Cuf2 Is a Novel Meiosis-Specific Regulatory Factor of Meiosis Maturation

Raphael Ioannoni¹, Jude Beaudoin¹, Luis Lopez-Maury², Sandra Codlin², Jurg Bahler², Simon Labbe¹*

¹ Département de Biochimie, Faculté de Médecine et des Sciences de la Santé Université de Sherbrooke, Sherbrooke, Québec, Canada, ²Department of Genetics, Evolution and Environment, University College London, London, United Kingdom

Abstract

Background: Meiosis is the specialized form of the cell cycle by which diploid cells produce the haploid gametes required for sexual reproduction. Initiation and progression through meiosis requires that the expression of the meiotic genes is precisely controlled so as to provide the correct gene products at the correct times. During meiosis, four temporal gene clusters are either induced or repressed by a cascade of transcription factors.

Principal Findings: In this report a novel copper-fist-type regulator, Cuf2, is shown to be expressed exclusively during meiosis. The expression profile of the cuf2 mRNA revealed that it was induced during middle-phase meiosis. Both cuf2 mRNA and protein levels are unregulated by copper addition or starvation. The transcription of cuf2 required the presence of a functional mei4 gene encoding a key transcription factor that activates the expression of numerous middle meiotic genes. Microscopic analyses of cells expressing a functional Cuf2-GFP protein revealed that Cuf2 co-localized with both homologous chromosomes and sister chromatids during the meiotic divisions. Cells lacking Cuf2 showed an elevated and sustained expression of several of the middle meiotic genes that persisted even during late meiosis. Moreover, cells carrying disrupted cuf2/cuf2 alleles displayed an abnormal morphology of the forespore membranes and a dramatic reduction of spore viability.

Significance: Collectively, the results revealed that Cuf2 functions in the timely repression of the middle-phase genes during meiotic differentiation.

Introduction

Meiosis is a specialized type of cell division by which sexually reproducing diploid organisms generate haploid gametes [1]. Gametogenesis starts with the pre-meiotic S-phase during which the DNA is replicated, thereby generating pairs of homologous chromosomes. Subsequently, these homologous chromosomes are subjected to genetic recombination, a process which is known to contribute to phenotypic diversity within a given population [2]. Homologous chromosomes and sister chromatids are then successively segregated, generating four haploid sets of chromosomes that are inheritable to the next generation. In the terminal stage of meiosis, a differentiation program is induced in order to generate four mature gametes ready for fertilization. Whereas these meiotic hallmarks have been extensively characterized, the molecular mechanisms that control meiotic progression and gamete maturation in higher eukaryotes remain less well understood.

Haploid gametes are present in very small numbers in most mammals, making molecular studies that require substantial amounts of material very arduous [3]. An additional difficulty comes from the fact that both animal models and tissue co-culture cells are not easy to synchronize with respect to their entry into meiosis [4,5]. Consequently, the use of model organisms has turned out to be a practical solution for the study of the molecular mechanisms that initiate and control meiosis [6,7]. Among these models, the fission yeast Schizosaccharomyces pombe has become particularly attractive for the study of the key molecular aspects of meiosis, from its initiation through to the generation of mature haploid cells [8]. S. pombe cells essentially undergo meiosis in a manner analogous to that of the germ line cells in higher eukaryotes, except that the gametes differentiate into spores that are enclosed in an ascus. Each ascus contains four haploid spores that are highly resistant to adverse environmental conditions [9].

Nitrogen availability triggers the decision to follow either the mitotic or the meiotic pathway in S. pombe. Under nitrogen-rich conditions, cells grow mitotically because the Pat1 kinase inhibits the initiation of meiosis by phosphorylating both the transcription factor Ste11 and the meiotic inducer Mei2 [10]. Conversely, under nitrogen-starved conditions, the Ste11 transcription factor becomes active and induces the expression of the mating type loci [11]. As a consequence, haploid cells of the opposite mating types conjugate, forming diploid zygotcs. A cascade of transcription factors then enables the expression of mei3, which encodes for an...
inhibitor of the Pat1 kinase [12]. Once, Mei3 inhibits Pat1, the latter becomes unable to phosphorylate its target proteins, including Ste11 and Mei2. As an active Ste11 fosters mei2+ expression, unphosphorylated Mei2 accumulates and triggers the initiation of zygotic meiosis [10]. Zygotes can be returned to a nitrogen-rich medium before commitment to meiosis and will resume vegetative growth, forming colonies of diploid cells. Conveniently, these cells will undergo asygotic meiosis in response to a nitrogen starvation shock in a more synchronous manner than zygotic meiosis [13]. The pat1-114 allele encodes a thermosensitive version of the Pat1 kinase. Consequently, cells harboring the pat1-114 mutation show temperature-sensitive growth and undergo meiosis and sporulation at the restrictive temperature (34°C), thus bypassing the Mei3-dependent inactivation pathway of Pat1. The advantage of pat1-induced meiosis is that it is more synchronous than asygotic meiosis [13].

In fission yeast, meiosis progression is driven by an extensive gene expression program during which the expression of several genes is either induced or repressed [14]. Transcriptional profiles have defined four successive waves of gene expression that are mainly controlled by key meiosis-specific regulators [15]. First, nitrogen starvation triggers the activation of Ste11, which in turn activates the expression of the nutrient-responsive genes so as to initiate meiosis [11]. The expression of the nutrient-responsive genes is subsequently repressed by the transcription factor Rep1, which in turn activates the expression of several early meiotic genes [15]. Once chromosome pairing and homologous recombination have been completed, the transcription factor Mei4 activates the expression of the middle meiotic genes and represses that of the early genes [16,17]. Rep1 and Mei4 function in both the activation and the repression of gene expression. Although the transcription factors Atf21, Atf31 and Rsv2 activate the expression of the late meiotic genes after the meiotic divisions, it is still unclear how the middle genes are repressed at the end of the middle meiotic phase, a step that precedes late meiosis [15].

When the previously identified potential meiotic transcriptional regulators were examined, one, denoted Cuf2 (SPCC584.02) was found to be a putative copper-fist-like transcription factor whose expression profile exhibited a peak that coincided with both the meiotic divisions and the forespore membrane formation (FSM) [14]. Based on sequence homologies with other metalloregulatory transcription factors, it is predicted that the amino-terminal residues 1 to 40 of Cuf2 contains a Zn2+ module that is required for DNA minor groove binding [18]. The Cuf2 protein exhibits 42% sequence identity with the N-terminal 61 amino acid residues of the copper-fist-like transcription factor Cuf1 [19,20]. In S. pombe, Cuf1 activates transcription of genes that encode for Ctr copper transporters (Ctr4, Ctr5 and Ctr6) that are involved in copper acquisition [19,21–23]. Structurally, the similarity between the Cuf1 and Cuf2 proteins resides exclusively within the amino-terminal 61-residue segment of the two proteins. As is suggested for Cuf1-related transcription factors, residues 41–60 of Cuf2 that display high similarity with the residues 41–61 of Cuf1 may be required in order to allow the transcription factor to make contact with the major groove of DNA [19,24].

To gain further insight into the role of Cuf2, its expression profile was characterized. It was discovered that Cuf2 was strictly expressed during meiosis. The expression of cuf2+ required the presence of a functional mei4+ gene, which encodes a key regulator of several middle-phase meiotic genes. Microscopic analyses revealed that a functional Cuf2-GFP protein co-localized with the chromosomes that underwent late anaphase I, metaphase II, anaphase II (early and late) and FSM formation. Furthermore, pan-S. pombe microarray analysis revealed that 247 genes were up-regulated in a cuf2A/cuf2A disruption strain. In the absence of Cuf2, forespore membrane formation was abnormal and spore viability was significantly reduced. Taken together, the results of these studies revealed that Cuf2 is a meiosis-specific transcriptional regulator that is required for the down-regulation of a large set of middle-phase meiotic genes during meiotic development.

**Results**

**S. pombe Cuf2 protein**

Analysis of genomic DNA sequences from the *S. pombe* Genome Project revealed an open reading frame (SPCC584.02) that encoded an uncharacterized protein which displayed an extended homology at its N terminus to the N terminus of the *S. pombe* copper-sensing transcription factor Cuf1. Because this common region was shared by the two proteins, the locus SPCC584.02 was named *cuf2+. The N-terminal 60 amino acid segment of Cuf2 displayed 42% sequence identity and 62% sequence similarity with the Cuf1 61 amino acid segment (Fig. 1). A putative zinc coordination domain (residues 1–40) lies within this region of Cuf2 and may be part of a minor groove DNA binding domain that is similar to those found in previously characterized copper metalloregulatory transcription factors [25]. Such regulators are mainly involved in either the copper detoxification or copper acquisition pathways. In particular, the Ace1 and Amt1 proteins from the yeasts *S. cerevisiae* and *C. glabrata*, respectively, promote copper detoxification via the induction of metallothionein gene transcription and possess such a domain [26]. In the yeasts *S. cerevisiae* and *S. pombe*, the Mac1 and Cuf1 proteins, respectively, promote copper acquisition through the induction of the copper transporting genes [20,27]. Similar to Ace1, Amt1, Mac1 and Cuf1, Cuf2 harbors a conserved (R/K)GRP motif (Fig. 1) that is known to be involved in the direct binding of the nucleotides located within the minor groove of DNA helix [18,24]. Interestingly, the N-terminal region of Cuf2, specifically residues 11 to 53, contained 9 basic amino acids (Arg10, Arg26, Arg28, Arg34, Arg36, Arg38, Lys36, Arg40 and Lys47) that are highly conserved in Cuf1 and whose presence (underlined amino acids) has been shown to be required for the targeting of Cuf1 to the nucleus [28]. The first N-terminal 60 amino acid residues of Cuf2 also shared a strong sequence homology with the N terminal sequences of the Ace1 and Amt1 proteins. However, Cuf2, as is the case of Cuf1, did not possess the second half of the Ace1/Amt1 copper regulatory domain in which two highly conserved Cys-X-Cys sequences of the Ace1 and Amt1 proteins are found [29]. The absence of these two Cys pairs in Cuf2 makes improbable the formation of Ace1/Amt1-like copper regulatory domain that consists of two lobes separated by a cleft in which a Cu₄S₄ center takes place in the presence of copper ions [18]. Moreover, as opposed to Cuf1 and Mac1, Cuf2 did not contain a Cys-rich domain (Cys-X-Cys-X₄-Cys-X₄-Cys-X₄-His) located near its C terminus that could sense and coordinate copper ions [29–31]. In fact, the C-terminal region of Cuf2 lacked any potential motif that could bind copper ions. Nevertheless, because some features of Cuf2 were reminiscent of copper-regulatory transcription factors, the *cuf2+* gene was isolated for further analysis.

**cuf2+ is expressed exclusively during meiosis**

Initial experiments using cells proliferating in mitosis failed to detect the *cuf2+* transcript regardless of the copper status (Fig. 2A). In contrast, as expected, the *ctr4+* copper transport mRNA levels (assayed as a control) were up- (~10-fold) and down- (~3-fold) regulated after treatment with the copper chelator tetrathiomolybdate (TTM) and copper, respectively, as compared to basal
conditions (Fig. 2A). The *cuf2* transcript was first detected in genome-wide studies in which cells were switched from mitosis to meiosis [14]. After cell entrance into meiosis, the expression profile of *cuf2* revealed that it reached a maximum 5 h after meiotic induction, indicating that *cuf2* was a middle meiotic gene potentially involved in the differentiation process [14].

*S. pombe* cells growing mitotically carry an active Pat1 kinase which inhibits cells from entering meiosis. Cells harboring the *pat1-114* mutation show a temperature-sensitive growth and undergo meiosis at the restrictive temperature of 34°C. The use of the *pat1-114* temperature-sensitive mutant permits the synchronization of cells in terms of their entry into the meiotic program [12]. To further investigate the expression profile of *cuf2* during meiosis, a *pat1-114/pat1-114* diploid strain that was pre-synchronized in G1 mitotic-phase by nitrogen starvation at 25°C was used. The temperature was then shifted to 34°C so as to inactivate Pat1 and allow the cells to undergo synchronous meiosis. The results obtained were consistent with data reported by others [14], namely that the expression of *cuf2* peaked between 4 and 6 h after meiotic induction (Fig. 2B). A homozygous *pat1-114/pat1-114 cuf2Δ/cuf2Δ* diploid strain was generated to validate the signal corresponding to the *cuf2* RNase protection product (data not shown). In all of the experiments the *cuf2* transcript was found to be absent in the disruption strain (*cuf2Δ/cuf2Δ*), irrespective of the copper status (data not shown). To examine if copper availability had any effect on the expression of *cuf2* during the meiotic program, the cells were either left untreated or were treated with either the copper chelator TTM (50 μM) or CuSO4 (50 μM) prior to the temperature shift (34°C). Aliquots of cultures were taken every 2 h following meiotic induction, and the steady-state levels of *cuf2* mRNA were analyzed by RNase protection assays. Results showed that the steady-state levels of *cuf2* mRNA under basal (untreated), copper-starved (50 μM TTM) or copper replete (50 μM CuSO4) conditions were primarily increased between 4 and 6 h following meiotic induction (Fig. 2B). In response to 50 μM copper, the *cuf2* mRNA levels were induced within 4 h, but were down-regulated to a lesser degree over time and were maintained at a level ~1.4-fold above the basal mRNA levels detected in untreated cells (Fig. 2B). To ascertain whether or not the steady-state levels of Cuf2 protein followed those of the *cuf2* mRNA, a *pat1-114/pat1-114 cuf2Δ/cuf2Δ* strain in which a functional *cuf2Δ-TAP* fusion allele was returned into the genome by integration was used. The results showed that, under basal, low or high copper conditions, Cuf2-TAP protein levels increased in a manner similar to that of the *cuf2* transcript levels, although it should be noted that steady-state levels of Cuf2 protein remained present for a longer period of time than did the mRNA (Fig. 2C).

To ensure that cell growth conditions (basal, 50 μM TTM or CuSO4) had no negative effect on meiotic progression and sporulation, a series of microscopic analyses were performed. *pat1-114/pat1-114* diploid cells were synchronously induced into meiosis and Hoescht 33342 was added to 0.5 μg/μl every hour to cell culture aliquots to visualize the DNA and monitor meiotic progression. Under basal and copper conditions, meiosis I occurred primarily between 3.5 and 6 h after meiotic induction, meiosis II between 6.5 and 8 h (Fig. 2D) and sporulation after 8 h (data not shown). Although meiotic progression of cells under mild copper starvation conditions (50 μM TTM) was slowed by approximately 1 h as compared to untreated (basal) cells, spore formation was clearly observed at the end of meiosis (data not shown). Globally, cells that were grown under basal, low or elevated (50 μM) copper concentrations displayed no significant changes in their ability to proceed meiosis since there was no apparent timing defect in their progression (Fig. 2D). Taken together, these results indicated that, under basal, copper-depleted and copper-replete conditions, the *cuf2* gene is effectively but transiently expressed during meiosis. Furthermore, the Cuf2 protein was mostly produced during meiotic divisions, with reduced levels of protein that persist towards the end of the meiotic program.

Figure 1. Comparison of Cuf2 with the *S. pombe* copper metalloregulatory transcription factor Cuf1. A. Schematic representations of Cuf1 and Cuf2. The amino acid sequences of both proteins are numbered relative to their initiator codons. The locations of the domains required for Cuf1 function are indicated, including the N-terminal nuclear localization signal (NLS) (11–53) that is located within the DNA-binding module (1–174), the C-terminal Cu-sensing module (C-rich) (328–342) and the C-terminal nuclear export signal (NES) (349–358). The positions of some of the Cys (C) and His (H) residues within both Cuf1 and Cuf2 are also indicated. B. Amino acid alignment of the N-terminal 61 amino acid residues of Cuf1 with the N-terminal 60-residue segment of Cuf2. The black boxes indicate identical amino acids, and the gray boxes indicate amino acids that are similar between Cuf2 and Cuf1. The asterisks highlight the 7 Cys residues that are conserved between Cuf2 and Cuf1.
Figure 2. Assessment of the mRNA and protein steady-state levels of Cuf2 during meiosis. 

A, Representative expression profiles of the cuf2+ and ctr4+ mRNAs in h+ haploid cells that were either left untreated (−) or were treated with either 50 μM TTM or 50 μM CuSO4 during mitosis. B, Cultures of pat1-114/pat1-114 diploid cells were either maintained in vegetative growth at 25°C, or were induced to initiate and proceed through meiosis at 34°C. pat1-114/pat1-114 diploid cells were either left untreated, or incubated in the presence of 50 μM TTM or 50 μM CuSO4. Total RNA was isolated at the indicated time points after the induction of meiosis. Shown are representative RNase protection assays of both the cuf2+ and the act1+ (internal control) mRNA steady-state levels during meiosis.

C, Cuf2-TAP protein expression during meiosis. pat1-114/pat1-114 cuf2/cuf2 diploid cells expressing Cuf2-TAP were either left uninduced (25°C), or were induced (34°C) under basal conditions or in the presence of 50 μM TTM or 50 μM CuSO4. Shown are Western blots of both Cuf2-TAP and α-tubulin (control loading) levels at different time points after meiotic induction.

D, Meiotic progression of cells under either basal (untreated) conditions, or in the presence of TTM (50 μM) or CuSO4 (50 μM). The values shown for each condition (TTM, basal or Cu) correspond to the percentage of cells with 1, 2, or 3–4 nuclei, and the percentage of cells with horse tails. The graphed values represent the averages of triplicate determinations +/− the standard deviations.

doi:10.1371/journal.pone.0036338.g002
The mei4+ gene is required for cuf2+ gene expression

Genome-wide studies of the global effects of the deletions of meiosis-specific transcription factors have revealed that the transcription regulator Mei4 is required for the induction of numerous of the middle meiosis-specific genes [15]. To independently assess whether or not Mei4 was necessary for the expression of the middle meiotic gene cuf2+, RNease protection assays in synchronous meiosis experiments were carried out using a pat1-114/pat1-114 mei4A/mei4A diploid strain and the results were compared to those obtained with a pat1-114/pat1-114 control strain. In the case of pat1-114/pat1-114 control cells, cuf2+ exhibited a typical middle meiosis gene time-dependent expression profile, peaking 4 h after meiotic induction (Fig. 3). In contrast, cuf2+ mRNA was absent in pat1-114/pat1-114 mei4A/mei4A cells throughout meiosis (Fig. 3). Furthermore, the cuf2+ transcript was not detected in the pat1-114/pat1-114 mei4A/mei4A mutant under all of the conditions tested, including basal, copper-replete and copper-depleted conditions (data not shown). The observation that the pat1-114/pat1-114 mei4A/mei4A deletion strain showed a loss of cuf2+ gene expression indicates that the meiotic-dependent expression of cuf2+ mRNA requires Mei4.

Subcellular localization of Cuf2 during meiosis

Based on the fact that Cuf2 was a meiosis-specific protein, the next step was the elucidation of its subcellular location during the meiotic program. Hence, a GFP coding sequence was fused in-frame with the 3′-end of the cuf2+ gene. When the Cuf2-GFP fusion protein was expressed in meiosis, it complemented the FSM formation deficiency and triggered the down-regulation of several fusion protein was expressed in meiosis, it complemented the FSM formation. Microarrays were hybridized with probes derived from RNA isolated from either cuf2Δ/cuf2Δ mutant cells or control (cuf2+ /cuf2+ ) cells. The analysis of gene expression profiling data obtained nine hours after meiotic induction revealed that 247 genes were expressed at higher levels in the cuf2Δ/cuf2Δ mutant cells (averaging ≥1.5-fold) (Fig. 5A; Table S1). Conversely, the data also revealed that 298 genes were expressed at lower levels in the cuf2Δ/cuf2Δ mutant cells (averaging ≤1.5-fold) (Fig. 5A). The reason why we selected the 9-h time point for microarray experiments was based on the fact that we wanted to leave time of the translational process and the protein product (Cuf2) to take place and operate after the production of the cuf2+ transcripts in cuf2Δ/cuf2Δ cells. As previously reported, the transcription profiles of the meiotic cell cycle have defined four successive waves of gene expression that coincide with the major meiotic phases. These are, wave 1 in response to nutrient starvation (nutrient-responsive genes), wave 2

![Figure 3](image-url)

Figure 3. cuf2+ gene expression is Mei4-dependent. pat1-114/pat1-114 (mei4Δ/mei4Δ) and pat1-114/pat1-114 mei4A/mei4A strains were pre-synchronized by nitrogen starvation at 25 C, and then were induced to undergo synchronous meiosis at 34 C. At the indicated time points, the cuf2+ and act1+ (internal control) mRNA levels were analyzed in both the control strain (mei4+/mei4+) and the isogenic strain lacking the mei4+ alleles. To validate the absence of the cuf2+ transcript during mitosis, total RNA was probed for the presence of cuf2+ mRNA in vegetative cells incubated at 25 C.

doi:10.1371/journal.pone.0036338.g003
that involves premeiotic replication and recombination (early meiotic genes), wave 3 during which the meiotic divisions occur (middle meiotic genes), and wave 4 that is associated with spore formation (late meiotic genes) [14]. Among the 247 genes that were expressed at higher levels in the absence of Cuf2, 149 were classified as either early (4 genes), middle (144 genes) or late (1 gene) meiotic genes (Table S2). The other 98 genes were unclassified with respect to the expression waves. Strikingly, 144 out of a total of 149 genes classified were middle meiotic genes (97%). The expression levels of these 144 genes were up-regulated and remained at high steady-state levels even during late meiosis when their expression levels normally were reduced in order to return them to a basal level of expression.

Some of the genes with increased expression levels in the absence of Cuf2 could be grouped together based on their predicted protein products or meiotic-specific profiles of expression. Some groups of proteins displayed conserved homologous domains that were potentially functionally important. One example of these groups included the meiotic genes encoding the family of uncharacterized DUF999 proteins of which 9 members were identified (Fig. 5B; Tables S2, S3 and S4). A second group of 15 genes, which encoded the Wt family of proteins, was also expressed at higher levels in cuf2A/cuf2A mutant cells. wtf genes are known to be flanked by intergenic regions that contained long terminal repeat fragments of retrotransposons, and are transcribed during meiosis [32]. A third group includes several members of the Mug (meiotically up-regulated gene) family of proteins that were abnormally up-regulated in the absence of Cuf2 (Table S4).

Because the results indicated that Cuf2 may have a role in repressing middle meiotic genes, we concentrated our efforts on this aspect in the present study. To assess whether the microarray analyses were successful in identifying the up-regulated genes in cuf2A/cuf2A mutant cells, RNases protection assays were performed using an independent biological trial to examine the relative expression levels of four middle-phase meiotic genes: SPAC1B2.03c, wtf13, SPBC1348.01 and meu14. In the case of the cuf2A/cuf2A mutant strain, at all points examined after 10 h, the levels of these four mRNAs were up-regulated as compared to those in control cuf2/cuf2 cells (Fig. 5C). As expected in experiments using either DNA microarray or RNase protection analysis, SPAC1B2.03c, wtf13, SPBC1348.01 and meu14 transcripts exhibited similar increases in transcript abundance in a strain lacking Cuf2. At the 10-h time point, RNase protection experiments revealed that the expression level of SPAC1B2.03c, wtf13, SPBC1348.01 and meu14 were increased 4.4-, 1.6-, 3.6- and 4.5-fold, respectively, while DNA microarray analysis showed increases of 3.1-, 2.4-, 2.5- and 2.2-fold, respectively (Fig. 5C and Table S2).

Interestingly, cuf2A/cuf2A cells showed sustained higher levels of meu5 expression (2.3-fold up-regulated according to the DNA microarray analysis) as compared with those observed in the control (cuf2/cuf2) cells. The meu5 gene encodes a RNA-binding protein that stabilizes meiosis-specific transcripts, especially those expressed in the middle meiotic phase [33]. To further investigate the relation between the cuf2 and meu5 genes, the meu5 regulon [33] was compared to that of cuf2. In all 93 genes were found to be common to both regulons (Fig. 6A; Table S3). Based on this observation, we hypothesized that, in the absence of Cuf2, meu5 expression was up-regulated. As a consequence, the Mei5 protein was present for a longer period of time, thereby stabilizing and extending the presence of several middle-phase meiotic transcripts to the later time points. To independently verify the microarray data, the diploid strains pat1-114+cuf2/cuf2 and pat1-114+cuf2/cuf2 were synchronized through meiosis and the meu5 mRNA levels were monitored at different time points after meiotic induction. Consistent with the microarray results, the meu5 mRNA levels were ~2.4-fold higher (in cuf2/cuf2 control cells) after 10 h of meiotic induction and remained elevated (~4.2-fold higher) even after both 12 and 14 h of meiotic induction (Fig. 6B). To further investigate the effect of Mei5 on the expression profile of Cuf2-regulated target genes, two additional diploid mutant strains, pat1-114 meu5A/meu5A and pat1-114+cuf2A/cuf2A meu5A/meu5A, were created. In the absence of Cuf2 (cuf2A/cuf2A cells), wtf13 and SPAC1B2.03 transcripts were found to be significantly increased after 10 h of meiotic induction (Figs. 5C, 6C and D). In contrast, only very low amounts of the wtf13 and SPAC1B2.03 transcripts were detected in pat1-114 meu5A/meu5A cells (Fig. 6, C and D). These results correlate well with the concept that Mei5 stabilizes and extends the presence of wtf13 and SPAC1B2.03 mRNAs to the later meiotic time points. In cells lacking both Cuf2 and Mei5 (cuf2A/cuf2A meu5A/meu5A), the levels of wtf13 and SPAC1B2.03 mRNAs were found to be slightly higher as compared with those observed in pat1-114 meu5A/meu5A, but lower as compared with

Figure 4. Analysis of Cuf2-GFP localization during both meiosis and sporulation. The Cuf2-GFP fluorescence signal (center left) was observed in Cuf2-GFP+ cells. The Cuf2-GFP fluorescence signal was detected during the FSM formation (i.e. at the end of meiosis), but disappeared during spore formation. Nomarski optics (far left) were used to visualize Cuf2-GFP fluorescence signal during the FSM formation. Cells at different stages of meiosis were stained using Hoechst 33342 to visualize the DNA (centre right). The merged images are shown in the far right panels. Nomarski optics (far left) were used to monitor cell morphology.

doi:10.1371/journal.pone.0036338.g004
those detected in \textit{pat1-114 \textit{cuf2}\Delta/cuf2}\Delta cells (Fig. 6, C and D). Taken together, the results strongly suggest that Cuf2 is a meiosis-specific regulator that functions in a timely, controlled repression of middle genes during meiotic differentiation. Furthermore, the results suggest that Cuf2 and Meu5 have opposite effects on common meiosis-specific genes, specifically that Cuf2 down-regulates middle-phase transcripts, while Meu5 stabilizes the same transcripts.

\section*{Deletion of cuf2\textsuperscript{+} leads to FSM defects}

The results shown above revealed that the expression levels of several middle-phase meiotic genes were sustained, even during late meiotic time points in the absence of Cuf2 (Fig. 5 and Table S2). One possible explanation for this is that Cuf2 could be specifically required for the down-regulation processes that normally occurred only during the divisions steps. The absence of Cuf2 would trigger errors that could jeopardize both the quality and quantity of forespores. To test this possibility the meiotic differentiation in \textit{h}\textsuperscript{+}/\textit{h}\textsuperscript{2} \textit{cuf2}\Delta/cuf2}\Delta diploid cells was compared to that of \textit{h}\textsuperscript{+}/\textit{h}\textsuperscript{2} \textit{cuf2}\textsuperscript{+}/cuf2}\textsuperscript{+} control cells (Fig. 7A). Both strains were pre-synchronized in G1 by nitrogen starvation and were then synchronously induced to undergo azygotic meiosis. In the case of the control cells, 4 FSMs were detected 8 to 9 h after meiotic induction (Fig. 7A). The presence of FSMs was confirmed using a GFP-tagged Psy1 protein as Psy1 is a well-established FSM marker [34]. An abnormal number of FSMs were observed in the case of the \textit{cuf2}\Delta/cuf2}\Delta mutant cells. Specifically, \textasciitilde42% of the mutant cells exhibited more than 4 FSMs per ascus (Fig. 7, A and B, group S2).
whereas ~18% of cells displayed 3 FSMs per ascus (Fig. 7, A and B, group iii). Many cuf2Δ/cuf2Δ cells showed FSMs of various sizes (~15% of cells), including elongated FSMs with small buds forming a shape reminiscent of a shmoo (Fig. 7, A and B, group iv) as previously reported [35]. To confirm that the meiotic FSM maturation defect was due to the inactivation of cuf2Δ+, a h+/h− cuf2Δ/cuf2Δ strain was created in which wild-type cuf2Δ+ -GFP/cuf2Δ+ alleles were returned by integration and expressed under the control of the cuf2Δ+ promoter. In these experiments, normal FSM formation was observed when the strain underwent azygotic meiosis in a manner similar to that observed in control cells (Fig. 7B).

Further insight into the phenotype resulting from loss of Cuf2 function was gained by determining the presence of chromosomal DNA within each FSM structure. All asci generated from cuf2Δ+/cuf2Δ- cells contained chromosomal DNA (Hoescht 33342-staining) and was surrounded by FSMs (Fig. 7C). Although 4 chromosomal DNA spots were also found in cuf2Δ/cuf2Δ mutant cells, the chromosomal DNA spots were not all packaged into FSM structures (Fig. 7C and data not shown). Taken together, these results suggest that loss of Cuf2 leads to the formation of asci containing unpackaged chromosomal DNA in addition to the anucleated FSM shells observed with the shmoo-like FSM structures.

Spore viability is reduced in cuf2Δ/cuf2Δ mutant cells

We next asked whether the inactivation of Cuf2 that caused abnormal FSM structures was intrinsically linked to a decrease of spore viability. To answer this question [h+/h− cuf2Δ/cuf2Δ diploid cells were used, and the results obtained compared to those from either h+/h− cuf2Δ/cuf2Δ or h+/h− cuf2Δ/cuf2Δ cells in which a functional integrative plasmid harboring the cuf2Δ-GFP allele expressed under the control of the cuf2Δ promoter was present. After 12 h of induced azygotic meiosis, the cells were analyzed by

Figure 6. cuf2Δ and meu5Δ gene disruptions have opposite effects on common meiosis-specific genes. A, Venn diagram representing the overlap between the cuf2Δ− and the meu5Δ− dependent genes. B, Total RNA from both the pat1-114/pat1-114 (cuf2Δ/cuf2Δ) and pat1-114/pat1-114 cuf2Δ/cuf2Δ disruption strains was analyzed throughout meiosis and sporulation. A representative RNase protection experiment of the effect of the absence of Cuf2 on the expression of the meu5 transcript (especially 10 to 14 h after meiotic induction) is shown. C–D, Cultures of pat1-114/pat1-114, pat1-114/pat1-114 cuf2Δ/cuf2Δ, pat1-114/pat1-114 meu5Δ/meu5Δ and pat1-114/pat1-114 cuf2Δ/cuf2Δ meu5Δ/meu5Δ diploid cells were synchronously induced into meiosis under basal conditions. Total RNA was isolated from culture aliquots taken at the indicated time points. After RNA preparation, the wtf13Δ and SPAC1B2.03cΔ steady-state mRNA levels were analyzed by RNase protection assays using actin (act1Δ) as an internal control. The results shown are representative of three independent experiments.

doi:10.1371/journal.pone.0036338.g006
performing tetrad dissection assays. Spores dissected from \( h^+/h^- \) \( cuf2\Delta/cuf2\Delta \) ascis exhibited a \( \sim 59\% \) decrease in viability as compared to those of both wild-type and \( h^+/h^- \) \( cuf2\Delta/cuf2\Delta \) ascis. When GFP-Psy1 localized to 4 circular FSM structures, these FSMs were classified as normal (i). Histograms showing the percentages of each normal (i) and abnormal (ii, iii, iv) FSM structure in both wild-type (\( cuf2^+/cuf2^+ \)) and \( cuf2\Delta/cuf2\Delta \) mutant cells, as well as in a diploid \( cuf2\Delta/cuf2\Delta \) disruption strain in which wild-type copies of the \( cuf2^+ \) fusion gene were reintegrated. C, Typical images of FSM structures 9 h after meiotic induction in both wild-type (\( cuf2^+/cuf2^+ \)) and \( cuf2\Delta/cuf2\Delta \) mutant cells (top panels). Each strain had previously been transformed with pJK210GFP-Psy1, which encodes GFP-Psy1 that is used as an FSM-resident marker. Hoechst 33342 staining was used to visualize the chromosomal DNA (middle panels). The merged images of the GFP-Psy1 and the Hoechst 33342 dye are shown in the bottom panels. Anucleated FSM structures, or unpackaged nuclei, are indicated by the white arrows.

**Discussion**

Blunt searches of the *S. pombe* proteome identified two proteins that contain a putative copper-fist-like domain. The first protein, Cuf1, activates the *ctr4, ctr5* and *ctr6* genes, which all encode copper transport proteins [20,21,23,31,36–38]. The second protein containing a putative copper-fist-like domain was Cuf2 for which relatively little data was available [14]. Curiously, Cuf2 was found to be strictly expressed during meiosis. Because of this, Cuf2 became highly interesting as it represented the first example of a meiosis-specific copper-fist-like transcription factor. Consequently, the *cuf2* gene was isolated and analyzed. Transcriptome
analyses of S. pombe cells undergoing synchronized meiosis permitted the identification of meiosis-specific transcripts. In these analyses, *cuf2* was consistently identified as being a meiosis-specific middle gene, exhibiting a peak of expression between 4 and 6 h after the induction of meiosis. Middle meiotic genes are transiently expressed during meiotic divisions when the segregation of the homologous chromosomes (MI), and ultimately of sister chromatids (M2) occurs. The meiosis-specific forkhead-type transcription factor Mei4 expresses the activity of the majority of the middle meiotic genes [15,17]. Previous microarray experiments have suggested that *cuf2* expression requires Mei4 [15]. In agreement, the results presented here demonstrate that *cuf2* transcription relies on Mei4, since *cuf2* expression was completely abolished in a *mei4Δ*/*mei4Δ* mutant strain (Fig. 3).

Analysis of the *cuf2* promoter revealed that it contains four putative consensus Mei4-binding FLEX sequences (positions −59 to −55, −335 to −341, −1104 to −1110 and −1209 to −1215) (the nucleotide numbers refer to the position relative to the A of the initiator codon of the *cuf2* gene) (data not shown). This observation is consistent with the genetic data and further suggests that Mei4 could directly activate *cuf2* expression.

The transcription of *cuf2* was unaffected when meiotic cells were treated with ≤50 μM TTM or ≤50 μM CuSO4. However, cells that had been treated with ≥75 μM CuSO4 consistently exhibited a timing defect in their meiotic progression (data not shown). Under toxic copper concentrations (e.g. ≥75 and 100 μM), *mei4* and *cuf2* expression profiles were delayed and overall reduced during the meiotic process (data not shown). These results suggested that toxic levels of copper could negatively affect the expression of several middle meiotic genes. Furthermore, we consistently observed that toxic copper levels (≥75 and 100 μM) affected the ability of meiotically induced cells to complete meiosis. Under these toxic levels of copper (≥75 and 100 μM), the ascospore maturation was abnormal (data not shown). These observations suggested that an excess of copper has a generic negative effect on meiotic gene expression, progression and maturation, rather than specifically affecting the expression of *cuf2* and/or *mei4* (data not shown). This effect may be explained by the fact that in excess (≥75 and 100 μM), copper may induce intracellular oxidative stress through the generation of hydroxyl radicals produced by the Fenton reaction [39]. Nevertheless, considering its copper-fist-like structure, it is not impossible that Cuf2 may be affected by the cellular status of copper.

The results presented indicate that the expression of *mei4* and *mei4* middle genes is up-regulated in a *cuf2Δ/cuf2Δ* mutant strain 10–14 h after meiotic induction. Moreover, analysis of genome-wide gene expression 9 h after meiotic induction revealed that the expression of ~75 genes was up-regulated (averaging ≥1.5-fold) in a *cuf2Δ/cuf2Δ* mutant strain. Interestingly, these genes were associated with a large spectrum of possible cellular functions (Fig. 5B and Table S4), indicating that Cuf2 could act as a middle phase meiotic regulator rather than as a regulator of the gene expression of a specific homeostatic pathway. The microarray results also showed that 298 genes were down-regulated in *cuf2Δ/cuf2Δ* mutant cells (Table S5). Because several of these genes encode ribosomal proteins (~67), which may reflect a global decrease of the cellular metabolic status, we first concentrated our efforts on the analysis of the genes that were up-regulated in the absence of Cuf2.

In all conditions, *cuf2* mRNA was primarily detected between 4 to 6 h after meiotic induction. At the protein level, Cuf2 was first detected at the 4-h time point and was then maintained up to ~12 h following induction of meiosis. Cuf2-GFP was consistently observed when the first meiotic division occurred ~5–6 h after meiotic induction. The Cuf2-GFP fluorescent signal progressively decreased 10 to 12 h after meiotic induction, coinciding with mature spore appearance. Interestingly, Cuf2 repression activity was only observed ~9–10 h following the induction of meiosis, coinciding with the end of the meiotic divisions and the process of spore formation. Although the possibility that Cuf2 could have other functions or target genes between 4 and 8 h following induction of meiosis cannot be excluded, the results suggest that Cuf2 activity is regulated through middle meiosis. This suggestion is based on the observation that Cuf2 became active only once the second meiotic division was completed. It is possible that Cuf2 is subjected to post-translational modifications, or that it interacts with potential co-repressors that are induced downstream of itself.

In the baker’s yeast *S. cerevisiae*, the transcriptional regulator Sun1 prevents the expression of meiosis-specific genes in cells undergoing vegetative growth (i.e. in the mitotic cell cycle) [40]. Furthermore, it has been shown that Sun1 represses the expression of a subset of the middle meiotic genes during both early meiosis [41] and at the beginning of late meiosis [42].
Interestingly, the data presented here concerning the repression of middle meiotic genes by Cuf2 was reminiscent of the results obtained with the S. cerevisiae protein Sum1. However, Cuf2 is likely to be different from Sum1 as the SUM1 gene is expressed during mitosis, whereas cuf2+ is not. During meiotic differentiation, SUMI is expressed in both the early- and the late-phases of meiosis, as well as during spore maturation, but not in the middle-phase [42,43]. In contrast, Cuf2 is expressed both in the middle-phase of meiosis and at the onset of late meiosis. In S. cerevisiae, it has been shown that the expression of Ndt80, the ortholog of S. pombe Mei4, is up-regulated in a sum1Δ null strain [42]. In contrast, in S. pombe, the meu5+ transcript was expressed at comparable levels in both the cuf2AΔ/cuf2A mutant and the wild-type parental strains (data not shown). An alignment of the amino acid sequence of Cuf2 with that of Sum1 revealed no sequence similarities between the two proteins (data not shown). Collectively, all of these observations suggest that the Cuf2 and Sum1 functions diverge.

The S. pombe meu5+ gene is a meiosis-specific middle gene that encodes a RNA-binding protein which stabilizes transcripts from numerous genes that are expressed throughout middle-phase meiosis [33]. This post-transcriptional regulatory mechanism enhances the translation of ∼188 transcripts, including that of cuf13+ [33]. In contrast, Cuf2 repressed the expression of several middle-phase genes at the conclusion of the meiotic divisions, thereby presumably reducing their translation rates during spore maturation (late-phase). Thus, Cuf2 and Mei5 have antagonistic roles with respect to the expression of middle-phase meiotic genes. Interestingly, a significant proportion (93/188) of the Mei5-stabilized transcripts was observed to be expressed at higher levels in cuf2AΔ/cuf2A mutant cells than in wild-type cells. Moreover, the meu5+ gene itself was expressed at higher levels in a cuf2AΔ/cuf2A mutant than in a wild-type strain, especially 10 to 14 h after meiotic induction. This suggests that Cuf2 represses not only meu5+ expression, but also that of approximately half of the genes encoding Mei5-stabilized transcripts. This synergetic gene regulatory mechanism may optimize the extinction of middle-phase meiotic gene expression at the onset of late meiosis.

The characterization of the molecular mechanisms underlying meiosis revealed that the loss of key meiotic genes may lead to severe defects and could impair spore viability. In the absence of Cuf2, FSM formation and ascospore maturation were abnormal. Considering the fact that during a given meiotic phase, global gene expression of the previous phase is down-regulated [14,15], the results suggest that the aberrant expression of the middle meiotic genes during late meiosis could have impaired both FSM formation and ascospore maturation. Furthermore, tetrad dissection experiments revealed that spore viability was reduced in a cuf2AΔ/cuf2A mutant strain. Surprisingly, 4 nuclei were systematically detected at the end of meiosis in cuf2Δ/cuf2Δ mutant asci, suggesting that the meiotic divisions were normal. However, a fraction of asci produced by cuf2Δ/cuf2Δ mutants revealed some anucleated FSMs. These results suggested that FSM defects could have reduced the spore viability in cuf2Δ/cuf2Δ mutant cells. Taken together, these results further suggested that the Cuf2-dependent timely repression of the middle meiotic genes is critical to providing the correct gene product at the correct time, thereby contributing to normal FSM biogenesis and optimal spore viability.

**Materials and Methods**

**Yeast strains and media**

The S. pombe strains used in this study are listed in Table 1. Standard methods were used for the growth, mating and sporulation of fission yeast cells [44]. Untransformed strains were maintained on yeast extract plus supplements (YES) containing 225 mg/L of adenine, histidine, leucine, uracil and lysine. The strains for which plasmid integration was required were grown on synthetic Edinburgh minimal medium (EMM) lacking the specific nutrients required for selection and maintenance of the recombinant plasmid. The h"h" diploid strains used for asci visualization were isolated as follows. Haploid cells of the opposite mating types were fused on a solid malt extract (ME) medium and the resulting zygotes were then returned to rich media (YES) prior to commitment to meiosis. At this point, the zygotes can resume vegetative growth as diploid cells and later on undergo asci visualization upon a nitrogen (N)-starvation shock. Asci visualization was induced using EMM lacking nitrogen (EMM-N) and supplemented with 10 mg/L of the required auxotrophic nutrients. Diploid strains homozygous for the mating type h"h" were generated by protoplast fusion as described previously [45].

**pat1-induced meiosis**

In order to synchronize pat1-114/pat1-114 diploid cells for their entry into the meiotic program, the cells were pre-cultured in EMM supplemented with adenine (225 mg/L) at 25°C. The cells were harvested at mid-log phase (∼107 cells/mL) and washed twice before being transferred to EMM-N supplemented with 10 mg/L of adenine. After incubating for 16 h at 25°C, 0.5 mg/mL of NH4Cl was added to the culture medium and the culture divided into three. The three fractions were either left untreated, or were treated with either 50 µM of TTM (Sigma-Aldrich) or CuSO4 concentrations that ranged from 25 to 100 µM. At this point, the temperature was shifted to 34°C so as to induce meiosis. Meiosis progression was monitored by adding Hoechst 33342 stain to 5 µg/mL (Invitrogen) at different times following meiotic induction.

**Plasmids**

As shown in the Results section, the cuf2+ gene was found to be exclusively expressed during meiosis. Furthermore, as reported by others [46], it was determined that the gene contained two introns located within its 5′-end′s coding region (data not shown). Because of these features, a synthetic DNA fragment possessing the first 52 codons of cuf2+ that was optimized for translation in both S. pombe and E. coli was fused in-frame to the genomic cuf2+ codons 53 through 177. To generate the synthetic intron-less DNA segment of cuf2+ (i.e. codons 1–52), four oligonucleotides that were partially complementary to each other were annealed in a pairwise manner, forming a partially double-stranded DNA molecule. This molecule was then made completely double-stranded by incubating it with the Klenow fragment of DNA polymerase 1 in the presence of the four deoxynucleotide triphosphates. The resulting synthetic DNA fragment was digested with PstI and AflIII, as these restriction sites had previously been inserted on either side of the desired fragment, and the fragment was then purified using a EZ-10 spin column (Bio Basic, Markham, ON). Polymerase chain reaction (PCR) amplification of the 3′-end DNA segment of cuf2+ (i.e. codons 53–177, excluding the stop codon) was carried out using primers designed to generate AflIII and SmaI restriction sites at the upstream and downstream termini of the segment, respectively. Genomic DNA from the FY435 S. pombe wild-type strain was used as DNA template in this step as no introns have been mapped within this region. The PstI-AflIII synthetic intron-less DNA fragment (codons 1–52) and the AflIII-SmaI PCR-amplified DNA fragment (codons 53–177) were then digested together in the PstI-SmaI sites of the pBluescript SK vector (Stratagene, La Jolla, CA), creating pSKcuf2+. To create a plasmid possessing the cuf2+ gene
in-frame with the TAP tag, the coding sequence of TAP was PCR amplified from pJK-148 TAP-epi F' [47]. The resulting DNA fragment was digested with SmaI and SacI, and then fused in-frame with cuf2 G' into the corresponding sites of pSK-cuf2 G', generating pSK-cuf2 G'-TAP. To generate the pSK-cuf2 G'-GFP plasmid, a SmaI-SacI PCR-amplified DNA segment containing the GFP coding sequence was isolated from the plasmid pBPctr4 G'-GFP [22], and was then substituted for the SmaI-SacI restriction fragment present in the plasmid pSK-cuf2 G'-TAP, thereby replacing the TAP epitope with the GFP fluorescent fragment. Subsequently, the cuf2 G' promoter, up to position −300 from the start codon of the cuf2 G' gene, was isolated by PCR using S. pombe FY435 genomic DNA as template [48]. Once purified, the DNA fragment was digested with ApaI and PstI and inserted immediately upstream of both the cuf2 G'-TAP and cuf2 G'-GFP fusion alleles, generating pSK-500cuf2 G'-TAP and pSK-500cuf2 G'-GFP, respectively. The resulting plasmids were subsequently digested with ApaI and SacI, and the DNA fragments containing the coding sequences of the cuf2 G'-TAP
and cuf2δ-GFP genes (both under the control of the cuf2δ promoter) then inserted into the corresponding sites of both pBPade6 and pJK148 [22,49]. The integrative plasmids were denoted pBP-500cuf2δ-TAP, pBP-500cuf2δ-GFP, pJK-500cuf2δ-TAP and pJK-500cuf2δ-GFP. To monitor the formation of the forespore membrane (FSM) in meiotic cells, an expression plasmid harboring the GFP-poy1 fusion allele was constructed as described previously [37].

RNA isolation and analysis
Total RNA was extracted using a hot phenol method as described previously [30] and was quantified spectrophotometrically. In the case of the RNase protection assays, 15 μg of RNA per reaction were used as described previously [51]. DNA templates for the antisense riboprobes (Table 2) were cloned into the BamHI and EcoRI sites of the Bluescript SK vector. The resultant constructs were linearized with BamHI for subsequent antisense RNA labelling with [35S]ATP and T7 RNA polymerase as described previously [51]. act1 mRNA was probed as an internal control for normalization during quantification of the RNase protection products.

Experimental design and microarray experiments
An experimental design based on the following node, h²/h² pat1-114/pat1-114 (cuf2δ/cuf2δ) versus h²/h² pat1-114/pat1-114 cuf2Δ/cuf2Δ was adopted. The meiotic time courses were performed in triplicate. Two of the trials were used in the microarray protocol in which the Cy dyes were swapped. The third trial was used for the analysis of the mRNAs using the RNase protection protocol. After 9 h of meiotic induction under basal conditions, cells corresponding to 5 optical density units (OD₅₀₀) were harvested by centrifugation and snap-frozen by immersion in liquid nitrogen. Total RNA was extracted using a hot phenol method [50]. RNA (20 μg) was labelled by directly incorporating Cy3- and Cy5-dCTP using Superscript (Invitrogen, Carlsbad, CA) reverse transcriptase as described previously [52]. The resulting cDNA preparation was hybridized onto glass DNA microarrays containing probes for 99.3% of all known and predicted S. pombe genes. The microarrays were scanned using a GenePix 4000B laser scanner (Axon instruments, Foster City, CA). Data were subsequently analyzed using the GenePix pro software. Unreliable signals were filtered out, and the data were analyzed using a customized Perl script [32]. This script applies cut-off criteria to discard the data from weak signals. Genes that did not yield reproducible results between trials were discarded. Similarly, genes for which 50% of the data points were missing were also discarded. Data acquisition, processing and normalization were performed using the GeneSpring GX software (Agilent Technologies, Cheshire, UK). Normalized signals were exported from GeneSpring into Microsoft Excel spreadsheets and the expression ratios of biological repeat experiments were averaged. A gene was classified as cuf2δ-dependent if its expression was up- or down-regulated ±1.5-fold in the cuf2δ/cuf2Δ strain, as compared to the wild-type strain grown under basal conditions. Gene annotations were retrieved from GeneDB at the Sanger Institute WEB site http://www.genedb.org/genedb/pombe/index.jsp.

Protein extraction and Western blot analysis
Whole cell extracts were prepared using a trichloroacetic acid extraction method [53], and equal amounts of each sample were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, the proteins were electrobotted onto nitrocellulose HYbond-ECL membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The immunoblots were analyzed for the steady-state levels of Cuf2-TAP and the α-tubulin protein using both polyclonal anti-mouse IgG antibody (ICN Biomedicals, Aurora, OH) and monoclonal anti-α-tubulin antibody B-5-1-2 (Sigma-Aldrich Canada, Oakville, ON). After a 1-h incubation with the above-mentioned primary antibodies in 1% powdered skimmed milk in phosphate buffered saline (10.1 mM Na₂HPO₄, 1.6 mM KH₂PO₄, pH 7.4, 138 mM NaCl, 2.7 mM KCl and 0.1% Tween 20), the membranes were washed three times with phosphate buffered saline, incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and visualized by chemiluminescence detection on X-ray films.

Microscopic analysis of Cuf2-GFP localization
h² cuf2Δ and h² cuf2Δ haploid cells expressing the cuf2δ-GFP allele were grown under low nitrogen conditions and were then crossed in order to produce diploid zygotes. After mating, the cells were quickly transferred to rich YES medium so as to stabilize their diploid state. The aszygotic meiosis of diploid cells was synchronously induced by transferring the cells to nitrogen-poor EMM as described previously [37]. At the zero time point when cells had just entered meiosis, they were maintained in nitrogen-poor EMM supplemented with 10 μg/L of adenine, histidine, leucine, uracil and lysine. Culture aliquots were taken up every hour and 5 μg/mL of Hoechst 33342 was added to analyze the

| Gene ID  | Gene name | Riboprobe length (bp) | Position relative to initiator codon | Source |
|----------|-----------|------------------------|--------------------------------------|--------|
| SPAC584.02 | cuf2δ | 208 | +173 to +380 | This study |
| SPBC32H12.11 | mei4δ | 200 | +171 to +370 | This study |
| SPAC1610.03c | meu5δ | 200 | +501 to +700 | This study |
| SPBC1347.03 | meu14δ | 210 | +351 to +560 | This study |
| SPAC182.03cδ | - | 202 | +565 to +766 | This study |
| SPCC162.04c | wt13δ | 198 | +1222 to +1419 | This study |
| SPBC1348.01c | - | 195 | +241 to +435 | This study |
| SPBC32H12.12c | act1δ | 151 | +334 to +485 | [51] |

Table 2. Riboprobes used to detect steady-state levels of transcripts.
accelerate ascus breakdown. More than 200 spores from each of when asci were observed, the ascospores were transferred from either an empty integrative vector or a plasmid containing an independent experiment. The viability counts reported in this study represent a minimum of four series 200; Singer Instrument, UK). Following spore dissection, the cell fields were dissected and sequentially separated approximately 10 mm apart with the aid of a micromanipulator (MS Instruments, Bridgewater, NJ) as described previously [54]. The cell fields shown in this study represent a minimum of five independent experiments. The merged images were obtained using the Simple PCI software version 5.3.0.1102 (Compix, Sewickly, PA).

Spore viability

h and p mutant cells and h and p cells harboring a cuf2A deletion with either an empty integrative vector or a cuf2-GFP allele were mated with their corresponding isogenic h and p and cuf2A/cuf2A and h and cuf2avs/cuf2-GFP, respectively) onto ME-agar plates. When ascii were observed, the ascospores were transferred from ME to YES-agar plates and incubated for 4 h at 36°C so as to accelerate ascus breakdown. More than 200 spores from each of the three strains (cuf2A/cuf2A, cuf2A/cuf2A and cuf2A/cuf2A/ cuf2-GFP) were dissected and sequentially separated approximately 10 mm apart with the aid of a micromanipulator (MS Instruments, UK). Following spore dissection, the isolated spores were examined to determine whether or not they retain viability on YES-agar plates. Spore viability was expressed as a percentage of the total number of spores dissected. Spore viability counts reported in this study represent a minimum of four independent experiments.

References

1. Handel MA, Schimenti JC (2010) Genetics of mammalian meiosis: Regulation, dynamics and impact on fertility. Nat Rev Genet 11: 124-136.
2. Marston AL, Anson A (2004) Meiosis: Cell-cycle control shuffles and deals. Nat Rev Mol Cell Biol 5: 983-997.
3. Kim AM, Vogt S, O’Halloran TV, Woodruff TK (2010) Zinc availability regulates exit from meiosis in mammalian oocytes. Nat Chem Biol 6: 674-681.
4. Staub C (2001) A century of research on mammalian male germ cell meiotic differentiation in vitro. J Androl 22: 911-926.
5. Hogarth CA, Griswold MD (2010) The key role of vitamin A in spermatogenesis. J Clin Invest 120: 956-962.
6. Davis L, Smith GR (2001) Meiotic recombination and chromosome segregation in Schizosaccharomyces pombe. Proc Natl Acad Sci U S A 98: 8395-8402.
7. Li Y, Lam KS, Dasgupta N, Ye P (2010) A yeast’s eye view of mammalian meiotic dynamics and impact on fertility. J Androl 22: 911-926.
8. Hogarth CA, Griswold MD (2010) The key role of vitamin A in spermatogenesis. J Clin Invest 120: 956-962.
9. Shirasawa C, Nakamura T (2004) Control of late meiosis and ascospore formation. In: Egel R, ed. The Molecular Biology of Schizosaccharomyces pombe. Berlin, Germany: pp 311-327.
10. Yamamoto M (2004) Initiation of meiosis. In: Egel R, ed. The Molecular Biology of Schizosaccharomyces pombe. Berlin, Germany: pp 297-309.
11. Mata J, Bähler J (2006) Global roles of Ste11p, cell type, and pheromone in the control of gene expression during early sexual differentiation in fission yeast. Proc Natl Acad Sci U S A 103: 15317–15322.
12. Bähler J, Schuchert P, Grimm C, Kohli J (1991) Synchronized meiosis and recombination in fission yeast: observations with pat1-114 diploid cells. Genetica 19: 445–451.
13. Doll E, Molnar M, Cunow G, October G, Latypov V, et al. (2008) Cohesion and recombination proteins influence the Gl-1 to S transition in asynaptic meiosis in Schizosaccharomyces pombe. Genetics 170: 727-740.
14. Mata J, Lyne R, Burns G, Bähler J (2002) The transcriptional program of meiosis and sporulation in fission yeast. Nat Genet 32: 143–147.

Supporting Information

Table S1 List of genes that showed elevated expression (averaging ≥1.5-fold) in a cuf2A/cuf2A mutant (pat1-114/pat1-114 synchronized cells).

Table S2 List of middle-phase meiotic genes that showed elevated expression (averaging ≥1.5-fold) in a cuf2A/cuf2A mutant (pat1-114/pat1-114 synchronized cells).

Table S3 List of genes that showed elevated expression (averaging ≥1.5-fold) in both cuf2A/cuf2A and meu5Δ/meu5Δ mutants (pat1-114/pat1-114 synchronized cells).

Table S4 List of genes that showed elevated expression (averaging ≥1.5-fold) in both cuf2A/cuf2A mutants (pat1-114/pat1-114 synchronized cells).

Table S5 List of genes that showed reduced expression in a cuf2A/cuf2A mutant (pat1-114/pat1-114 synchronized cells).

Acknowledgments

We are grateful to Gilles Dupuis, Alexandre Mercier and William Home for critical reading of the manuscript and for their valuable comments. We thank Raymund Welling for the use of his micromanipulator in the tetrad dissection. We also thank Anghelique Lanthier for her technical assistance.

Author Contributions

Conceived and designed the experiments: RI JB LLM SC JB SL. Performed the experiments: RI JB LLM SC JB SL. Analyzed the data: RI JB LLM SC JB SL. Contributed reagents/materials/analysis tools: RI JB LLM SC JB SL. Wrote the paper: RI JB LLM SC JB SL.
27. Labbé S, Zhu Z, Thiele DJ (1997) Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. J Biol Chem 272: 15951–15958.

28. Beaudoin J, Labbé S (2006) Copper induces cytoplasmic retention of fission yeast transcription factor Cuf1. Eukaryot Cell 5: 277–292.

29. Jensen LT, Winge DR (1998) Identification of a copper-induced intramolecular interaction in the transcription factor Mac1 from Saccharomyces cerevisiae. EMBO J 17: 5400–5408.

30. Zhu Z, Labbé S, Pería MMO, Thiele DJ (1998) Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. J Biol Chem 273: 1277–1280.

31. Beaudoin J, Mercier A, Langlois R, Labbé S (2003) The Schizosaccharomyces pombe Cuf1 is composed of functional modules from two distinct classes of copper metallorregulatory transcription factors. J Biol Chem 278: 14565–14577.

32. Bowen NJ, Jordan IK, Epstein JA, Wood V, Levin HL (2003) Retrotransposons and their recognition of pol II promoters: A comprehensive survey of the transposable elements from the complete genome sequence of Schizosaccharomyces pombe. Genome Res 13: 1984–1997.

33. Amorim MJ, Cotobal C, Duncan C, Mata J (2010) Global coordination of transcriptional control and mRNA decay during cellular differentiation. Mol Syst Biol 6: 380.

34. Nakamura T, Asakawa H, Nakase Y, Kashiwazaki J, Hiraoka Y, et al. (2008) Live observation of forespore membrane formation in fission yeast. Mol Biol Cell 19: 3544–3553.

35. Shigeoka A, Okazaki D, Kasama T, Tsuchi H, Hirata A, et al. (2010) Mug28, a meiosis-specific protein of Schizosaccharomyces pombe, regulates spore wall formation. Mol Biol Cell 21: 1955–1967.

36. Ioannoni R, Beaudoin J, Mercier A, Labbé S (2010) Copper-dependent trafficking of the Ctr4-Ctr5 copper transporting complex. PLoS One 5: e11964.

37. Ioannoni R, Ioannoni R, Lopez-Maury L, Böhrer J, Ait-Mohand S, et al. (2011) Mcl1 is a novel forespore membrane copper transporter in meiotic and sporulating cells. J Biol Chem 286: 34356–34372.

38. Beaudoin J, Labbé S (2007) Crm1-mediated nuclear export of the Schizosaccharomyces pombe transcription factor Cufl during a shift from low to high copper concentrations. Eukaryot Cell 6: 764–775.

39. Halliwell B, Gutteridge JM (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update. FEBS Lett 307: 108–112.

40. Xie J, Pierce M, Gailus-Durner V, Wagner M, Winter E, et al. (1999) Sum1 and Ubf1 repress middle sporulation-specific gene expression during mitosis in Saccharomyces cerevisiae. EMBO J 18: 6448–6454.

41. Pak J, Segall J (2002) Regulation of the premiddle and middle phases of expression of the NDT80 gene during sporulation of Saccharomyces cerevisiae. Mol Cell Biol 22: 6417–6429.

42. Klusmann M, Siegried Z, Giupsan A, Farzad-Kam A, Zsinn G, et al. (2010) Combination of genomic approaches with functional genetic experiments reveals two modes of repression of yeast middle-phase meiosis genes. BMC Genomics 11: 478.

43. Lindgren A, Bungard D, Pierce M, Xie J, Vershon A, et al. (2000) The pachytene checkpoint in Saccharomyces cerevisiae requires the Sum1 transcriptional repressor. EMBO J 19: 6489–6497.

44. Sahatçuoğlu A, Forshburg SL (2010) Molecular genetics of Schizosaccharomyces pombe. Methods Enzymol 470: 739–795.

45. Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol 194: 795–823.

46. Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, et al. (2002) The genome sequence of Schizosaccharomyces pombe. Nature 415: 871–880.

47. Jo M, Mercier A, Pelletier B, Beaudoin J, Labbé S (2009) Iron activates in vivo DNA binding of Schizosaccharomyces pombe transcription factor Fep1 through its amino-terminal region. Eukaryot Cell 8: 649–664.

48. Pelletier B, Beaudoin J, Mokai Y, Labbé S (2002) Fep1, an iron sensor regulating iron transporter gene expression in Schizosaccharomyces pombe, J Biol Chem 277: 22950–22958.

49. Keeney JB, Boeke JD (1994) Efficient targeted integration at leu1-32 and ura4-294 in Schizosaccharomyces pombe. Genetics 136: 849–856.

50. Chen D, Toone WM, Mata J, Lyne R, Burns G, et al. (2003) Global transcriptional responses of fission yeast to environmental stress. Mol Biol Cell 14: 214–229.

51. Mercier A, Watt S, Bähler J, Labbé S (2008) Key function for the CCAAT-binding factor Pfp4 to regulate gene expression in response to iron deficiency in fission yeast. Eukaryot Cell 7: 493–508.

52. Lyne R, Burns G, Mata J, Penkett CJ, Rustici G, et al. (2003) Whole-genome microarrays of fission yeast: Characteristics, accuracy, reproducibility, and processing of array data. BMC Genomics 4: 27.

53. Foiani M, Marini F, Gamba D, Lucchini G, Plevani P (1994) The B subunit of the DNA polymerase alpha-primase complex in Schizosaccharomyces pombe DNA binding of Schizosaccharomyces pombe transcription factor Fep1 through its amino-terminal region. Eukaryot Cell 8: 649–664.

54. Ioannoni R, Ioannoni R, Lopez-Maury L, Böhrer J, Ait-Mohand S, et al. (2011) Mcl1 is a novel forespore membrane copper transporter in meiotic and sporulating cells. J Biol Chem 286: 34356–34372.