.

Phosphorylation by Cig2

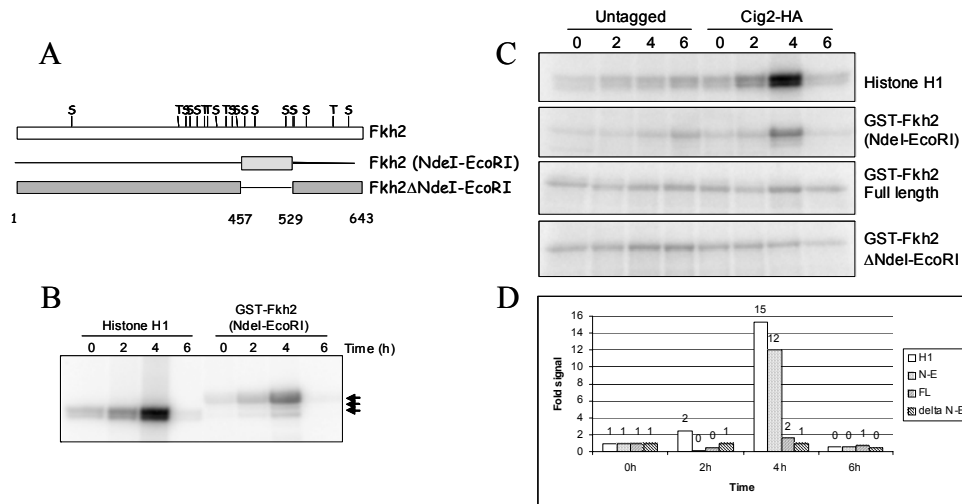


Figure 24. Phosphorylation of Fkh2 by Cig2. (A) Schematic of the constructs used. (B) Kinase assay of immunopurified Cig2-HA at the indicated meiotic time-points over Histone H1 and recombinant GST-Fkh2 (NdeI-EcoRI). (C) Kinase assay of the immunopurified from an untagged strain or a strain with tagged Cig2-HA at the indicated meiotic time-points over Histone H1, recombinant GST-Fkh2 (NdeI-EcoRI), recombinant GST-Fkh2 (Full length) and recombinant GST-Fkh2ΔNdeI-EcoRI. (D) Quantification of the signal obtained in (C, “Cig2-HA”).

To study if the phosphorylation of Fkh2 was important for the splicing function we tried to find which the kinase responsible for this phosphorylation was. A good candidate was the Cig2/Cdc2 kinase complex, because its activity peaks at 4 hours of meiosis. We constructed several truncated proteins of recombinant GST-Fkh2 to check if there was specific phosphorylation by immunoprecipitated Cig2-HA from meiotic extracts. Fkh2 has 18 putative phosphorylation sites for cyclin/cdks complexes. After many constructs assayed we finally could limit a region whose peptide presented specific phosphorylation. The 73 aminoacids fragment (from Ser457 to Asn529) has 3 putative phosphorylatable residues: Ser432, Ser481 and Ser522. The two former present the extended consensus phosphorylation site [S/T-P-X-R/K] while the third one is just SP.

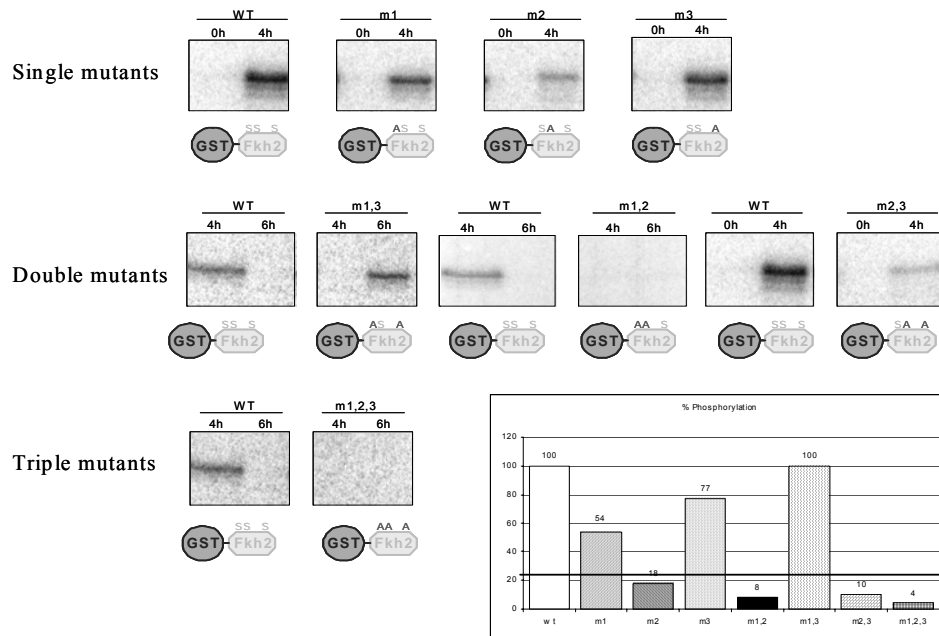


Figure 25. Cig2 phosphorylates Fkh2 at Ser481 *in vitro*. Kinase assay of immunopurified Cig2-HA at the indicated meiotic time-points over recombinant GST-Fkh2 (NdeI-EcoRI) carrying different point mutations. Quantification of the signal is shown. Data is expressed as relative to the wild-type.

In Figure 24, *top left panel*, we can observe a map of phosphorylation sites for Fkh2 and the constructs used. Meiotic extracts of an untagged strain and a strain expressing a tagged version of Cig2 (Cig2-HA) were obtained and an α -HA immunoprecipitation was done to purify Cig2. The different GST-Fkh2 proteins were incubated with the immunoprecipitated Cig2-HA in presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. We used Histone H1 as a positive substrate control. We can observe specific phosphorylation of

Fkh2[457-529] (NdeI-EcoRI fragment) at 4 hours of meiosis. Furthermore, we do not observe specific phosphorylation of the complementary protein, Fkh2 Δ 457-529. In the full length protein we can observe a moderate phosphorylation. In the *bottom right panel* of figure 24 quantification of the assay is shown.

To check which of the three Serines was/were specifically phosphorylated by Cig2, we performed site-directed mutagenesis in the GST-Fkh2[457-529] fragment to mutate them to the non-phosphorylatable aminoacid Alanine (S432A \rightarrow m1, S481A \rightarrow m2 and S522A \rightarrow m3). We assayed the single, double and triple mutants with extracts from active or inactive Cig2 and, as shown in figure 25 (see quantification), and conclude that the main substrate of Cig2/Cdc2 phosphorylation was Ser481 (m2). In the absence of S481, Ser432 (m1) was also phosphorylated while mutation in S522 (m3) behaved nearly as the wild-type. Regarding the double and triple mutants, in all the cases that S481 (m2) remained as wild type the construct presented wild type phosphorylation levels while if it was mutated the phosphorylation levels decreased more than 90%.

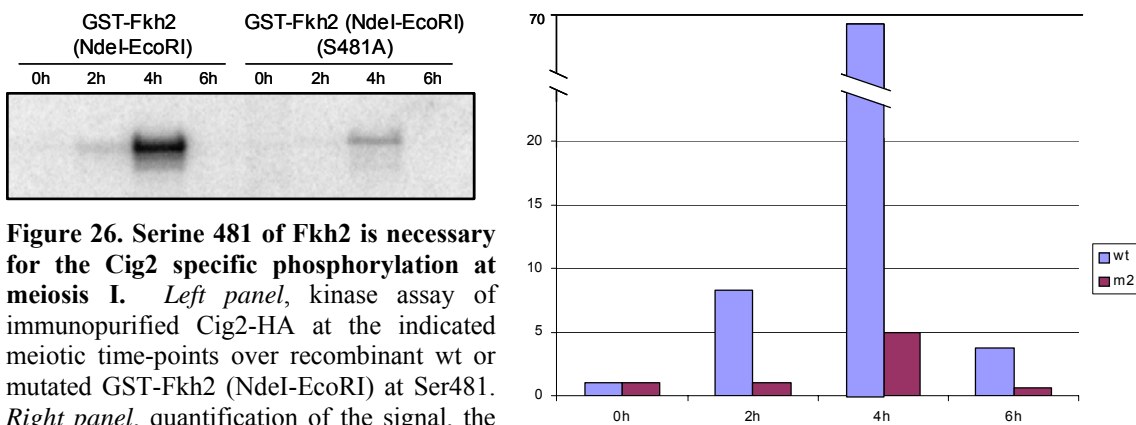


Figure 26. Serine 481 of Fkh2 is necessary for the Cig2 specific phosphorylation at meiosis I. *Left panel*, kinase assay of immunopurified Cig2-HA at the indicated meiotic time-points over recombinant wt or mutated GST-Fkh2 (NdeI-EcoRI) at Ser481. *Right panel*, quantification of the signal, the data is represented as relative to the 0h.

To confirm this result, we performed a complete time-course of meiosis and assayed Cig2-HA kinase activity with the wt and the S481A mutant GST-Fkh2[457-529] fragment. As shown in figure 26, the mutant fragment suffered a 15-fold reduction in the 32 P intake indicating an *in vitro* specific phosphorylation of Cig2/Cdc2 over this residue at mid-meiosis.

in silico structure of Mei4 and Fkh2

To try to understand the differences between these two transcription factors regarding their binding to the DNA, we compared the crystal structure of the DNA-binding domain. The structure of the transcription factor FoxK1a was determined by Kuang-Lei Tsai and coworkers (Tsai et al., 2006). This transcription factor has important aminoacid sequence homology with Mei4 and Fkh2, especially in the DNA-binding domain (Figure 23, A). Thus, we decided to model Mei4 and Fkh2 Forkhead DNA-binding domain by comparison and overlapping with the crystal structure of the human FoxK1a transcription factor. Using PyMol v0.99 we obtained a 3D structure of our proteins. For both of them, we obtained a well-delimited helix-loop-helix motif,

```

1      10      20      30      40      50      60      70      80      90      100
fkh2  KPPYSYVMI AQAILSSSEC MMTLSNIYSW ITHYPPYRT TKSGWQNSIR HNLSLNKAFR KVPRKSGEQG KGMKWSIVPE FREEEIAKTR KTPKRSPSS
mei4  KPPCSYATLI GLAILQSHNK QLTLSGLIYW IRNTFRYYLN HDGGWQNSIR HNLSLNKAFI KVEKPKGKTL KGHYWTIDPD HMQNFVSVRL HRSBSTDSNS
ilf   KPPSYAQLI VQAITMAPDK QLTNLNLIYTH ITKNYPYIRT ADKGWQNSIR HNLSLNRYFI KVPRSQEEPG KGSFWRIDPA SESKLEQAF RKRPRGVPC
hnf3g KPPYSYISLI TMAIQAPGK MLTLSEIYQW IMDLFPYRE NQQRWQNSIR HSLSFNDCFV KVARSPDKPG KGSYWALHPS SGNMFENGCY LRRQKRFKLE
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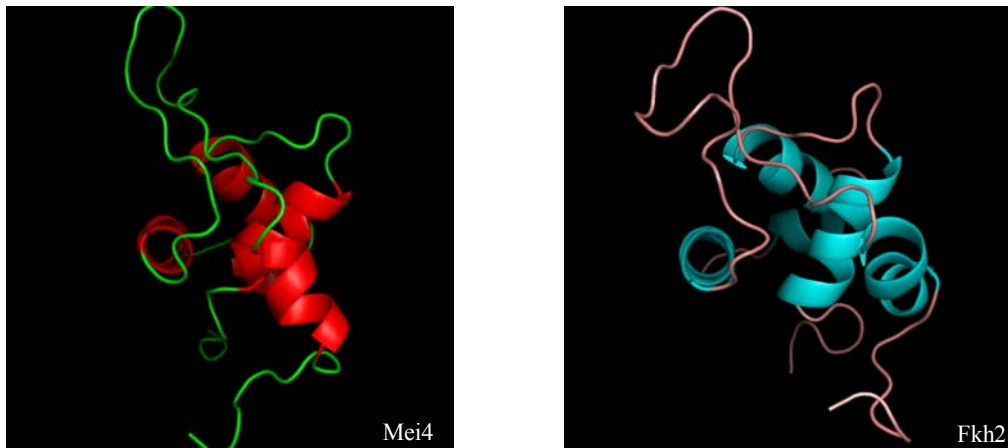


Figure 27. 3D structure of the DNA-binding domain of Mei4 and Fkh2. Alignment of the sequences used to perform the modelling. Bottom panel, result of the modelling for the two transcription factors.

with no significant differences of the core of the domain binding the DNA, which function is to recognize it unspecifically (Figure 27). The two “wings” of the motif, though, have differences regarding the structure and the way they should recognize the DNA.

As can be observed in Figure 28, these differences could explain the different specificity of promoters of the two factors. We could determine the DNA base pairs that can contact the factors but this must be validated experimentally since the modelling is only *in silico*. The theoretical DNA-binding box as far as this model is

concerned is TAAACAANACA as shown in Figure 28. Although this experiments do not allow us to conclude anything regarding the binding of the transcription factors to the FLEX boxes, they just give us some hints and possible explanations of the differences of gene specificity for both of them.

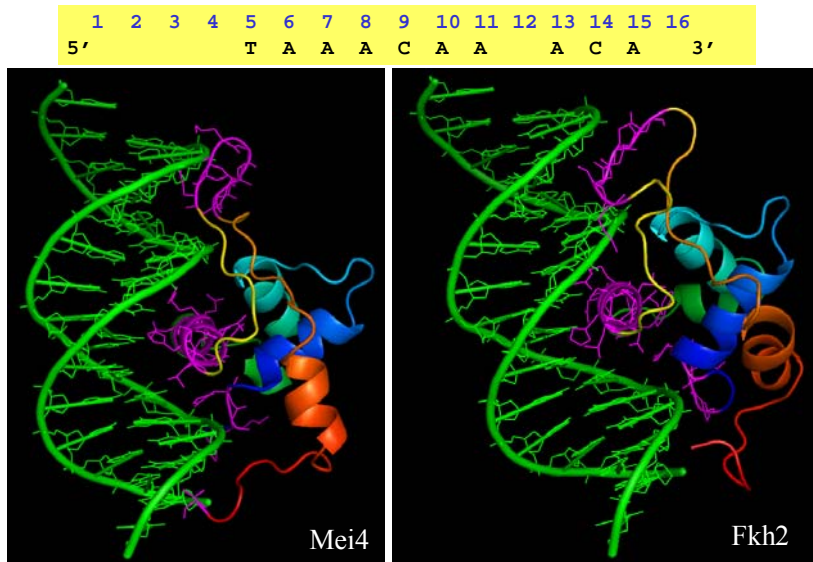


Figure 28. Model of the Mei4 and Fkh2 binding to the DNA. *Top panel*, nucleotides that directly contact the Forkhead domain. *Bottom panel*, model of the binding. The differences in how the wings touch the DNA can be appreciated.

Rem1 function

Rem1 was initially isolated *in silico* by homology with other cyclins (Malapeira et al., 2005). The cyclin domain was localized entirely in the second exon of the gene, meaning that only when the mRNA is processed, the cyclin domain will be expressed. The first exon, has no sequence homology with any other known protein and does not seem to have any secondary structure.

Cyclin function

We started studying the full length protein, its association with Cdks and the associated kinase activity.

To check if Rem1 was associated with de *S. pombe* CDK Cdc2, we performed Co-Immunoprecipitations with cell expressing HA C-terminus tagged Rem1 from its own *locus*. Cells were harvested at 0 and 4.5h of meiosis and native extracts were incubated with α -HA antibody and Protein G-Sepharose. Immunoprecipitated proteins were separated by SDS-PAGE and western blotted with α -PSTAIR antibody to detect Cdc2.

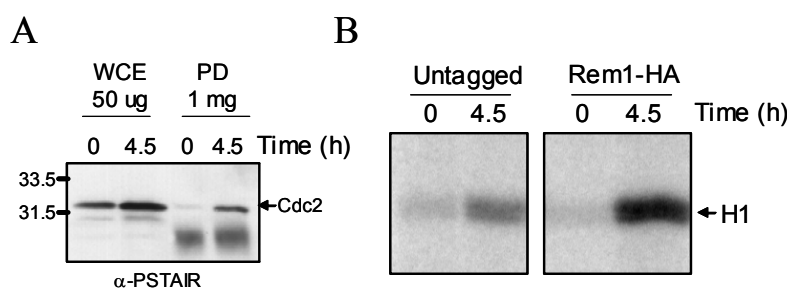


Figure 29. Cyclin function of Rem1. (A) Immunoprecipitation of Rem1-HA and Western Blot to detect Cdc2. (B) α -HA immunoprecipitations from Untagged and Rem1-HA tagged strains were used for a kinase assay over Histone H1 (H1).

As shown in Figure 29, Cdc2 can be detected when Rem1-HA is Immunoprecipitated. This shows that Cdc2 and Rem1 form a complex in mid-meiosis. To know if this complex could have kinase activity, *in vitro* kinase assays were performed with Rem1 purified from cells at different hours of meiosis. Specific phosphorylation of Histone H1 could be detected at 4.5h of meiosis indicating that this complex has specific kinase activity (Figure 29 and (Malapeira et al., 2005)).

Recombination function

While searching for a phenotype of cells lacking Rem1 we found that it was necessary for meiotic recombination, that is to say, $\Delta rem1$ cells presented 50% of intragenic recombination compared to wild type cells (Figure 30 and (Malapeira et al., 2005)).

It was really surprising to find a phenotype limited at 2-3h of meiosis (a moment of meiosis when the mRNA is still not processed and the full length protein was not still expressed). This pointed the possibility that the mRNA itself was participating in the recombination events or that a protein encoded by the first exon, without being processed was participating in recombination.

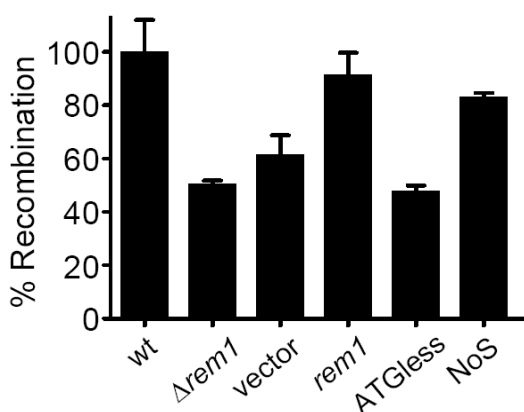


Figure 30. The second exon of Rem1 is not needed to have normal recombination levels. Levels of recombination were measured in wild-type and $\Delta rem1$ strains and in a $\Delta rem1$ strain carrying an empty vector (vector), a full length copy of *rem1* (*rem1*), a mutant *rem1* without the initiation codon (ATGless) and a *rem1* with the 5' Splice Site mutated (NoS). The values are the mean and standard deviation of five independent experiments

To assay these possibilities recombination of various strains were quantified. We compared the recombination efficiency in the *ade* locus in different strains, a wild-type strain, a $\Delta rem1$ strain and $\Delta rem1$ strains transformed with different plasmids: an empty vector or vectors carrying a complete copy of *rem1* genomic region, either with the wild-type sequence, carrying a mutation in the start codon (ATG→TAG) or carrying a mutation in the 5' splice donor site (GU→AC). As shown in Figure 30, $\Delta rem1$ cells have decreased recombination efficiency compared to wild-type. If we transform it with a vector carrying a copy of *rem1*, the phenotype is reverted. The recombination is also affected if the protein cannot be expressed (ATGless) meaning that the mRNA directly has no effect on recombination. Even though, if we transform $\Delta rem1$ cells with a copy lacking the 5' splice site, we can recover the recombination efficiency (Figure 26). To elucidate if the full length protein could also recover the phenotype, we transformed the cells with a copy of *rem1* lacking the intron, we could observe a mating

with spore viability bottom than 90%, the spore formation was aberrant with *asci* containing only two spores or empty *asci*. This could be due to Rem1 expression toxicity in vegetative growing cells (Malapeira et al., 2005) so we could not conclude anything from this experiment.

MATERIALS AND METHODS

Methodology

Growth and maintainance of *S. pombe*

All *S. pombe* strains used are isogenic to wild type 972 h⁻. Media were prepared as described previously (Moreno et al., 1991).

Cell fixation and DAPI staining

4',6'-diamine-2-phenylindole (DAPI) is used to stain the DNA, this way, cells' nuclei can be easily observed under the microscope. Aproximately 10⁷ cells are harvested from the culture, centrifuged and resuspended in 70% Ethanol (EtOH). This way cells can remain fixed for a long time. To stain the cells, they should be previously rehidratated. When the desired amount of cells are put on the slide they are dried and 5mg/ml DAPI is applied. Under a florescence lamp in the microscope and with a UV filter, nuclei can be easily observed.

Flow citometry

It is used to mesure the relative DNA content of every single cell. 1 ml of Sodium citrate (50 mM, pH 7) is added to 100 µl of 70% EtOH fixed cells to wash them. 0.5 ml of Sodium citrate (50 mM, pH 7) with 50 mg/ml RNase are added and incubated over night (o/n) at 37°C. 0.5 ml of Sodium citrate (50 mM, pH 7) with Propidium Iodide is added, mixed vigorously and sonicated smoothly to separate cellular agregates. Samples are now ready to be analyzed in the flow citometer.

Transformation of *S. pombe*

Cell culture should be grown in rich media until a concentration of 10⁷ cells/ml. Cells should be washed with water and resuspended in Litium Acetate (LiAc-TE: 0.1 M Litium Acetate, 10 mM Tris pH 7.5, 1mM EDTA). 2x10⁶ cells are mixed with 4 µg of carrier DNA (SIGMA Herring Sperm DNA (D-7290)) and 1 µg of the transformant DNA. After incubating the cells for 10 minutes at room temperature, 260 µl of

PEG/LiAc-TE (LiAc-TE, 40% PolyEthilenGlycol) are added and incubated for 1 hour at 25-30°C. 43 µl Dimethyl Sulfoxide are added and cells are heat shocked at 42°C for 5 minutes. After 2 water washes, cells are plated on selective media.

Synchronous meiosis

Nearly all the cells used in this study were temperature sensitive *pat1-114* strains so meiosis was performed as follows: 5×10^6 cells/ml were starved of nitrogen o/n (EMM without NH₄Cl, with 1% of glucose and 50 µg/ml of Leucine) so that cells block at G1. To induce meiosis to the cells, 25 mM NH₄Cl and 50 µg/ml of Leucine are added and the culture is shifted to 34.5°C in a water bath with vigorous shaking. This way Pat1 is inactivated synchronously and meiosis starts.

Sometimes, when cells were used for proteic assays, cultures were not nitrogen starved but directly shifted to 34.5°C asynchronously to increase protein recovery.

RNA purification

Cells harvested at a concentration of 10^7 cells/ml were resuspended in 400 µl of AE buffer (50 mM NaAcO pH 5.3, 10 mM EDTA pH 8.0). 40 µl of 10% SDS, 350 µl of Acid Phenol and 350 µl of Chloroform are added. The mix is vortexed and incubated for 1 hour at 65°C, remixing the cells every 10 minutes. Samples are washed twice with 400 µl of premixed Acid phenol-Chloroform. To precipitate the RNA, sample were mixed with 2 volumes of 100% EtOH and 1/10 of 3M Sodium Acetate pH 5.3 and incubated for 30 minutes at -20°C. After centrifugation, supernatant (SN) was discarded and RNA pellet was resuspended in DEPC-treated water.

To eliminate the DNA contamination from the samples, 2 µg of RNA are incubated with RNase-free DNase for 30 minutes at 37°C. RNA was extracted with the Acid phenol-Chloroform method explains above.

Northern blot

3-10 µg of RNA are separated in 1% agarose gel with Formaldehyde. RNA was transferred by capilarity to a GeneScreen Plus (NEN Life Science Products) o/n.

Hybridization and washes are performed as explained by the manufacturer. DNA probes of the desired genes were labelled radioactively with the Roche random primer labelling kit. Detection was performed in parallel with Phosphorimager Screen and with films.

Reverse Transcriptase-PCR (RT-PCR)

8 µg of DNase I-treated RNA is used for the reaction. The RNA is diluted up to 19 µl and the samples are incubated at 65°C for 10 minutes. Then, the sample is split in two tubes and the reagents from the Reverse Transcription System (Promega) kit are added (4 µl of 10x Reaction Buffer, 1 µl of RNAsin, 8 µl of MgCl₂, 4 µl of 10mM dNTPs and 2 µl of 100 mM oligo dT). Then Reverse Transcriptase is added just to one of the duplicates and the reaction is performed as indicated by the manufacturer (60 minutes at 42°C, 30 minutes at 52°C and 3 minutes at 94°C).

For the PCR reaction 1 µl of the cDNA from the previous reaction is used and the oligos are chosen depending on the target gene. Reactions are loaded on a Ethidium Bromide gel to analyze the results.

Native protein extraction and immunoprecipitations

Extracts were prepared in NET-N buffer (Malapeira et al., 2005) and immunoprecipitations were performed with 30 µl of IgG sepharose. Immunoprecipitates (8 mg) were done in IPP buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, 5 µg/ml leupeptin, and 5 µg/ml aprotinin] for TAP purification or NET-N for the rest of purifications, washed 3 times with the same buffer, resolved in 8% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with the indicated antibody.

Boiled protein extraction

Pellet from the culture is resuspended in 200 µl of HB Buffer (25 mM MOPS pH 7.2, 60 mM β-glycerol phosphate, 15 mM P-nitro phenil phosphate, 15 mM MgCl₂, 15 mM EGTA, 1% Triton X-100, 1 mM DTT and 170 mg/l PMSF). The mixture is

boiled for 10 minutes and put back on ice. Glass beads are added and cells are broken up to 80% in a Minibead-beater. Glass beads are separated and extracts are quantified by Bradford.

Western blot

Desired quantity of protein extract is mixed with loading buffer and run on a SDS-PAGE gel (10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, 30:0.8 acrylamide:bisacrylamide), gel is transferred to a nitrocellulose membrane 1 hour at 400 mA. The membrane is blocked with 5% BSA or milk (depending on the primary antibody) for 1 hour and is incubated over night with the antibody. After 3 washes with TBS-T, the membrane is incubated with the secondary antibody, washed 3 times again with TBS-T and developed with ECL reagent kit.

Phosphatase assay

Phosphatase assay was performed with 30 µg of cell native extracts and alkaline (Roche) or lambda phosphatase (New England Biolabs) was used as indicated by the manufacturer.

Electrophoretic mobility shift assay

Incubate the desired protein or extracts with 1x EMSA Buffer (5x: 50mM HEPES (pH 7.9), 375 mM KCl, 12.5 mM MgCl₂, 0.5 mM EDTA, 5 mM DTT and 15% Ficoll), 1 µg of polydI/dC, 10 µg BSA and 1 µl of ³²P-labeled oligonucleotide probe (10K c.p.m.) in a final volume of 20 µl. Incubate for 20 minutes and load it on a 5% polyacrylamide gel with 0.5x TBE. Dry the gel and develop with autoradiographies.

Chromatin Immunoprecipitation

90 ml of cells at OD₆₀₀ 0.5 (or equivalent) were crosslinked using 1% formaldehyde (final concentration) for 10 minutes at 25 °C. The pellet was washed twice with PBS. Cells were resuspended in Breaking Buffer (0.1M Tris pH 8.0, 20%

glycerol and 1mM PMSF) and broken in a Minibead-beater 3 times at medium intensity. The pellet was washed twice with Lysis Buffer (50 mM Hepes pH7.5, 140 mM NaCl, 1 mM EDTA 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, 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 15 minutes at 14 krpm and the pellet was discarded. Immunoprecipitations were performed overnight with the same amount of protein (measured by Bradford) with 15µl IgG-Sepharose (Amersham) or a specific antibody [9E10 for Myc-tagged proteins, 12CA5 for HA-tagged proteins, α -RNA pol II CTD monoclonal antibody (8WG16, Abcam), α -RNA pol II CTD (phospho-Ser5) polyclonal antibody (Abcam) or α -RNA pol II CTD (phospho-Ser2) polyclonal antibody (Abcam)]. Immunoprecipitated extracts were incubated with 15 µl of Protein G Sepharose for 5 hours. Beads were washed once with Lysis buffer, twice with Lysis buffer 500 mM NaCl, twice with Wash Buffer (10 mM Tris pH 8, 0.25M LiCl, 0.5% NP-40, 0.5% Sodium deoxycholate, 1 mM EDTA, 1 mM PMSF) and once with TE (10 mM Tris pH 8, 1mM EDTA). The beads were resuspended in 0.1 ml of Elution Buffer (50 mM Tris pH 8, 10mM EDTA, 1% SDS) and incubated at 65°C for 20 minutes. Then the beads were washed with 0.15 ml of TE/0.67% SDS and the crosslinking was reversed overnight at 65°C. To clean up the DNA, 0.25 ml of TE were added, 1 µl of 20mg/ml glycogen and 7.5 µl 20mg/ml of Proteinase K and incubated 2 hours at 37 °C. DNA was extracted with 1:1 Phenol/Chloroform and precipitated with EtOH and sodium acetate for 30 min at -20°C. DNA was pelleted 30 min, 14 krpm at 4°C. The pellet was air dried and resuspended in 100 µl of TE. To perform the PCR, SYBR Green kit was used and quantitative real time PCR was performed in an Applied Biosystems Cyclor. Data was expressed as percentage of the input.

In vitro kinase assay

0.5-1 mg of total extract is purified conveniently depending on the protein and/or the tag. After the washes described previously, an additional wash with 1x Kinase Buffer (2x Kinase Buffer: 100 mM HEPES pH 7.5, 20 mM MgCl₂, 4 mM EGTA, 2 mM DTT) is performed. Then, 1x Kinase Buffer with the substrate and 10 mCi of γ -³²P ATP are added and incubated for 20 minutes at 30°C. To stop the reaction loading

buffer is added and put it on ice. The samples are then boiled and proteins are separated by SDS-PAGE. The gel is Coomassie-stained and dried. The signal is detected by autoradiographies.

Recombination assays

Recombination efficiency was determined as described (Malapeira et al., 2005). Basically, intragenic *ade6* recombination (gene conversion) was measured in wild-type and $\Delta rem1$ strains with the *ade6* alleles M26 and 52. Ade⁺ prototrophs/106 viable spores were measured and plotted the efficiency relative (in %) to a wild type strain.

Microarray experiments and data evaluation

Global expression analysis utilized custom designed *S. pombe* microarrays. Array construction, sample labeling, and hybridization, were carried out as described previously (Sinibaldi et al., 2001). The arrays consisted of 8785 70-mer oligonucleotides representing 6918 exons, 1521 introns, and 333 3' UTRs (http://research.stowers-institute.org/microarray/S_pombe/). Briefly, total RNA was prepared from wild type, and $\Delta mei4$ strains, at 0 and 4 hours into meiosis. Polyadenylated RNA was extracted from total RNA by purification with an oligo(dT) cellulose column. RNA quality was assessed on a Bioanalyzer 2100 machine (Agilent). 2 μ g of RNA 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 printed with 70-mers 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 analyzed using GenePix Pro 6.0 software (Molecular Devices, Union City, CA). Data analysis was performed with the R programming language. Differential expression was assessed using the limma package (Smyth, 2004).

Eight microarrays were used, with two experimental factors: time points 0 and 4 hours from meiotic induction and strains wild type and $\Delta mei4$. Data within arrays was normalized using the print-tip loess method. Data between arrays was normalized using Aquantile normalization. Linear models were fit with coefficients estimated for wt

0/mei4 0, wt 4/mei4 4, wt 0/mei4 4, and mei4 0/mei4 4. Microarray data are available at ArrayExpress (www.ebi.ac.uk/arrayexpress/) with accession number: E-TABM-465.

Modelling of protein structure

The modelling of the 3D structure of the DNA-binding domain of Mei4 and Fkh2 was performed with the programme PyMol v0.99. The modelling was based on the crystal structure presented elsewhere (Tsai et al., 2006).

Strains

Strain	Genotype	Figure
JA95	pat1-114/pat1-114 rem1::rem1-HA kan ⁺ /rem1 ⁺ leu1-32/leu1-32 ade6-M210/ade6-M216 h ⁻ /h ⁻	17, 25
JA124	ade6-M26 leu1-32 ura4-D18 h ⁻	26
JA128	ade6-M52 leu1-32 ura4-294 h ⁺	26
JA163	nmt41x(-130)rem1-kan ⁺ leu1-32 h ⁻	5
JA167	nmt41x(ATG) rem1-kan ⁺ leu1-32 h ⁻	5
JA247	pat1-114 leu1-32 h ⁻	2, 6, 7, 9, 10, 11, 12, 14, 18, 20, 25, 27, 28
JA249	pat1-114 rem1::ura4 ⁺ leu1-32 h ⁻	2, 3, 4, 5, 8
JA397	pat1-114 mfr1::ura4 rem1-HA-Kn leu1-32 ura4-D18 h ⁻	17
JA406	pat1-114 rem1::ura4 ⁺ rem1 _{genomic} @leu1 leu1-32 h ⁻	
JA416	pat1-114 mei4::ura4 leu1-32 ura4-D18 h ⁺	6, 7, 9, 11, 12, 14, 27, 28
JA417	pat1-114 rem1-GST:Kn leu1-32	2
JA463	pat1-114 sep1::ura4 leu1-32 ura4-D18 h ⁺	9
JA480	pat1-114 fh1::kan ⁺ leu1-32 h ⁻	9
JA489	rem1::kan leu1-32 ade6-M26 h ⁻	26
JA494	rem1::kan leu1-32 ade6-52 ura4-294 h ⁺	26
JA497	pat1-114 fkh2-HA: kan ⁺ leu1-32 h ⁺	13, 19
JA508	pat1-114 fkh2::kan ⁺ leu1-32 h ⁻	10, 11, 28
JA509	pat1-114 mei4-13Myc-kan ⁺ leu1-32 h ⁻	13, 19
JA536	pat1-114 cig2-HA leu1-32 h ⁻	20
JA544	pat1-114 prp11-TAP- kan ⁺ leu1-32 h ⁺	15, 16
JA545	pat1-114 mei4:ura4 pap11-TAP- kan ⁺ leu1-32	15
JA546	pat1-114 cdc5-TAP-kan ⁺ leu1-32 h ⁺	15, 16
JA548	pat1-114 mei4::ura4 cdc5-TAP-kan ⁺ leu1-32 ura4-D18 h ⁻	15
JA551	pat1-114 mei4-13Myc-kan ⁺ cdc5-TAP-kan ⁺ leu1-32	16
JA552	pat1-114 mei4-13Myc-kan ⁺ prp11-TAP-kan ⁺ leu1-32	16
JA558	pat1-114 fkh2-HA-kan ⁺ prp11-TAP-kan ⁺ leu1-32	16
JA559	pat1-114 fkh2-HA-kan ⁺ cdc5-TAP-kan ⁺ leu1-32	16
JA661	pat1-114 U1Δ70K-2XTAP-kan ⁺ leu1-32 h ⁺	15, 16
JA662	pat1-114 U1Δ70K-2XTAP-kan ⁺ mei4:ura4 leu1-32	15
JA728	pat1-114 U1Δ70K-2XTAP-kan ⁺ mei4-13Myc-Nat ⁺ leu1-32 h ⁺	16
JA733	pat1-114 U1Δ70K-2XTAP-kan ⁺ fkh2-HA-kan ⁺ leu1-32 h ⁺	16
JA699	pat1-114 fkh2::kan ⁺ mei4::ura4 leu1-32 ura4-D18 h ⁻	11

Plasmids

Plasmid	Description	Figure
pAY043	pJK148	26
pAY155	pAY168-1.2 kbp rem1 promoter-rem1 Δ 652nt	2
pAY168	pJK148-1.2 kbp rem1 promoter-rem1-500 bp 3'UTR	3, 4, 8, 26
pAY169	pJK148-1.2 kbp rem1 promoter-rem1 (ATG \rightarrow TAG)-500 bp 3'UTR, ATGless	4, 26
pAY171	pJK148-1.2 kbp rem1 promoter-rem1 (GT1676AC)-500 bp 3'UTR, NoS	3, 26
pAY278	pREP-1.2 kbp rem1 promoter +UTR-HA- rem1	26
pAY322	pREP-rem1 prom + UTR (PstI/XhoI)-HA (XhoI/BamHI)-rec6 (BamHI/SmaI)	6
pAY324	pREP-rem1 prom + UTR (PstI/XhoI)-HA (XhoI/BamHI)-cdc2 (BamHI/SmaI)	6
pAY334	pREP-rem1 promoter-cdc2-nmt term	6, 7
pMV100	pREP-prom res2-rem1	5
pAY344	pGEX-4T-1 Fkh2 (BamHI/SmaI)	20,
pAY361	pGEX-4T-1 Fkh2 NdeI to EcoRI	20, 21, 22
pAY363	pGEX-4T-1 Fkh2 Δ NdeI-EcoRI	20
pAY365	pGEX-4T-1 Fkh2 NdeI to EcoRI (Ser462Ala) m1	21
pAY366	pGEX-4T-1 Fkh2 NdeI to EcoRI (Ser481Ala) m2	21, 22
pAY367	pGEX-4T-1 Fkh2 NdeI to EcoRI (Ser522Ala) m3	21
pAY368	pGEX-4T-1 Fkh2 NdeI to EcoRI (Ser481,522Ala) m2,3	21
pAY369	pGEX-4T-1 Fkh2 NdeI to EcoRI (Ser462,481Ala) m1,2	21
pAY370	pGEX-4T-1 Fkh2 NdeI to EcoRI(Ser462,522Ala) m1,3	21
pAY371	pGEX-4T-1 Fkh2 NdeI to EcoRI (Ser462,481,522Ala) m1,2,3	21
pAY372	pAY168 Δ FLEX1 (has a deletion of the core GTAAACAAA)	8
pAY374	pAY168 FLEX2mut (GTAAACA \rightarrow CTAATGA)	8
pAY375	pAY168 Δ FLEX1,FLEX2mut (GTAAACA \rightarrow CTAATGA)	8

Oligonucleotides

Oligo	Sequence	Description
JA44	GCAGCTTGAATGGGCTTCCATAG	nmt terminator
JA71	CAAAGTCCTAATTGCAGTGTTTCGG	rem1 (seq +300, RT-PCR)
JA174	GGAGGATGGATCTCTTCATACTTGC	rem1 (RT-PCR)
JA218	CCATATTGAGTAAGGAATGTCC	5' rec6
JA219	GTCGAGAAGGGAAGACTCACTG	3' rec6
JA286	CACCGTTCACTAGCTGGTAGGTAAC	3' cdc2
JA310	GCTAATAGCGGATCCATGGAG	5' for RT-PCR rem1-cdc2
JA311	GGCAATTTGAAGCTAGCAGATTTTGG	5' mid cdc2 (downstream)
JA312	GCTAGGTTGCTTCTTTTAGTGGTTTGC	5' UTR cdc2
JA314	CGACCGTTTCAGCAACGAGAACCAATTC	5' UTR rem1 (RT-PCR)
JA348	CAGTACAGCGCAACCATAAGC	5' RT-PCR SPAC4G9.05
JA349	CCGCATATTATTTGGTCCATGTTG	3' RT-PCR SPAC4G9.05
JA360	ATGGTGTCTAGATTTTTTTCTTGG	5' RT-PCR SPCC1919.11
JA361	GATTGGCTTTTCCTAATTGCGAC	3' RT-PCR SPCC1919.11
JA368	GGCTATCTTTGTAGCGTTCATG	5' RT-PCR SPBC17D1.07c
JA369	CTTCTAACTAAATCTTTCGTCTCG	3' RT-PCR SPBC17D1.07c
JA376	CTCAGTGTTAGATTTGACACAAGTC	5' RT-PCR mde3
JA377	GTCCATAAACGTCTCCAAGAGATCG	3' RT-PCR mde3
JA388	CATCCTCAATTGATGTGTGGTCAG	5' RT-PCR spo4
JA389	CTGTTTTGGCCTTTACTCGTTTCG	3' RT-PCR spo4
JA394	GGTCATTCAAGGCTCATTTGGG	5' RT-PCR mde1
JA395	GACGGTGCATCACGTTCCAGG	3' RT-PCR mde1
JA398	CCATTGACCTACCCACTGGTG	5' RT-PCR meu6
JA399	GTTTCCAGTACCGGGTTCTATGG	3' RT-PCR meu6
JA400	GAGAAAACGGTCTCGTGAAGGG	5' RT-PCR meu14
JA401	CTCAGATTACTGGATTTTGCTCCC	3' RT-PCR meu14
JA406	GCGCAACACACACGAATTGTTAGC	5' RT-PCR ppk35
JA407	GTATTCAGGAGATCCAACAACGG	3' RT-PCR ppk35
JA412	GGAAGAAAAGGAGAACGTAAACTTG	5' RT-PCR SPBC32H8.06
JA413	GTCTTAACTGCCATGTTTCTGCAC	3' RT-PCR SPBC32H8.06
JA453	AGGTAAAGTTTAGTTTTGGGTTGTG	5' rem1 FLEX box ChIP
JA454	AAGTTGATGGTGAAAATGTTTGT	3' rem1 FLEX box ChIP
JA455	GATTTATGCCTGATTACCGACAT	5' rem1 Intron ChIP
JA456	ATGTATGCACCCCCACAATC	3' rem1 Intron ChIP
JA459	GGATTAGTCGACAAGTGCATGA	5' rem1 3'-end ChIP
JA460	CTTCTCTCTTTTTCCCTCCTTTTT	3' rem1 3'-end ChIP
JA488	TTTGTCAAAAAGTTTTCCACATAC	5' Intergenic region ChIP
JA489	GCGGTTCCCTTTCTAAGAGACT	3' Intergenic region ChIP
JA559	GCCGATAAAAAGATACAAAAGTGAGA	5' ChIP mde2
JA560	TTTTCTATAACAAGCACATCATTG	3' ChIP mde2

DISCUSSION

Rem1 regulation

Rem1 levels and function are regulated at multiple levels. Here we have studied some of them. We have determined that it is regulated at the level of transcription, splicing and protein stability; moreover, we have some hints that it may be also regulated by mRNA degradation. Some other possibilities still not studied are the regulation during the translation of the protein and regulation by post-translational modifications.

We have determined that the promoter is necessary and enough to regulate *rem1* expression (Fig. 6 and 7) and that neither the ORF nor the 5'UTR have any role in this regulation (Fig. 5, 6 and 7). Regarding the 3' end of the gene, we could not detect any significant difference at the level of transcription and splicing between a wild type strain and a strain lacking part of the second exon and the 3' end of the gene. Even though, there is a slight stabilization of the mRNA at late meiosis that should be studied deeper to assure whether it has a role in mRNA stability (Fig. 2).

The elements found to be important in the promoter are two FLEX boxes. They bind Forkhead transcription factors *in vitro* (Fig. 12). We have analysed the effects of the deletion of these boxes, in the absence of FLEX1, we can observe lower levels of transcription although the splicing seems to be normal. In the absence of FLEX2, we could observe an advancement regarding the timing of the transcription and higher levels of mRNA while splicing of *rem1* is nearly normal. In the double mutant, the transcription is seriously impaired and the splicing becomes very inefficient. These results give us some hints about the function of each FLEX box (FLEX1 inducer and FLEX2 repressor) but is not easy to conclude anything probably because they can have some overlapping affinity for the *trans* factors binding to them. Another possibility is that they are not directly implied in the regulation but we detect the effects derived from the de-structuration of the promoter, like the system reported by the group of John Warner (Rudra et al., 2007).

In figures 6 and 7 we can observe that the regulation is not only positive at mid-meiosis but also negative during vegetative growth and early stages of meiosis. That is to say, in mitosis the transcription and splicing are repressed and in mid-meiosis the transcription and splicing are induced. This means that the system has at least two regulators. It is very important that *rem1* has this self-lock mechanism of regulation,

rem1 is not under the regulation of the *mmi1* system (Yuriko Hariyaga, personal communication) and it is very toxic even at low levels in mitotic cycle. Avoiding the splicing of the mRNA, the cell has no mature Rem1 protein protecting itself of the consequences of Rem1 expression (mitotic catastrophe) (Malapeira et al., 2005).

The main regulators of *rem1* are the forkhead transcription factors Mei4 and Fkh2 (Fig. 9). In the absence of Mei4, we have some residual transcription of *rem1* but we cannot detect spliced mRNA. This way, Mei4 seems to be directly or indirectly (promoting the transcription of other regulators) the main inducer of Rem1 expression. To differentiate between these two possibilities we performed ChIPs of Mei4 to show that it binds to *rem1* promoter (Fig. 13). In fact, we can detect Mei4 at *rem1* ORF pointing the possibility that Mei4 can be travelling with the RNA polymerase II. This technic also allowed us to study RNA polymerase II binding to *rem1* gene. In Figure 14 we show that RNAPol II binding to *rem1* is *mei4*-dependent. We could detect some binding and accumulation of the initiation isoform of the RNAPol II (Ser5 of the CTD repeats phosphorylated) in a Δ *mei4* strain but no elongating isoform (Ser2 of the CTD repeats phosphorylated) could be detected in the absence of Mei4. The strongest evidence that Mei4 is playing a direct role in the recruitment of the transcription machinery and the spliceosome is shown in figures 15 and 16. We could coimmunoprecipitate Mei4 pulling down some components of the spliceosome (U1-70k, Prp11 and Cdc5). This binding is probably indirect because of the weak signal of the CoIPs and because experiments using *in vitro* translated (*ivt*) Mei4 cannot bind these factors *in vitro*. The link between both is probably the RNAPolymerase II whose CTD repeats is reported to bind the spliceosome (Kim et al., 1997).

The second component of the system is Fkh2, in the absence of this factor, we can detect spliced mRNA at early stages of meiosis (Fig. 9) and, as shown in figure 10, this is not due to an ectopic expression of Mei4. In the absence of Fkh2 the meiotic induction of *rem1* transcription is also lost. This indicates that Fkh2 is necessary together with Mei4 to induce transcription of *rem1*.

Fkh2 also binds directly to *rem1* gene but its localization seems to be more extended in time and narrower in space. In figure 13, we can detect an increase in the binding of Fkh2 to *rem1* gene slightly earlier than Mei4, moreover, if we observe the results in figure 9, they could explain an earlier, even constitutive binding of Fkh2 to the gene. The ChIP experiment also shows no binding of Fkh2 to the *rem1* ORF.

Regarding localization of the RNAPol II and the spliceosome on *rem1* gene in cells lacking *fkh2*, preliminary results indicate that the binding of RNAPol II is even lower than in a $\Delta mei4$ strain (data not shown) and for the spliceosome there are still no results. Interaction of Fkh2 and the spliceosome could not be detected (Figure 16). This can be related to the fact that Fkh2 seems to stay in the promoter instead of travel with the RNAPol II in opposition to Mei4. The way Fkh2 is inhibiting the recruitment of the spliceosome is still not known.

It is not well understood the relationship and interactions between Mei4 and Fkh2 in *rem1* promoter. Analyses of *rem1* splicing in double mutant $\Delta mei4\Delta fkh2$ cells by RT-PCR are shown in figure 11. This result was very surprising for us because we expected a similar behaviour to the single $\Delta fkh2$ mutant but the result was different. It behaves as single mutants in a time-dependent manner: during the first three hours of meiosis it behaves exactly as a $\Delta fkh2$ strain but on the onset of meiosis (concomitantly to Mei4 expression in a wild type strain) it starts behaving as a $\Delta mei4$ strain, losing the spliced mRNA and accumulating non-processed mRNA (compare single mutants and double mutant pannels before and after 3h of meiosis at figure 11).

Future work would consist in determine the different domains of the Mei4 implicated in this regulation and in search other components of this mechanism of regulation.

At the protein level, we have seen that Rem1 is actively degraded by the proteasome since the absence of meiosis-specific E3-ubiquitin ligase Mfr1, Rem1 is tabilize until the end of meiosis (Fig. 17).

No studies have been done about post-tranlational modifications on Rem1.

Regulation of other meiotic intron-containing genes

We have shown that intron retention is a common regulation system for meiotic protein expression in *S. pombe*. There are at least ten more genes that behave as *rem1*. (Fig. 19). All of them have at least one FLEX motif in their promoters whose consensus seems to be longer (Fig. 21) than the one reported before (Horie et al., 1998). Although *cis* differences between FLEX motifs with regulated and constitutive splicing could not be determined, future studies about chromatin structure and interaction of the Forkhead transcription factors between them and the DNA will give us more answers about it.

Meiosis, as a differentiation process, has a completely alternative gene expression pattern to the normal mitotic cell cycle. To assure that overlapping of both gene expression programs is avoided, the transcriptional regulation seems not to be enough. Other save-lock mechanisms exist such as the *mmi1* system (Harigaya et al., 2006) or the active repression of intron-containing meiotic genes (Averbeck et al., 2005; Malapeira et al., 2005). We have studied the molecular basis of the latter determining that Fkh2 and Mei4 regulate not only *rem1* but other meiotic-specific genes. It is still not determined if all the other genes have different functions depending on the mRNA intron removal.

Searching for genes containing FLEX motifs in their promoters in the *S. pombe* genome allowed us to develop basic software able to be adapted for future searching of any other motif in all *S. pombe* promoters.

Mei4 and Fkh2 regulation

During meiosis, *fkh2* transcription increases slowly while *mei4* has an abrupt induction at mid-meiosis (Fig. 22). At the level of protein Mei4 parallel the pattern of its mRNA while Fkh2 seems to have more or less the same quantity of total protein although we can observe a dynamic pattern of slower migrating bands peaking at mid-meiosis.

Taking into account the hypothesis that Fkh2 is constitutively bound to *rem1* promoter, there are two possibilities to explain its different behaviour without leaving the promoter. One of them is that there could be other factors binding the promoter, the other is that Fkh2 could be suffering post-translational modifications. As shown before, Fkh2 is hyperphosphorylated at mid-meiosis (Fig. 22). A candidate to produce this phosphorylation is the complex Cdc2/Cig2, which has high levels of kinase activity at mid-meiosis (Borgne et al., 2002). Fkh2 has 18 putative phosphorylatable sites by Cyclin/Cdks complexes so we checked if Cig2 purified at different time points of meiosis could phosphorylate Fkh2. Using different truncated proteins we could find a fragment (NdeI-EcoRI) that contains three S/T-P residues which were putative candidates for the modification (Fig. 24). Mutations to Alanine at residue Ser522 had no significant effect on the phosphorylation, mutating Ser432 has minor effects but Ser481Ala mutation resulted in a decrease of 90% in the phosphorylation levels (Fig. 25 and 26). All these assays were performed in vitro with recombinant Fkh2 so the future work consists on validating these results in vivo and study the relevance of Ser481 on Fkh2 function.

Regarding Mei4, it seems to have constitutive phosphorylation with a lower mobility band of unknown characteristics (it does not disappear with alkaline phosphatase or lambda phosphatase).

The interaction of the two transcription factors at *rem1* promoter is still not clear. Physical interaction of both factors could not be demonstrated, the strain used was tagged in both genes with Myc and HA. This strain suffered a slight delay in meiosis progression but not the single tagged strains (data not shown). Maybe this is pointing the possibility that both tags are interfering with Mei4 and Fkh2 function.

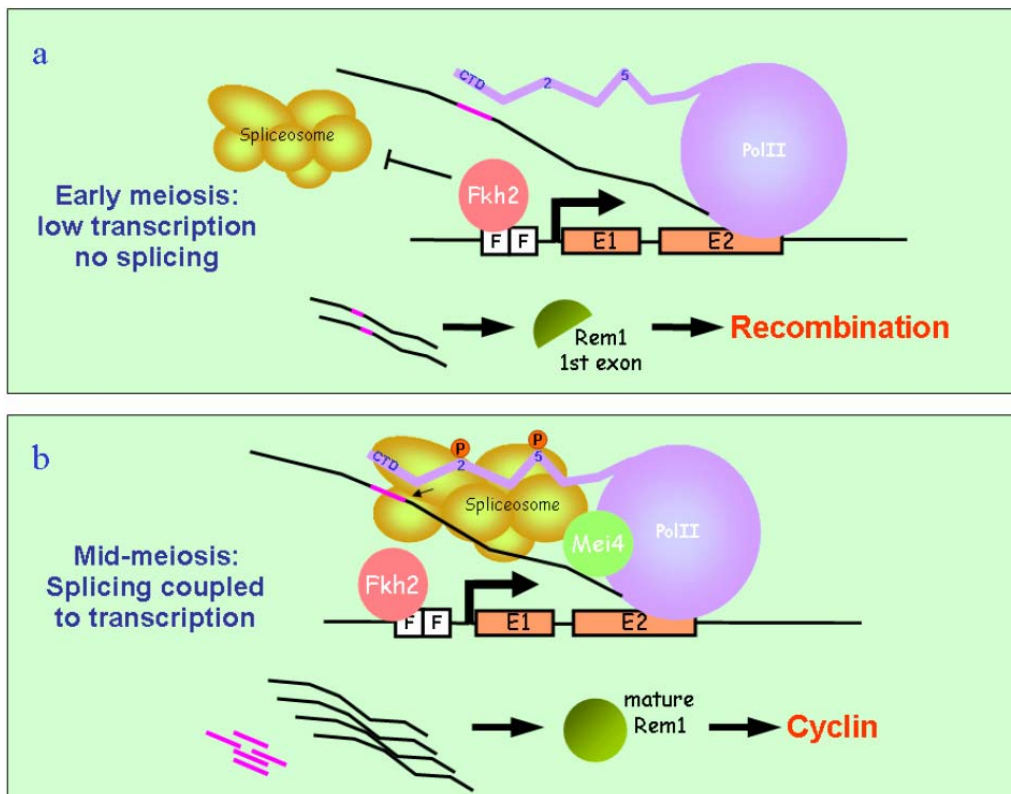


Figure 1. Model of the Fkh2-Mei4 regulation of *rem1*

The *in silico* modelling of the DNA-binding domain of both factors (Fig. 27 and 28) indicate that they bind differently the FLEX box.

A possible explanation is that Mei4 binding to the promoter FLEX boxes is weaker and the interaction with Fkh2 can stabilize it (Fkh2 seems to be constitutively bound to the promoter) through the Fkh2 Forkhead-Associated Domain (FHA) [this domain is known to mediate protein-protein interactions in other proteins]. At the beginning of meiosis Fkh2 alone (or in a homodimer form) would avoid RNAPol II and spliceosome machinery recruitment, achieving low transcription levels of unprocessed mRNA. This mRNA will be translated to a truncated protein with a recombination function. At mid-meiosis, Mei4 is expressed and could form a heterodimer Fkh2-Mei4 at *rem1* promoter. This way Mei4 would stabilize the RNA pol II and promote the recruitment of the spliceosome. Now, transcription will be induced and mRNA will be spliced, coding for the mature Rem1 protein with a function as a cyclin. The hypothesis is shown in the discussion figure 1

Rem1 function

We have shown that *rem1* gene can have two different protein products with different functions depending on the splicing of its only intron. If the mRNA remains unprocessed, a truncated protein is translated with a function in intragenic recombination (Fig. 30). At the onset of meiosis I, a transcription induction coupled to splicing is produced and the intron containing a stop codon in frame is removed. This processed mRNA is translated to the mature Rem1 with a function as a cyclin (Fig. 29). Although direct targets have not been found for the Cdc2/Rem1 complex, our group saw that Rem1 promotes the degradation of Cdc18. This could induce a cycle without DNA replication what would explain the mitotic catastrophe of cells overexpressing Rem1 and why is so important that Rem1 is only expressed in Meiosis I.

The system reported here could account for the first case of alternative splicing in a unicellular eukaryote, which when the mRNA is not processed (during the pre-meiotic S phase) will render a 17 KDa protein that will have a role in recombination and when the mRNA is processed will produce a protein with a described function as a cyclin during meiosis I.

CONCLUSIONS

rem1 promoter is the most important region for the gene regulation of transcription and splicing, specifically the two FLEX boxes found there. *rem1* promoter is necessary and enough to regulate *rem1* expression.

Rem1 regulation is based on, at least, a two factor model. One factor repressing the splicing in vegetative growth and early meiosis and another one inducing the splicing at mid-meiosis.

Fkh2 is the repressor factor, which binds *rem1* promoter constitutively.

Mei4 is the activator factor, which binds *rem1* promoter only in mid-meiosis and is able to travel with the RNAPolymerase II, stabilize it and interact physically with the spliceosome to recruit it.

There are at least other 10 genes that are regulated as *rem1*, which means that intron retention is a general mechanism of regulation for meiotic intron-containing genes.

Mei4 and Fkh2 are phosphoproteins. Cdc2/Cig2 purified from mid-meiosis is able to phosphorylate *in vitro* Fkh2.

Fkh2 is specifically phosphorylated by Cdc2/Cig2 in Serine 481.

Rem1 has two different functions: at pre-meiotic S phase, *rem1* mRNA is not processed and a truncated 17 kDa Rem1 is translated with a function in intragenic recombination and at mid-meiosis *rem1* intron is removed and the mature 46 kDa Rem1 is expressed with a cyclin function.

Rem1 is degraded at late-meiosis by the E3 ubiquitin-ligase Mfr1.

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