PHP4 Is a Key Player for Iron Economy in Meiotic and Sporulating Cells

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ABSTRACT Meiosis is essential for sexually reproducing organisms, including the fission yeast Schizosaccharomyces pombe. In meiosis, chromosomes replicate once in a diploid precursor cell (zygote), and then segregate twice to generate four haploid meiotic products, named spores in yeast. In S. pombe, PHP4 is responsible for the transcriptional repression capability of the heteromeric CCAAT-binding factor to negatively regulate genes encoding iron-using proteins under low-iron conditions. Here, we show that the CCAAT-regulatory subunit PHP4 is required for normal progression of meiosis under iron-limiting conditions. Cells lacking PHP4 exhibit a meiotic arrest at metaphase I. Microscopic analyses of cells expressing functional GFP-PHP4 show that it colocalizes with chromosomal material at every stage of meiosis under low concentrations of iron. In contrast, GFP-PHP4 fluorescence signal is lost when cells undergo meiosis under iron-replete conditions. Global gene expression analysis of meiotic cells using DNA microarrays identified 137 genes that are regulated in an iron- and PHP4-dependent manner. Among them, 18 genes are expressed exclusively during meiosis and constitute new putative PHP4 target genes, which include hry1+ and mug14+. Further analysis validates that PHP4 is required for maximal and timely repression of hry1+ and mug14+ genes. Using a chromatin immunoprecipitation approach, we show that PHP4 specifically associates with hry1+ and mug14+ promoters in vivo. Taken together, the results reveal that in iron-starved meiotic cells, PHP4 is essential for completion of the meiotic program since it participates in global gene expression reprogramming to optimize the use of limited available iron.

KEYWORDS iron-sparing response iron-regulated genes CCAAT-binding factor meiosis fission yeast

Eukaryotic organisms that sexually reproduce have a specialized type of cell division that enables the formation of haploid gametes from diploid germ cells. This specialized mode of cell division is called meiosis (Marston and Amon 2004; Handel and Schimenti 2010; Ohkura 2015). The early stage of meiosis involves a round of DNA synthesis during which chromosomal material is duplicated, generating pairs of homologous chromosomes. The subsequent step consists of meiotic recombination between homologous chromosomes that increases genetic diversity and the potential appearance of new phenotypic traits. This step is followed by two successive meiotic divisions (denoted meiosis I and II, in which homologous chromosomes and then sister chromatids are separated to generate four haploid sets of chromosomes that are inheritable by the next generation. In fungi, including Schizosaccharomyces pombe, the terminal stage of meiosis involves a differentiation program that induces compartmentalization of the genetic material into four spores (or gametes) that are enclosed into an ascus (Sabatinos and Forsburg 2010; Shigehisa et al. 2010).

Studies using different model organisms have shown that micronutrients, including transition metals such as zinc and copper, are required for the normal progression of meiosis (Kim et al. 2010; Beaudoin et al. 2011). In mice, zinc insufficient oocytes proceed through segregation of homologous chromosomes (meiosis I) but fail to further segregate sister chromatids, therefore blocking meiotic progression past telophase I (Kim et al. 2010). In addition, maturation of
porcine oocytes under conditions of zinc insufficiency is blocked at metaphase I, leading to a failure to segregate homologous chromosomes (Jeon et al. 2015). Similarly, studies with the fission yeast *S. pombe* have revealed that copper deficiency arrests meiosis by blocking the process at metaphase I (Beaudoin et al. 2011). On the basis of these observations, it is reasonable to suggest that iron, one of the most used transition metals in biology, may also be required during meiotic differentiation.

*S. pombe* was used here as a model to characterize iron requirement during meiosis since it is one of the best understood model systems to investigate the eukaryotic cell cycle by way of conventional mode of division (mitosis) or meiotic cell division program (meiosis) (Navarro et al. 2012; Hoffman et al. 2015). In this context, growth conditions and temperature-sensitive strains have been developed that allow the synchronization of cells prior to their entry into the meiotic program (Mata et al. 2002; Harigaya and Yamamoto 2007). For instance, haploid cells arrest in the G1 phase of the cell cycle under low-nitrogen conditions. When cells of the opposite mating type interact during the G1 block, haploid cells conjugate to form diploid zygotes. If the resulting zygotes are maintained under nitrogen-starved conditions, they undergo meiosis by a process called zygotic meiosis. Alternatively, zygotes freshly formed can be returned to a nitrogen-replete medium before their commitment to meiosis and they will grow as diploids for a period of time. Over this period of time, if these diploid cells undergo a second switch from sufficient to insufficient nitrogen, their passage to meiosis occurs very rapidly and in a more synchronous manner than zygotic meiosis. Mitotically growing cells produce an active protein kinase called Pat1 that inhibits cells from entering meiosis. When active, Pat1 phosphorylates the transcription factor Ste1 and the meiosis-specific inducer Mei2. This Pat1-mediated posttranslational modification blocks their activity. A mutant strain containing a temperature-sensitive *pat1-114* allele produces a thermostable Pat1 kinase. When *pat1-114* cells undergo a transition from low (25°C) to elevated (34°C) temperature, Pat1 is readily inactivated, triggering a cell cycle switch from mitosis to meiosis in a highly efficient and synchronous fashion. This latter system, termed *pat1*-induced meiosis, is more synchronous than aszygotic meiosis (Yamamoto 2004; Doll et al. 2008).

In *S. pombe*, Fep1 and Php4 are two iron-dependent regulatory proteins that play a critical role in maintaining cellular iron homeostasis (Labbé et al. 2013; Brault et al. 2015). Their roles have traditionally been investigated in dividing cells that grow mitotically. In response to high concentrations of iron, the GATA-type transcription factor Fep1 binds to GATA elements and represses several genes encoding proteins that are involved in iron acquisition (Jbel et al. 2009). Fep1 also represses the expression of Php4, which is a negative iron-dependent regulatory subunit of the heteromeric CCAAT-binding factor (Mercier et al. 2006). In contrast, when iron levels are low, Fep1 becomes inactive and loses its ability to interact with chromatin. This situation leads to transcriptional activation of the Fep1 regulon, which includes the *php4* gene. Under low-iron conditions, Php4 is produced and becomes competent to associate with the CCAAT-binding core complex that is composed of Php2, Php3, and Php5. The Php4/CCAT complex reprograms the cells for iron economy (Mercier et al. 2006). At the molecular level, Php4 is responsible for the transcriptional repression capability of the CCAAT complex (Mercier et al. 2008). The Php4/Php2/Php3/Php5 heteromeric complex coordinates the repression of 86 genes in cells that grow mitotically (Mercier et al. 2008). Among these, the majority encode proteins involved in iron-dependent metabolic pathways such as the tricarboxylic acid cycle (TCA), mitochondrial electron transport chain, heme biosynthesis, and iron-sulfur cluster assembly. Microarray analyses have also revealed that the *fep1* gene is under the transcriptional control of Php4 being repressed in response to iron deficiency in a Php4-dependent manner (Mercier et al. 2008). Overall, Php4 and Fep1 mutually control each other’s expression at the transcriptional level in response to changes in iron levels.

Previous studies have used *S. pombe* mutants to distinguish between the effects of iron on Php4 protein and its transcriptional regulation by Fep1 (Mercier and Labbé 2009; Khan et al. 2014). This approach using cells proliferating in mitosis led to the discovery that Php4 exhibits a differential cellular localization as a function of iron availability. For instance, Php4 accumulates in the nucleus under low-iron conditions, whereas it transits from the nucleus to the cytoplasm in response to high levels of iron (Mercier and Labbé 2009). Although nuclear import of Php4 is independent of the other CCAAT-regulatory subunits Php2, Php3, and Php5, it is a cargo for the karyopherins Imp1, Cut15, and Sal3 (Khan et al. 2014). In mitotically growing cells undergoing a transition from low to high iron, Php4 transits from the nucleus to the cytoplasm in a process that is dependent on monothiol glutaredoxin Grx4 and exportin Crm1 (Mercier and Labbé 2009). When cells are exposed to iron-poor conditions, nuclear localization of Php4 is reestablished through a mechanism of import. A rationale for the Php4-mediated iron-sparing response is to prevent a futile expenditure of energy in producing iron-requiring proteins when iron is absent or present in insufficiently low concentrations. In the case of *php4* mutant cells, iron-using genes are expressed continuously, rendering these cells hypersensitive to low-iron conditions (Mercier et al. 2008; Khan et al. 2014). This is presumably due to the lack of optimization of iron utilization when iron concentrations are insufficient to meet the metabolic needs of the cell.

Although it is known that deficiencies in iron-dependent proteins culminate in meiotic cell developmental defects and subfertility (Narisawa et al. 2002; Nordstrand et al. 2010; Kipp et al. 2011), iron homeostasis during meiotic differentiation has not been extensively studied. Here, we have combined the use of DNA microarray analysis and *S. pombe* aszygotic and *pat1*-driven meiotic models to investigate whether iron deficiency and inactivation of Php4 would perturb the meiotic program. Results showed that iron is required for the normal progression of meiosis. Iron insufficient zygotes experienced a meiotic block at metaphase I. Similarly, zygotic cells carrying disrupted *php4Δ/php4Δ* alleles were arrested at metaphase I when iron was limited. The *php4* gene was expressed at higher levels in iron-starved meiotic cells in comparison with cells treated with iron. Furthermore, *fep1Δ/PHP1Δ* meiotic cells exhibited increased levels of *php4* mRNA under low- and high-iron conditions. Microscopic analyses revealed that a functional GFP-Php4 protein colocalizes with chromosomes/chromatids in meiotic and sporulating cells under low-iron conditions. Using DNA microarrays, we identified a first set of genes whose transcription is expressed at higher levels in iron-replete meiotic cells. Second, we identified genes whose transcription is induced in a *php4Δ/php4Δ* mutant strain under low-iron conditions. These two combined data sets globally identified 137 genes that are regulated in an iron- and Php4-dependent manner, including 18 genes that are meiosis-specific. Experiments were designed to validate a direct role for Php4 in participating in the regulation of newly identified meiosis-specific target genes. Results of ChiP assays showed that the *hry1* and *mug14* promoters are directly bound by Php4 in response to iron starvation. Taken together, the results demonstrate that Php4 is required during the meiotic differentiation program to repress
Iron concentrations are low, revealing a meiotic role for Php4 in the optimization of iron use under iron starvation conditions.

MATERIALS AND METHODS

Yeast strains and growth conditions

The *S. pombe* strains used in this study are listed in Table 1. Standard yeast genetic methods were used for growth, mating, and sporulation of cells (Sabatinos and Forsburg 2010). Under nonselective conditions, strains were grown on yeast extract medium (YES) containing 0.5% yeast extract and 3% glucose that was further supplemented with 225 mg/L of adenine, histidine, uracil, leucine, and lysine. Strains for which plasmid integration was required were grown in synthetic Edinburgh minimal medium (EMM) lacking specific amino acids required for plasmid selection and maintenance. After mating, zygotic haploids were returned to nonselective yeast extract medium before commitment to meiosis. Diploid cells underwent asyzyotic meiosis following a synchronized nitrogen-starvation shock in which EMM lacking nitrogen (EMM-N) was supplemented with 10 mg/L of adenine or 10 mg/L of adenine, histidine, leucine, uracil, and lysine. Diploid strains homozygous for the mating type (h*/h*) were generated by incubating haploid cell cultures with carbendazim (20 μg/ml) (Sigma-Aldrich) as described previously (Zhang et al. 2013).

To synchronize *pat1*-114/pat1-114 diploid cells (Bähler et al. 1991) for their entry into meiosis, liquid cultures were seeded to an A_{600} of 0.2 and grown to midlog phase (A_{600} of 0.5) in EMM supplemented with adenine (225 mg/L) at 25°C. Cells were harvested, washed twice, and transferred to EMM minus nitrogen (EMM-N) that was supplemented with adenine (10 mg/L). At this point, cells were separated into two different lots that were pretreated with 2,2′-dipyridyl (Dip) (50 μM) and FeCl₃ (Fe) (0.74 μM) for 16 hr at 25°C, unless otherwise stated. After pretreatment of the cells, NH₄Cl (0.5 g/L) was added to each lot and cells were further exposed to Dip (75 μM) and FeCl₃ (100 μM), respectively. At this time, the temperature was shifted to 34°C to induce meiosis as described previously (Beaudoin et al. 2011). Meiosis progression was monitored using the Hoechst 33342 stain (5 μg/ml) added at various times following meiotic induction.

Plasmids

The sad1+/-mCherry chimeric gene was isolated from pJK210sad1+/-mCherry (Beaudoin et al. 2011) by PCR using primers that contained BsmHI and SacI restriction sites at their ends. The purified DNA fragment was digested with BsmHI and SacI and then cloned into the corresponding sites of pJK148 (Keeney and Boeke 1994). The resulting plasmid was denoted pJK148sad1+/-mCherry and the fluorescent protein product served as a spindle pole body marker. Plasmid pJK194+promphp4 harbors a 194 bp DNA segment of the php4+ promoter (Mercier and Labbé 2009). The asterisk (in the plasmid name) indicates that the promoter contains multiple point mutations in the iron-responsive GATA sequences (positions −188 to −183 and −165 to −160), rendering the promoter constitutively expressed irrespective of the iron status (Mercier and Labbé 2009). The wild-type php4+ open reading frame was isolated by PCR from genomic DNA of the parental FY435 strain. The PCR product was digested with BamHI and Asp718 and then cloned into the corresponding sites of pJK194+promphp4+.

The resulting plasmid was denoted pJK194+promphp4+php4+.

The wild-type version of php4+ promoter up to position −194 (from the start codon of the php4+ gene) was isolated by PCR. After amplification, the purified DNA fragment (−194 to −1) was digested with SacI and BamHI and then was exchanged with the SacI-BamHI mutated php4+ promoter region in plasmid pJK194+promphp4-GFP-php4+ (Mercier and Labbé 2009). The resulting plasmid was named pJK194+promphp4-GFP-php4+. A similar strategy was used to create the plasmid pJK194promphp4-TAP-php4+, except that the SacI-BamHI PCR-amplified DNA segment containing the wild-type version of php4+ promoter (−194 to −1) was exchanged with the mutated php4+ promoter DNA fragment into the plasmid pJK194+promphp4-TAP-php4+ (Mercier and Labbé 2009). Plasmid pJK194promphp4-TAP-php4+ was used as a template to amplify a DNA fragment encompassing the TAP-php4+ fusion gene and its promoter region up to −194. This PCR amplification was performed using primer pairs that incorporated unique 5′ and 3′ SacI and ApaI restriction sites, respectively. The PCR product was purified, digested with SacI and ApaI, and then cloned into the corresponding sites of pBPAde6+ (Beaudoin et al. 2006). The resulting plasmid was denoted pBP194promphp4-TAP-php4+.

RNA analysis

Total RNA was extracted using a hot phenol method as described previously (Chen et al. 2003). Gene expression profiles were analyzed using RNase protection assays as described previously (Mercier et al. 2008). Plasmids pSKphp4+, pSKisat1+, and pSKact1+ (Mercier et al. 2006) were used to produce antisense RNA probes that served to determine php4+, isat1+, and act1+ mRNA levels, respectively. Plasmid pSKhry1+ was constructed by inserting a 196-bp BamHI-EcoRI fragment from the hry1+ gene into the same sites of pBluescript SK. The antisense RNA hybridizes to the region between positions +66 and

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**Table 1. S. pombe strain genotypes**

| Strain | Genotype | Source or Reference |
|--------|----------|---------------------|
| FY435  | h+ his7-366 leu1-32 ura4-Δ18 ade6-M210 | Beaudoin et al. 2011 |
| FY436  | h+ his7-366 leu1-32 ura4-Δ18 ade6-M216 | Beaudoin et al. 2011 |
| AMY15  | h+ his7-366 leu1-32 ura4-Δ18 ade6-M210 php4Δ::KAN' | Mercier et al. 2006 |
| ABY60  | h+ his7-366 leu1-32 ura4-Δ18 ade6-M216 php4Δ::KAN' | This study |
| JB484  | h+ pat1-114 ade6-M210 | Bähler et al. 1991 |
| JB485  | h+ pat1-114 ade6-M216 | Bähler et al. 1991 |
| ABY61  | h+ pat1-114 ade6-M210 php4Δ::KAN' | This study |
| ABY62  | h+ pat1-114 ade6-M216 php4Δ::KAN' | This study |
| ABY63  | h+ pat1-114 ade6-M210 pep1Δ::KAN' | This study |
| ABY64  | h+ pat1-114 ade6-M216 pep1Δ::KAN' | This study |
| FY435/FY436 | h+/h+ his7-366/his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 | This study |
| php4ΔΔ | h+/h+ his7-366/his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 php4Δ::KAN/php4Δ::KAN' | This study |

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+262 downstream of the initiator codon of hry1+. Plasmid pSKug14* was generated by inserting a 193 bp fragment from the mug14* gene (corresponding to the coding region between positions +214 and +407 downstream of the A of the start codon of mug14*). 32P-labeled antisense RNA probes were produced from the above BamHI-linearized plasmids and with the use of [α-32P]UTP and T7 RNA polymerase. act1* mRNA was probed as an internal control for normalization during quantification of RNase protection products.

Microarray experiments
We adopted an experimental design that involved two nodes: pat1-114/pat1-114 php4*/php4* (WT) iron replete (+Fe) vs. pat1-114/pat1-114 php4*/php4* (WT) iron-starved (+Dip) and pat1-114/pat1-114 php4Δ/php4Δ iron-starved (+Dip) vs. pat1-114/pat1-114 php4*/php4* iron-starved (+Dip). Meiotic time courses were performed as three independent biological repeats. All of them were used in the microarray protocol for which the Alexa Fluor 555 and 647 dyes were swapped. A fourth independent biological repeat was used for quantification of mRNAs using RNase protection assays. Total RNA was isolated from cells that had undergone synchronous meiosis for 7 hr under the indicated iron status (replete or starved conditions). The preparation of cDNA libraries from samples of RNA was performed as described previously (Lyne et al. 2003). cDNAs were hybridized onto glass DNA microarrays (Agilent Technologies) containing 15,000 spots that were ~60-mer probes. Together, these probes result in ~2x3-times coverage for each S. pombe locus, representing all known and predicted protein-coding genes and some noncoding RNA genes. Microarrays were scanned using a GenePix 4000B laser scanner (Axon instruments). Data were analyzed using the GenePix pro software. Unreliable signals were filtered out and data were normalized using an R script as described previously (Marguerat et al. 2012). The script applies cut-off criteria to discard data from weak signals. Genes that did not yield reproducible results of biological repeats were eliminated. Furthermore, genes with 50% of their data points missing were also discarded. Data acquisition and processing were further analyzed using GeneSpring GX software (Agilent Technologies). Normalized signals were exported from GeneSpring into Excel software (Microsoft) and analyzed. To determine ratios of expression levels, gene values from the php4*/php4* (WT) (+Fe) and php4Δ/php4Δ (+Dip) were divided by the corresponding value of php4*/php4* (WT) (+Dip), which was set as the reference sample. The expression ratios of biological repeat experiments were averaged. Genes were classified as php4* dependent if their expression changed 2.0-fold more than the average of two repeats during iron repletion vs. iron starvation and if they were induced 2.0-fold more than the average of two repeats in the php4Δ/php4Δ strain during iron starvation compared with the php4*/php4* (WT) strain under the same conditions. Gene annotations were retrieved from the PomBase website (Wood et al. 2012).

Fluorescence microscopy
Assessment of GFP-Php4 localization during meiosis and sporulation was performed by using h* php4Δ and h* php4Δ haploid cells expressing a functional GFP-php4* allele and crossing the two strains to produce diploid zygotyes. After mating, the diploid state of cells was stabilized by incubation in YES medium. Subsequently, diploid cells were precultured in the presence of Dip (50 μM) in EMM containing nitrogen supplemented with 75 mg/L of adenine, histidine, uracil, leucine, and lysine. The aszygotic meiosis of diploid cells was synchronously induced by transferring the cells to nitrogen-poor EMM in the presence of Dip (75 μM) or FeCl3 (100 μM). After the cells had just entered meiosis, aliquots were withdrawn at various time points and stained with Hoechst 33342 (5 μg/ml) to assess progression of meiosis of individual cells. At the indicated meiotic phase, the cells were examined by fluorescence microscopy using a 1000 × magnification as described previously (Beaudoin et al. 2013). Fields of cells shown in this study correspond to a minimum of five independent experiments.

ChiP assays
h*/h* php4Δ/php4Δ cells expressing untagged (from pJK148-194 ‘promphp4-4-php4’) or TAP-tagged Php4 (from pJK148-194 ‘promphp4-4-TAP-php4’) were induced to synchronously enter aszygotic meiosis and then fixed (formaldehyde) after 7 hr. After formaldehyde cross-linking and neutralization with glycine, cell lysates were prepared by glass bead disruption in lysis buffer containing 100 mM HEPES-KOH pH 7.5, 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM EDTA, 140 mM NaCl, 2 × complete ULTRA Tablets (protease inhibitors, Roche), 1 mM phenylmethylsulfonil fluoride (PMSF), 50 mM NaF, and 0.2 mM Na3VO4, as described previously (Larochelle et al. 2012). Samples were then sonicated using a Branson 450 sonicator to shear chromatin DNA into fragments of ~500–1000 bp. Immunoprecipitation of TAP-Php4 bound to chromatin was performed using immunogolubin G (IgG)-Sepharose beads. Handling of beads, including washings and elution, reversed cross-linking, and DNA precipitation were performed as described previously (Adam et al. 2001; Jbel et al. 2009). Quantification of immunoprecipitated DNA was carried out by real-time PCR (qPCR) using different sets of primers that spanned hry1* and mug14* promoter regions. TAP-Php4 density at hry1* and mug14* promoters was calculated as the enrichment of specific genomic hry1* and mug14* promoter regions relative to an 18S ribosomal DNA coding region in which no functional CCAAT box was present. Primers were designated by the name of the gene promoter, followed by the position of their 5′ ends relative to that of the translational initiation codon: hry1-412 (5′-GAATGCTGGTACGATACAGAGAAGA-3′), hry1-323 (5′-GGCGACGTCAGCGTGTG-3′), mug14-692 (5′-GGATGCTTCTTATTATAGTGTCTAGTAA-3′), and mug14-577 (5′-CTGCTGTTTACCCGACGTCCCT-3′). Two primers derived from an 18S ribosomal DNA coding region were used as internal background controls: 18S-a (5′-AGCCATGACTGAGACGAGAG-3′) and 18S-b (5′-AGCCATGACTGAGACGAGAG-3′). Each qPCR was run in triplicate using Perfecta SYBR Green Fast mix (Quanta) on a LightCycler 96 Real-Time PCR instrument (Roche). All ChiP experiments were repeated at least three times using independent chromatin preparations.

Protein extraction and analysis
pat1-114/pat1-114 php4Δ/php4Δ cells expressing the TAP-php4*/ TAP-php4* allele were synchronized to initiate and proceed to meiosis. Every hour over a time period of 9 hr following meiotic induction, cells were fixed (formaldehyde) in the presence of Dip or FeCl3. For each time point, 15 min before cells were harvested, PMSF (1 mM) was added directly to the cultures. Cell lysates were prepared by glass bead disruption in the same lysis buffer as described for ChiP assays. TAP-Php4 was enriched using immunoglobulin G (IgG)-Sepharose beads and equal amounts of each sample preparation were resuspended in sodium dodecyl sulfate loading buffer and proteins were resolved by electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels. Proteins were electroblotted onto nitrocellulose membranes for 1 hr at 4°C. Membranes were blocked by treatment with 5% powdered skimmed milk (Difco) in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% bovine serum albumin) containing 0.1% Tween 20 (TBST). Following washings with TBST, membranes were incubated with primary antibodies diluted in 1% powdered skimmed milk in TBST for 16 hr at 4°C. The following antibodies were used for immunodetection of TAP-Php4 and α-tubulin: polyclonal anti-mouse IgG antibody (ICN Biomedicals) and monoclonal...
anti-α-tubulin antibody (clone B-5-1-2; Sigma-Aldrich), respectively. After incubation, the membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), developed with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences), and visualized by chemiluminescence using an ImageQuant LAS 4000 instrument (GE Healthcare) equipped with a Fuji film High Sensitivity F0.85 43 mm camera.

Data availability
All data are included in the present article and in the Supplemental Material. Strains and plasmids used for this study are also available if requested.

RESULTS
Iron deficiency leads to a meiotic block at metaphase I
Although iron fulfills essential functions in eukaryotes, little is known about its role in meiosis. To investigate whether insufficient concentrations of iron would perturb the meiotic program, diploid cells were precultured in the presence of the iron chelator Dip (50 µM) or FeCl₃ (0.74 µM) for 16 hr. At this point, Dip- and Fe-pretreated diploid cells underwent asynchronous meiosis in the presence of Fe (100 µM). In the case of iron insufficient zygotes, diploid cells were precultured in the presence of Dip (50 µM) for 16 hr and then transferred to media lacking nitrogen to initiate synchronous meiosis. Following the nitrogen-starvation shock, cells were treated with Dip (250 µM). (C) Aliquots of cells used in (B) (blocked at metaphase I) were incubated in the presence of exogenous Fe (300 µM), which resulted in release from metaphase I. The graphics (right) depict the meiotic profiles of cells after meiotic induction. Numbers of cells with 1, 2, or 3–4 nuclei were determined by counting Hoechst-stained nuclei after meiotic induction. At least 200 cells were counted every hour and under each above-mentioned condition. The reported values of cells are the means of three independent repeats ± SD. Dip, 2,2’-dipyridyl; Fe, iron(III) chloride; SD, standard deviation; T, time point (hours).

Figure 1 Iron insufficient zygotes undergo a meiotic arrest at metaphase I. Wild-type diploid cells expressing Sad1-Cherry were synchronously induced into azygotic meiosis. Shown are four representative stages of the meiotic program that occurred after 1, 5, 6, and 12 hr of meiotic induction. The spindle pole bodies’ marker Sad1-Cherry is in red (center left). The chromosomal material was probed by Hoechst 33342 staining (blue; center right). Cell morphology was examined by Nomarski optics (far left). Merged images of Hoechst dye and Sad1-Cherry are shown next to schematic representations of the meiotic steps on the far right. (A), Diploid cells underwent azygotic meiosis in the presence of Fe (100 µM). (B), In the case of iron insufficient zygotes, diploid cells were precultured in the presence of Dip (50 µM) for 16 hr and then transferred to media lacking nitrogen to initiate synchronous meiosis. Following the nitrogen-starvation shock, cells were treated with Dip (250 µM). (C) Aliquots of cells used in (B) (blocked at metaphase I) were incubated in the presence of exogenous Fe (300 µM), which resulted in release from metaphase I. The graphics (right) depict the meiotic profiles of cells after meiotic induction. Numbers of cells with 1, 2, or 3–4 nuclei were determined by counting Hoechst-stained nuclei after meiotic induction. At least 200 cells were counted every hour and under each above-mentioned condition. The reported values of cells are the means of three independent repeats ± SD. Dip, 2,2’-dipyridyl; Fe, iron(III) chloride; SD, standard deviation; T, time point (hours).
supplementation with iron restored the meiotic developmental program, including the two meiotic divisions and the generation of four haploid spores per ascus (Figure 1C). As positive controls, zygotes incubated in the presence of iron proceeded through meiosis and formed asci containing four spores after 10–12 hr of meiotic induction (Figure 1A). Percentages of cells with 1, 2, or 3–4 nuclei were quantitatively determined by counting Hoechst-stained nuclei (Figure 1). Taken together, the results showed that iron is required for normal progression of meiosis, based on the observation that the lack of iron leads to meiotic arrest at metaphase I.

**Meiotic cells harboring inactivated php4Δ/php4Δ alleles are arrested at metaphase I under low-iron conditions**

When iron levels are low, proliferating S. pombe cells that grow mitotically express the CCAAT-binding subunit Php4 (Mercier et al. 2006). Upon its biosynthesis, Php4 fosters repression of several genes encoding iron-using proteins as a means to minimize cellular iron consumption (Mercier et al. 2008). Taking into account the facts that Php4 is required for iron economy during mitosis and that iron plays an essential role during meiotic differentiation, we hypothesized that Php4 could also be important for normal progression of meiosis under conditions of iron starvation. To test this hypothesis, php4Δ/php4Δ diploid cells were used and results compared to php4+/php4+ control cells. Diploid strains were precultured in the presence of Dip (50 μM) and were synchronously induced by transferring the strains at the same time to nitrogen-poor medium, thus allowing strains to undergo azygotic meiosis (Figure 1A). Percentages of cells with 1, 2, or 3–4 nuclei were quantitatively determined by counting Hoechst-stained nuclei (Figure 1). Taken together, the results showed that iron is required for normal progression of meiosis, based on the observation that the lack of iron leads to meiotic arrest at metaphase I.

**Temporal expression profile of Php4 during meiosis**

To further investigate the meiotic function of Php4, we first assessed its transcription profile during meiosis as a function of time and iron availability. pat1-114/pat1-114 php4+/php4+ diploid cells were synchronously
induced into meiosis and treated with either Dip (75 μM) or FeCl₃ (100 μM). Aliquots of cultures were taken after meiotic induction and the steady-state levels of php4⁺ mRNA analyzed by RNase protection assays. Under low-iron conditions, results showed that steady-state levels of php4⁺ transcripts were constitutively present between 1 and 9 hr after meiotic induction, exhibiting a small peak of expression at middle meiosis (e.g., 5 hr time point) (Figure 3A). In the case of iron-starved cells, php4⁺ transcript levels were expressed to a higher degree over time compared to transcript levels observed in iron-treated cells. Relative expression values were 42%, 51%, 66%, and 51% higher in the presence of Dip than iron after 1, 3, 5, and 7 hr of meiotic induction, respectively. The 9 hr time point represented an exception in which case php4⁺ mRNA levels were expressed to a similar degree under iron-starved and iron-replete conditions (Figure 3A).

To determine whether the steady-state protein levels of Php4 followed those of php4⁺ mRNA, we used a pat1-114/pat1-114 php4Δ / php4Δ strain in which a TAP-php4 fusion allele was returned into the genome by integration. In this strain, the expression profile of TAP-php4⁺ mRNA was nearly identical to that of the php4⁺ transcript in the wild-type (control) strain (Figure 3A and Figure 4A). Using the same culture conditions as for php4⁺ or TAP-php4⁺ mRNA analysis (Figure 3A and Figure 4A), results showed that TAP-Php4 protein levels were exclusively detected in iron-starved meiotic cells (Figure 4B). After cell entrance into meiosis (1 hr time point), the levels of Php4 protein were very low. Subsequently, a strong increase of Php4 protein levels was observed 3, 5, and 7 hr after meiotic induction. This was followed by a reduction of Php4 protein levels within 9 hr (Figure 4B). In contrast, under iron-replete conditions, the signal corresponding to TAP-Php4 was lost throughout the meiotic program, suggesting iron-mediated extinction of TAP-Php4 steady-state levels in response to high iron concentrations (Figure 4B).

To ensure that the in-frame TAP insertion did not interfere with Php4 function, the untagged (php4⁺) and tagged (TAP-php4⁺) coding sequences were separately integrated into php4Δ mutant cells. Integrants were analyzed for their ability to repress isa1⁺ transcript levels in response to low concentrations of iron. Results showed that php4Δ cells expressing TAP-Php4 conferred iron starvation-dependent repression of isa1⁺ expression in a manner similar to that of wild-type (untagged) Php4 protein (Figure 4C). In contrast, deletion of php4⁺ (php4Δ) resulted in sustained expression of isa1⁺ mRNA levels and a lack of response to iron starvation (Figure 4C). Taken together, these results revealed that TAP-Php4 is present in meiotic cells under low-iron conditions, whereas the protein steady-state levels are dramatically decreased in response to high concentrations of iron.

**Analysis of Php4 localization during meiosis under iron-limited and iron-replete conditions**

We next determined the subcellular location ofPhp4 during meiosis and sporulation as a function of iron availability. As we previously showed, when GFP-Php4 is expressed in php4Δ mutant cells, the repression of isa1⁺ mRNA occurs in response to iron starvation conditions in a manner identical to that observed in cells expressing the untagged (wild-type) version of Php4 (Mercier and Labbé 2009; Khan et al. 2014). These results demonstrated that GFP does not interfere with Php4 function. The fully functional GFP-php4⁺ allele under the control of the php4⁺ promoter was integrated in h¹ php4Δ and h¹ php4Δ cells, and localization of GFP-Php4 in zygotes and asci was determined.
A. Brault

immunoglobulin G; mRNA, messenger RNA; RNase, ribonuclease; SD, standard deviation; WT, wild-type.

Diploid cells had undergone azygotic synchronous meiosis and they had been pretreated with Dip (50 μM) to trigger nuclear import of Php4. Results showed that GFP-Php4 was primarily detected in the nucleus of zygotic cells at the start of the observations (Figure 5). Once the cells were induced to undergo meiosis, one half of the cultures was further incubated with Dip (75 μM), whereas the other half was treated with FeCl3 (100 μM). Under conditions of iron starvation, GFP-Php4 colocalized with chromosomal material that was marked by Hoechst staining. This colocalization was observed through all different stages of meiosis, including prophase I, horse tail, metaphase I, and anaphase I and II (Figure 5). GFP-Php4 fluorescence in meiotic cells was observed as a single spot in each cell during prophase I and metaphase I in a manner similar to that observed for chromosomal material (Figure 5). Fluorescence associated with GFP-Php4 was seen as an elongated spot in each cell during prophase I and metaphase I in a manner similar to that observed for chromosomal material (Figure 5). Following metaphase I, GFP-Php4 fluorescence was successively observed as a pair of spots per cell (anaphase I) and two pairs of spots per cell (anaphase II) (Figure 5). This result was interpreted to correspond to the elongated nucleus of the "horse tail" stage (Figure 5). Following metaphase I, GFP-Php4 fluorescence was successively observed as a pair of spots per cell (anaphase I) and two pairs of spots per cell (anaphase II) (Figure 5). This result was interpreted to correspond to the elongated nucleus of the "horse tail" stage (Figure 5).

Figure 4  Assessment of the transcript and protein steady-state levels of a functional TAP-Php4 during meiosis. (A) Representative expression profile of TAP-Php4* mRNA in pat1-114/pat1-114 php4A/php4A Δ TAP-Php4*/TAP-Php4* cells that were induced to undergo synchronous meiosis. Following induction of meiosis, cells were incubated in the presence of Dip (75 μM) or Fe (100 μM) and total RNA was isolated at the indicated time points. After RNA preparation, TAP-Php4* and act1* steady-state mRNA levels were analyzed by RNase protection assays. 0 hr: zero time point refers to onset of meiotic induction. Graphics (right) represent quantification of results of three (n = 3) independent RNase protection assays, including experiments shown on the left side of the figure. The histogram values represent the averages ± SD. (B) Cell lysates from aliquots of the meiotic cultures expressing TAP-Php4 shown in (A) were analyzed by immunoblotting using anti-IgG and anti-α-tubulin antibodies. The positions of molecular weight standards are indicated on the right. (C) Mitotic WT and php4A strains were left untreated (−) or were incubated with either Dip (250 μM) or Fe (100 μM) for 90 min. php4A cells were transformed with integrative plasmids encoding php4* and TAP-Php4* alleles or an empty integrative plasmid (vector alone). Total RNA prepared from midlogarithmic cells was assayed by RNase protection assays. Steady-state levels of isa1* and act1* mRNAs are indicated with arrows. Dip, 2,2'-dipyridyl; Fe, iron(III) chloride; IgG, immunoglobulin G; mRNA, messenger RNA; RNase, ribonuclease; SD, standard deviation; WT, wild-type.

Effects of iron status and Php4 on S. pombe meiotic transcriptome

Given the fact that inactivation of php4+ (php4Δ/php4Δ) altered the process of meiosis under iron-limiting conditions, we used a microarray approach to identify additional genes that were potentially under the control of Php4 and/or regulated as a function of iron availability during meiosis. The following conditions were used in the case of genes that are differentially regulated in response to changes in iron levels. Microarrays were hybridized with probes derived from RNA isolated from iron-replete vs. iron-starved pat1-114/pat1-114 cells that had been synchronously induced to undergo meiosis. Differentially expressed genes were analyzed after 7 hr of meiotic induction. In this first set, 246 genes with high expression levels (averaging >2.0-fold) in the presence of iron were detected (Figure 6, A and C and Supplemental Material, Table S1). Among these genes, several of them encoded iron-using proteins that are involved in iron-dependent biochemical pathways, including the TCA cycle (e.g., sdh1+/2+/3+/4+), mitochondrial respiration (e.g., cyt1+, qcr7+, cox3+, and cyt1+), heme biosynthesis (e.g., hem3+ and SAP14E8.05c), and iron-sulfur cluster...
were identified, including frp1+, frp2+, and mug30+ (Table S1). Under conditions of iron deficiency, we determined that 57 genes were expressed at high levels (averaging >2.0-fold). We consistently noticed that genes encoding proteins involved in reductive iron uptake were induced such as frp1+, frp2+ (Figure 6 and Table S2). The shu1+ gene encoding a cell-surface protein involved in iron acquisition from heme was also induced (Mourer et al. 2015). Furthermore, we observed significant changes in the transcriptional profiles of other genes, including SPBPP2B2.06c (a putative metal-dependent phosphatase), frp2+ (a putative ferrireductase), and ecl2+ (a putative metal-dependent extender of chronological lifespan) (Ohtsuka et al. 2015) (Figure 6 and Table S2).

In the case of genes that are potentially under the control of Php4, a second set of microarrays were hybridized with fluro-c-CDNA preparations purified from meiotic cells of pat1-114/pat1-114 php4Δ/php4Δ mutant cells vs. iron-starved pat1-114/pat1-114 php4+/php4+ cells. In this second set, 225 genes exhibited high levels of expression (averaging >2.0-fold) in the absence of Php4 (php4Δ/php4Δ) under low-iron conditions (Figure 6, B and C and Table S3). These potential Php4 target genes included genes already characterized, including pcl1+, is1+, and sbd4+ (Mercier et al. 2006), as well as several uncharacterized genes, especially those that are expressed exclusively during meiosis (24 meiotic genes were identified) (Table S3).

Overall, 137 genes were expressed at high levels in both sets of data, revealing that they shared a common trait. Transcription of these genes was iron- and Php4-dependent (Table 2). Consistently, the majority of these genes (119 of 137) had one or more copies of the CCAAT consensus sequence within their promoters (Table 2). Furthermore, several of these genes could be regrouped based on their predicted protein or RNA products or meiosis-specific profiles of expression. Interestingly, among genes derepressed by both iron repletion and php4Δ/php4Δ deletion, 18 of them were meiosis-specific (Table 2). Taken together, these results identified 246 genes that are expressed at high levels in iron-replete meiotic cells. Among them, 137 genes (including 18 meiosis-specific) exhibit decreased transcript abundance in iron-starved meiotic cells and are potentially under the control of Php4.

Iron deficiency affects expression profiles of hry1+ and mug14+ meiosis-specific transcripts in a Php4-dependent manner

The microarray data suggested that 18 genes encoding meiosis-specific proteins were differentially expressed in relationship with the presence of Php4 under low-iron conditions (Table 2). To confirm that the results of the microarrays identified Php4-regulated genes, we performed RNase protection assays (using an independent biological repeat) to assess the relative expression of two meiosis-specific genes, hry1+ (SPAC69606c) and mug14+ (Table 2). Although both genes encode proteins of unknown function, the sequence of Hry1 contains a putative hemerythrin domain that may directly bind iron, suggesting that Hry1 may function as an iron-using protein. pat1-114 php4+/php4+ and pat1-114 php4Δ/php4Δ diploid cells were synchronously induced to undergo meiosis under iron-starved and iron-replete conditions. At different time points after meiotic induction, steady-state levels of hry1+ mRNA were analyzed. Results showed that hry1+ mRNA levels were primarily detected in php4+/php4+ cells treated with iron after 7 and 9 hr of meiotic induction. At these time points, levels of hry1+ mRNA increased 19.2-
in iron-starved cells during meiotic differentiation. (A) Cultures of pat1-114/pat1-114 php4+/php4+ cells were precultivated in the presence of Dip (50 μM) and then immediately induced to undergo synchronous meiosis. After 7 hr of meiotic induction, total RNA was extracted and used for microarray experiments. The graph represents a genome-wide picture of differentially expressed genes (X axis) in iron-replete and iron-limited cells. For simplicity, only a few differentially expressed transcripts are labeled on the graph. (B) Genome-wide picture of differentially expressed genes in pat1-114/pat1-114 php4Δ/php4Δ vs. pat1-114/pat1-114 php4+/php4+ cells that had been precultivated (50 μM) and cultured (75 μM) in the presence of Dip. Genome-wide transcripts (X axis) were analyzed by DNA microarrays. All differentially expressed genes are depicted, although only a few of them are labeled on the graph for the sake of clarity. (C) Venn diagram representing the number of genes that were induced in iron-treated php4+/php4+ cells compared to php4+/php4+ cells grown under iron-limiting conditions and the number of genes that exhibited higher expression levels in iron-starved php4Δ/php4Δ compared to php4+/php4+ cells grown under the same conditions. Dip, 2,2′-dipyridyl; Fe, iron(III) chloride.

Figure 6 Transcriptomic response of S. pombe as a function of changes in iron levels and effect of php4Δ/php4+ disruption relative to wild-type cells during meiotic differentiation. (A) Cultures of pat1-114/pat1-114 php4+/php4+ cells were precultivated in the presence of Fe (0.74 μM) and Dip (50 μM) for 16 hr. Pretreated cells were further exposed to Fe (100 μM) and Dip (75 μM), respectively, and then immediately induced to undergo synchronous meiosis. After 7 hr of meiotic induction, total RNA was extracted and used for microarray experiments. The graph represents a genome-wide picture of differentially expressed genes (X axis) in iron-replete vs. iron-limited cells. For simplicity, only a few differentially expressed transcripts are labeled on the graph. (B) Genome-wide picture of differentially expressed genes in pat1-114/pat1-114 php4Δ/php4Δ vs. pat1-114/pat1-114 php4+/php4+ cells that had been precultivated (50 μM) and cultured (75 μM) in the presence of Dip. Genome-wide transcripts (X axis) were analyzed by DNA microarrays. All differentially expressed genes are depicted, although only a few of them are labeled on the graph for the sake of clarity. (C) Venn diagram representing the number of genes that were induced in iron-treated php4+/php4+ cells compared to php4+/php4+ cells grown under iron-limiting conditions and the number of genes that exhibited higher expression levels in iron-starved php4Δ/php4Δ compared to php4+/php4+ cells grown under the same conditions. Dip, 2,2′-dipyridyl; Fe, iron(III) chloride.

16.4-fold, respectively, as compared to hry1+ mRNA levels observed in iron-starved php4+/php4+ cells (Figure 7A). Under iron starvation conditions, inactivation of php4Δ/php4Δ triggered an increase of hry1+ expression after 3, 5, 7, and 9 hr of meiotic induction (4.6-, 2.6-, 3.5-, 3.8-fold, respectively) as compared to hry1+ mRNA levels in php4+/php4+ cells that had been exposed to identical conditions (75 μM Dip). This observation showed that Php4 was required for maximal repression of hry1+ in response to iron starvation. Total RNA isolated from mitotically growing cells revealed that hry1+ mRNA was undetectable regardless of cellular iron and Php4 status (Figure 7C). These observations were expected in view of the function of a gene predicted to be expressed exclusively during meiosis.

The meiotic expression profile of mug14+ was first analyzed in pat1-114 php4Δ/php4Δ cells incubated in the presence of iron (FeCl3, 100 μM). Under these conditions, results showed that mug14+ mRNA levels were markedly elevated after 5 and 7 hr of meiotic induction (Figure 7B). When pat1-114 php4Δ/php4Δ cells were synchronized through meiosis but under low levels of iron (75 μM Dip), mug14+ mRNA levels were mainly detected after 5 and 7 hr of meiotic induction but to a lesser extent (6.4- and 2.1-fold less, respectively) in comparison with transcript levels observed in iron-replete cells (Figure 7B). When a pat1-114 php4Δ/php4Δ mutant strain was examined under iron-replete conditions, mug14+ transcript levels were primarily detected at the 5 and 7 hr meiotic time points as observed in the case of iron-treated pat1-114 php4+/php4+ cells (Figure 7B). However, under iron starvation conditions, disruption of php4Δ/php4Δ resulted in induced mug14+ mRNA levels after 5, 7, and 9 hr of meiotic induction (6.1-, 2.5-, and 20.0-fold, respectively) compared to those recorded in the case of php4+/php4+ cells incubated under the same conditions (Figure 7B). As observed in the case of the hry1+ gene, expression of mug14+ was detected exclusively during meiosis and was not seen in cells proliferating in mitosis (Figure 7C). Taken together, these results indicated that the repression of meiotic hry1+ and mug14+ genes occurs to a certain degree through the activity of the CCAAT-binding factor Php4, which represses transcription from these loci in response to iron starvation.

**Php4 interacts with the hry1+ and mug14+ promoters in vivo in an iron-dependent manner**

In previous studies, we had developed a biological system in which php4+ and TAP-php4+ alleles were expressed under the control of a GATA-less php4+ promoter (Mercier and Labbé 2009). We showed that a php4Δ mutant strain expressing php4+ or a functional TAP-php4+ allele was disengaged from transcriptional regulation by Fep1, therefore ensuring its constitutive expression irrespective of the cellular iron status. We took advantage of this system to test whether TAP-Php4 could be detected at the hry1+ and mug14+ promoters in vivo using a ChiP approach. In the case of the S. pombe CCAAT-binding complex, its capacity to associate with chromatin is conferred by the Php2/Php3/Php5 subunits that are required for the formation of a DNA binding complex at the CCAAT box promoter element (McNabb et al. 1997; Mercier et al. 2006). In response to iron starvation, Php4 associates with the Php2/Php3/Php5 heteromeric complex (Mercier et al. 2006). In contrast, when cells undergo a transition from low to high iron, Php4 is regulated at the posttranslational level via a multistep mechanism resulting in its inactivation (Mercier and Labbé 2009). php4Δ php4+ diploid cells expressing either an untagged or a TAP-tagged version of Php4 under the control of a GATA-less php4+ promoter were synchronized to initiate and proceed through asyngiotic meiosis under iron-deficient or iron-replete conditions. After 7 hr of meiotic induction, results showed that TAP-Php4 occupied the hry1+ and mug14+ promoters at high levels in response to iron starvation (Figure 8, A and B, respectively). The association of TAP-Php4 with hry1+ and mug14+ promoters exhibited 177- and 97-fold enrichment, respectively, relative to a 18S ribosomal DNA coding region that does not contain any CCAAT element (used as a negative control) (Figure 8). Promoter...
## Table 2 Transcripts derepressed by both Fe-repletion and a php4Δ deletion

| Gene ID | Gene Name | GeneDB Annotation | Fold Changes | PUTATIVE CCAAT BOXES |
|---------|-----------|-------------------|--------------|---------------------|
| SPBC869.06c | hry1+ | HHE domain cation binding protein (predicted) | 6.826 | 4.734 | 386, 250* |
| SPBC359.06 | mug14+ | Adducin | 5.127 | 2.229 | 649 |
| SPCC1235.12c | mug146+ | Schizosaccharomyces specific protein Mug46 | 3.715 | 4.370 | 854*, 804*, 753, 414* |
| SPBC6B1.03c | Pa1 family protein | 2.680 | 2.636 | 709*, 539* |
| SPCC1281.04 | Pyridoxal reductase (predicted) | 2.658 | 3.033 | 102 |
| SPAC3F10.05c | mug113+ | T5orf172 family protein | 2.505 | 2.309 | 792*, 597, 340, 251, 153 |
| SPAPB1A10.08 | Schizosaccharomyces specific protein | 2.501 | 2.682 | 874*, 365*, 47* |
| SPBC21.07c | ppp24+ | Serine/threonine protein kinase Ptk24 | 2.484 | 3.687 | 741*, 655*, 294 |
| SPAC3F10.07c | erf4+ | Palmitoyltransferase complex subunit Erf4 | 2.275 | 2.914 | None |
| SPCC320.07c | mde7+ | RNA-binding protein Mde7 | 2.193 | 2.727 | 951, 818*, 264, 107 |
| SPBC28E12.02 | RNA-binding protein | 2.129 | 2.936 | 970*, 754*, 271, 50 |
| SPSC562.03c | mug86+ | Acetate transmembrane transporter (predicted) | 2.224 | 2.736 | 848*, 70, 32 |
| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
| SPBC22E12.02 | RNA-binding protein | 2.094 | 2.205 | 665*, 267*, 9 |
| SPCC1259.12c | mug146+ | Schizosaccharomyces pombe specific protein | 2.157 | 2.617 | 158, 85 |
| SPBC28E12.02 | RNA-binding protein | 2.129 | 2.936 | 970*, 754*, 271, 50 |
| SPCC562.03c | mug86+ | Acetate transmembrane transporter (predicted) | 2.224 | 2.736 | 848*, 70, 32 |
| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
| SPBC22E12.02 | RNA-binding protein | 2.129 | 2.936 | 970*, 754*, 271, 50 |
| SPCC562.03c | mug86+ | Acetate transmembrane transporter (predicted) | 2.224 | 2.736 | 848*, 70, 32 |
| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
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| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
| SPBC28E12.02 | RNA-binding protein | 2.129 | 2.936 | 970*, 754*, 271, 50 |
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| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
| SPBC28E12.02 | RNA-binding protein | 2.129 | 2.936 | 970*, 754*, 271, 50 |
| SPCC562.03c | mug86+ | Acetate transmembrane transporter (predicted) | 2.224 | 2.736 | 848*, 70, 32 |
| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
| SPBC28E12.02 | RNA-binding protein | 2.129 | 2.936 | 970*, 754*, 271, 50 |
| SPCC562.03c | mug86+ | Acetate transmembrane transporter (predicted) | 2.224 | 2.736 | 848*, 70, 32 |
| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
| SPBC28E12.02 | RNA-binding protein | 2.129 | 2.936 | 970*, 754*, 271, 50 |
| SPCC562.03c | mug86+ | Acetate transmembrane transporter (predicted) | 2.224 | 2.736 | 848*, 70, 32 |
| SPCC1259.12c | mde7+ | UPF0300 family protein | 2.157 | 2.617 | 158, 85 |
| Gene ID       | Gene Name                | GeneDB Annotation                                      | Fold Changes          |
|---------------|--------------------------|--------------------------------------------------------|-----------------------|
|               |                          |            | WT (+Fe vs. −Fe) | php4Δ vs. WT (−Fe) | Putative CCAAT Boxes |
|               |                          |            |                 |                     |                        |
| Electron transport chain/mitochondrial respiration |                          |            |                 |                     |                        |
| SPCC191.07   | cyc1*                    | Cytochrome c                                      | 6.953                 | 5.821              | 907, 612, 525, 438, 380, 216†, 211†, 178† |
| SPBC16H5.06  | rip1*                    | Ubiquinol-cytochrome-c reductase complex subunit 5  | 3.480                 | 3.197              | 906, 769†, 759, 657, 584, 453†, 313† |
| SPCC737.02c  | qcr7*                    | Ubiquinol-cytochrome-c reductase complex subunit 6 (predicted) | 2.622                 | 2.715              | 729†, 611, 591, 462, 119†, 90† |
| SPCC338.10c  | cox5*                    | Cytochrome c oxidase subunit V (predicted)          | 2.511                 | 2.422              | 947†, 696†, 562†, 112, 90† |
| SPBC29A3.18  | cyt1*                    | Cytochrome c1 Cyt1 (predicted)                      | 2.509                 | 2.195              | 813, 457, 435†, 145, 65† |
| SPAC15A10.17 | coa2*                    | Cytochrome C oxidase assembly factor Coa2 (predicted) | 2.501                 | 2.102              | 838, 146†, 139†, 132† |
| SPBC947.15c  | nde1*                    | Mitochondrial NADH dehydrogenase (ubiquinone) Nde1 (predicted) | 2.496                 | 2.357              | 624, 489, 272† |
| SPBC16C6.08c | qcr6*                    | Ubiquinol-cytochrome-c reductase complex subunit 8, hinge protein (predicted) | 2.372                 | 2.100              | 158† |
| SPAC20G8.04c | cir2*                    | Mitochondrial electron transfer flavoprotein-ubiquinone oxidoreductase Cir2 (predicted) | 2.158                 | 2.420              | 520†, 479†, 437† |
| Carbohydrates metabolic process |                          |            |                 |                     |                        |
| SPBC32H8.13C | mok12*                   | α-1,3-glucan synthase Mok12 KRE9 family cell wall 1,6-β-glucan biosynthesis protein (predicted) | 3.096                 | 3.371              | None                        |
| SPBC11C11.05 |                          |            | 3.838                 | 3.237              | 836                        |
| SPAC5H10.11  | gmh1*                    | α-1,2-galactosyltransferase Gmh1 (predicted)         | 2.751                 | 3.462              | 495                        |
| SPAC23H3.11C | gld1*                    | Glucosidase (predicted)                             | 2.449                 | 2.194              | 902†, 654†, 642†           |
| SPAC13F5.03c | gto1*                    | α-glucosidase (predicted)                            | 2.249                 | 2.387              | 706, 666†, 498†, 339†      |
| SPAC1039.11C | gtc6*                    | Mannan endo-1,6-α-mannosidase (predicted)           | 2.218                 | 2.865              | 879†                        |
| SPCC970.02   |                          |            | 2.158                 | 2.420              | 520†, 479†, 437†          |
| SPBC19C7.12c | omh1*                    | α-1,2-mannosyltransferase Omh1 (predicted)           | 2.182                 | 2.102              | 175†, 167†                  |
| SPAC9E9.03   | leu2*                    | 3-isopropylmalate dehydratase Leu2 (predicted)       | 5.012                 | 5.971              | 815†, 750†, 293†           |
| SPAPB1E7.07  | gtn1*                    | Glutamate synthase Glt1 (predicted)                 | 2.982                 | 3.254              | 913†, 881†, 266†, 199†     |
| SPAC17G8.06c |                          | Dihydroxy-acid dehydratase (predicted)              | 2.781                 | 3.534              | 327, 284†                  |
| SPBC21H7.07c | his5*                    | Imidazoleglycerol-phosphate dehydratase His5         | 2.636                 | 2.623              | 994, 269, 165†             |
| SPAC13G7.06  | met16*                   | Phosphoadenosine phosphosulfate reductase            | 2.463                 | 2.461              | 820†, 636†, 186, 63†       |
| SPCC622.12c  | gdh1*                    | NADP-specific glutamate dehydrogenase Gdh1 (predicted) | 2.232                 | 2.610              | 917†, 650, 528, 487†       |
| SPCC1442.09  | trp3*                    | Anthranilate synthase component I (predicted)        | 2.149                 | 2.136              | 686, 138†                  |
| Amino acid biosynthesis |                          |            |                     |                     |                        |
| SPBC1718.03  | kerl*                    | DNA-directed RNA polymerase I complex subunit Ker1    | 2.984                 | 3.601              | None                        |
| SPBC17D1.01  |                          | Transcriptional regulatory protein Spp41 (predicted) | 2.870                 | 2.079              | None                        |
| SPAC3F10.06c | rit1*                    | Initiator methionine tRNA 2'-O-ribosyl phosphate transferase (predicted) | 2.480                 | 2.776              | 390†, 328*, 244*, 112       |
| SPAC4G8.07c  |                        | tRNA (m5U54) methytransferase Trm2 (predicted)       | 2.472                 | 3.223              | 924*, 10†                  |

(continued)
| Gene ID | Gene Name | GeneDB Annotation | Fold Changes | Putative CCAAT Boxes |
|---------|-----------|-------------------|--------------|----------------------|
| SPCC757.09c | mc1* | RNA-binding protein that suppresses calcineurin deletion Rnc1 | 2.376 | 23 |
| SPCC11E10.06c | elp4* | Elongator complex subunit Elp4 (predicted) | 2.091 | 614, 41, 19 |
| SPCC320.11C | nip7* | RNA-binding protein involved in ribosome biogenesis Nip7 (predicted) | 2.034 | 839, 608a |
| TCA cycle | | | | |
| SPBP23A10.16 | sdh4* | TIM22 inner membrane protein import complex anchor subunit Tim18 | 23.700 | 897a, 532, 77, 53 |
| SPAC140.01 | sdh2* | Succinate dehydrogenase (ubiquinone) iron-sulfur protein subunit (predicted) | 20.160 | 100, 28 |
| SPAC1556.02c | sdh1* | Succinate dehydrogenase Sdh1 (predicted) | 6.471 | 178, 43 |
| SPAC24C9.06c | aco1* | Aconitate hydratase Aco1 (predicted) | 5.997 | 440, 135 |
| SPCC330.12c | sdh3* | Succinate dehydrogenase (ubiquinone) cytochrome b subunit (predicted) | 5.923 | 868a, 822a, 708a, 375, 253a, 175, 144, 11 |
| SPBC3H7.03c | | 2-oxoglutarate dehydrogenase (lipoamide) (e1 component of oxoglutarate Dehydrogenase complex) (predicted) | 2.674 | None |
| Fe-S cluster biogenesis/Fe-S cluster-containing proteins | | | | |
| SPAC26F1.14c | aif1* | Apoptosis-inducing factor homolog Aif1 (predicted) | 11.380 | 951a, 355, 161 |
| SPCC645.03c | isa1* | Mitochondrial iron-sulfur protein Isa1 | 11.350 | 762, 207 |
| SPCC1235.02 | bio2* | Biotin synthase | 5.342 | 259 |
| SPBC14F5.06 | rli1* | Iron-sulfur ATPase involved in ribosome biogenesis and translation Rli1 (predicted) | 3.537 | 980a, 393a, 255, 200a, 46a |
| mRNA metabolic process | | | | |
| SPBC609.01 | | Ribonuclease II (RNB) family, involved in nuclear-transcribed mRNA Catabolic process (predicted) | 2.899 | 646a, 424a, 386 |
| SPAP8A3.05 | ski7* | Ski complex interacting GTPase Ski7 | 2.481 | 586, 198, 126 |
| SPBC16H5.10c | prp43* | ATP-dependent RNA helicase Prp43 | 2.129 | 678, 671a, 616 |
| SPBC2F12.08c | ceg1* | mRNA guanylyltransferase Ceg1 | 2.008 | None |
| SPBC3B9.19 | mge1* | Mitochondrial GrpE domain chaperone protein (predicted) | 3.076 | 971, 684, 415 |
| SPCC1235.11 | mpc1* | Mitochondrial pyruvate transmembrane transporter subunit Mpc1 (predicted) | 2.938 | 434a, 273, 141 |
| SPBC27B12.14 | | Mitochondrial membrane protein complex assembly protein (predicted) | 2.122 | 810a |
| Oxidative stress response | | | | |
| SPCC757.07c | ctt1* | Catalase | 9.384 | 575a, 480 |

(continued)
| Gene ID      | Gene Name                  | GeneDB Annotation                          | Fold Changes            | Putative CCAAT Boxes |
|-------------|----------------------------|--------------------------------------------|-------------------------|----------------------|
| **SPAC1486.01** | Manganese superoxide dismutase |                                           | WT (+Fe vs. −Fe)         | 2.073                |
|             |                            |                                            | php4Δ vs. WT (−Fe)       | 2.746                |
| **Sterol and fatty acid biosynthesis** |                           |                                            |                         | None                |
| **SPCC16A11.10c** | oca8*                      | Cytochrome b5 (predicted)                 | 3.357                   | None                |
| **SPAC1671.16c** | erg31*                     | C-S sterol desaturase Erg31              | 3.238                   | None                |
| **Nitrogen assimilation** |                           |                                            |                         | 3.671                |
| **SPCPB11C.01** | atm1*                      | Ammonium transmembrane transporter Amt1   | 4.256                   | 941, 941, 301        |
| **SPAC23H4.06** | gln1*                      | Glutamate-ammonia ligase Gln1             | 2.555                   | 742, 690, 680, 640   |
| **Heme biosynthesis** |                           |                                            |                         | 3.026                |
| **SPAC24B11.13** | hem3*                      | Hydroxymethylbilane synthase Hem3 (predicted) | 2.988                   | None                |
|             |                            |                                            |                         | 3.212                |
| **Other functions** |                           |                                            |                         | 977, 501             |
| **SPB21E7.02c** | pcm2*                      | Phosphoglycerate mutase family Protein-L-isooaspartate O-methyltransferase Pcm2 (predicted) | 3.711                   | 349, 153             |
| **SPAC869.08c** |                            |                                            |                         | 3.633                |
|             |                            |                                            |                         | 2.488                |
| **SPAC186.02c** |                            | Hydroxylacid dehydrogenase (predicted)    | 3.626                   | 465                 |
| **SPCC663.13C** | nna50*                     | NatA N-acetyltransferase subunit Naa50 (predicted) | 3.471                   | 948, 726             |
| **SPCC663.14c** | trp663*                    | TRP-like ion channel (predicted)          | 3.449                   | 782                 |
| **SPAC17G8.08c** |                            | Human TMEM165 homolog, implicated in calcium transport | 3.443                   | 441, 435             |
| **SPAC1486.11** | fmc1*                      | Mitochondrial matrix protein, F1F0 ATP synthase assembly factor Fmc1 (predicted) | 2.959                   | 312, 174             |
| **SPBC1711.12** |                            | Serine-type peptidase activity            | 2.943                   | 316                 |
| **SPBC725.03** |                            | Pyridoxamine 5'-phosphate oxidase (predicted) | 2.923                   | 270                 |
| **SPBC152.02** |                            | APC amino acid transmembrane transporter (predicted) | 2.814                   | 929, 323             |
| **SPBC1711.11** |                            | Autophagy associated protein (predicted)   | 2.657                   | 316, 212, 39, 26    |
| **SPCC152.01c** | pma2*                      | P-type proton ATPase, P3-type Pma2        | 2.654                   | 119, 74, 42         |
| **SPAC869.02c** |                            | Nitric oxide dioxygenase (predicted)      | 2.612                   | 315, 301             |
| **SPAC1556.03** | azr1*                      | Serine/threonine protein phosphatase Azr1 | 2.560                   | 755, 620             |
| **SPBC1703.06** | pof10*                     | F-box protein Pof10                       | 2.520                   | 814                 |
| **SPBC1703.12** | ubp9*                      | Ubiquitin C-terminal hydrolase Ubp9       | 2.520                   | 612                 |
| **SPBC830.08c** | yop1*                      | ER membrane protein DP1/Yop1              | 2.398                   | None                |
| **SPBC19C7.09c** | uve1*                      | Endonuclease Uve1                         | 2.314                   | 1000, 789, 242      |
| **SPBC1711.05** |                            | Nucleocytoplasmic transport chaperone Srp40 (predicted) | 2.287                   | 479, 356             |
| **SPAC8C9.03** | cgs1*                      | CAMP-dependent protein kinase regulatory subunit Cgs1 | 2.234                   | 667                 |
| **SPBC26H8.02c** | sec9*                      | SNAP-25 homolog, t-SNARE component Sec9   | 2.066                   | 582, 461             |
| **SPBC16B3.10c** | pcl1*                      | Ferrous iron/manganese transmembrane transporter Pcl1 | 2.034                   | 205                 |
| **SPBC20D10.10** | bdc1*                      | Bromodomain containing protein 1, Bdc1    | 2.005                   | None                |

Unknown functions

(continued)
occupancy by TAP-Php4 was detected using primers amplifying DNA regions located between positions —412 and —323 (hry1+ and positions —692 and —577 (mug14+) relative to the initiator codons of hry1+ and mug14+, respectively. These two amplified promoter regions were predicted to contain a putative functional trimeric DNA-binding complex, which becomes competent to repress repression through a specialized subunit of the CCAAT-binding factor, HapX (Figure 8). When meiotic cells were incubated in the presence of iron, TAP-Php4 chromatin occupancy of hry1+ and mug14+ promoters decreased drastically, exhibiting 17.7- and 2.6-fold TAP-Php4 enrichment, respectively, relative to a 18S ribosomal DNA coding sequence. These levels of enrichment were 10- and 37-fold weaker, respectively, compared to those of cells incubated under low-iron conditions. Results showed that untagged Php4 immunoprecipitated only background levels of hry1+ and mug14+ promoter regions (Figure 8). Taken together, these results showed that Php4 is recruited to the hry1+ and mug14+ promoters primarily in response to low concentrations of iron. Furthermore, the results further validated the microarray data that has revealed the existence of novel meiosis-specific Php4 target genes.

**DISCUSSION**

Meiosis is a critical biological process whereby genetic information is transmitted to the next generation in sexually reproducing species. In mice, zinc ions acquired during the early stage of meiosis are critical for egg development. In the case of severe zinc deficiency, oocytes undergo a meiotic arrest at telophase I, preventing the second meiotic division (MII) (Kim et al. 2010). In S. pombe, studies have shown that copper-insufficient zygotic cells experience a meiotic block at metaphase I (Beaudoin et al. 2011). Here, we report a similar result for S. pombe zygotic cells that were synchronously induced into meiosis under severe conditions of iron starvation. Iron insufficiency led to an arrest at metaphase I. The observation that iron can be a limiting factor for normal progression of meiosis suggests that its homeostatic status may be under the control of a regulatory mechanism that prevents futile expression of iron-using proteins in response to iron deficiency.

In S. pombe and several filamentous yeasts, mechanisms of iron-sparing include downregulation of iron-using proteins by transcript repression through a specialized subunit of the CCAAT-binding factor, which is synthesized under low-iron conditions (Mercier et al. 2006, 2008; Hortschansky et al. 2007; Jung et al. 2010; Schrett et al. 2010; Brault et al. 2015). This subunit, called Php4 (S. pombe) or HapX (Aspergillus species and Cryptococcus neoformans), binds to a heterotrimeric DNA-binding complex, which becomes competent to repress target gene expression. In A. fumigatus, inactivation of HapX (hapXΔ) decreases asexual reproduction under iron starvation conditions (Schrett et al. 2010). The production of conidia is significantly reduced, exhibiting 62% less formation compared to wild-type cells (Schrett et al. 2010). Although the step where conidio genesis is blocked remains unclear, the absence of HapX results in attenuation of A. fumigatus to produce conidia, making this fungus less effective to disperse conidia into new environments such as a host organism. In the case of Php4, its
disruption in meiotic cells led to an arrest at metaphase I under low-iron conditions. This meiotic block may be due to lack of optimization of iron utilization when iron is limited. To confirm that the absence of Php4 resulted in a constitutive expression of iron-using genes during meiosis, DNA microarray experiments were performed. Transcripts corresponding to 225 genes were up-regulated (>twofold) in the absence of php4 (Table S3). Out of these 225 genes, 21% were predicted to encode proteins involved in iron-dependent biochemical pathways. The number of 225 genes was higher than the 56 genes (>twofold) and 132 genes (>1.5-fold) previously identified in iron-starved cells proliferating in mitosis (Mercier et al. 2008). The higher number of identified Php4 target genes may be due to the experimental approaches used here as opposed to those of previous microarray results (Rustici et al. 2007; Mercier et al. 2008). First, the microarray gasket slide from Agilent Technologies was improved in that it contained a larger number of probes (15,000) which allowed a ~2–3 × increased coverage for each S. pombe ORF. Second, all S. pombe ORFs were represented, including multiple sequence orphan genes and several small S. pombe specific ORFs that were not known in previous genome-wide microarray screens. Third, a large number of meiosis-specific genes identified in the present study could not be detected in previous screens due to the fact that they were not expressed in dividing cells that grew mitotically. In a second set of experiments, we identified 246 genes that were expressed at high levels under iron-replete conditions in php4+/php4+ cells (Table S1). Based on the hypothesis that Php4 target genes would be expressed at higher levels in iron-replete php4+/php4+ cells than in iron-starved php4+/php4+ cells, and that they would be expressed at higher levels in iron-deficient php4Δ/php4Δ cells than in iron-deficient php4+/php4+ cells, the overlap of the two sets of arrays included 137 genes (Table 2). Among these 137 genes loci, 23 genes corresponded to noncoding RNAs, whereas 113 genes were predicted or known to encode proteins. In the group of gene-encoded proteins, 35% of these had been assigned a known or probable function in iron-related processes. We also noted that 18 genes encoded meiosis-specific proteins (Table 2). Microarray results showed that the meiotic hry1+ gene was the most highly expressed (6.8-fold) of all of the meiotic mRNAs detected under iron-replete conditions after 7 hr of meiotic induction. A relationship between Php4 and expression of hry1+ was observed when php4+ was deleted (php4Δ). This observation revealed that hry1+ was subjected
to Php4-dependent repression under low-iron conditions. Interestingly, hry1* encodes a protein that is predicted to possess a hemerythrin-like (Hr) domain (Stenkamp 1994; Xiong et al. 2000; French et al. 2008). Hr domains contain a di-iron center that often reversibly binds oxygen (Xiong et al. 2000). Proteins that contain Hr domains were first identified in some marine invertebrates (Stenkamp 1994). Subsequently, Hr domain-containing proteins have been found in bacteria, animals, and plants (French et al. 2008; Salahudeen et al. 2009; Kobayashi et al. 2013). Potential functions of Hr domains include the detection/transport of oxygen and the detoxification, storage, and sensing of iron (French et al. 2008). In humans, a Hr-like domain has been uncovered in the FBXL5 protein (Thompson et al. 2012; Ruiz and Bruick 2014). Elegant studies have demonstrated that the FBXL5 hemerythrin domain acts as an iron sensor and fosters degradation of iron regulatory protein 2 under iron-replete conditions through the ubiquitin-proteasome system (Salahudeen et al. 2009). Interestingly, Hry1 represents the first example of a hemerythrin-like protein in yeast. Since this putative iron-using protein may participate in regulating iron homeostasis during meiosis, it represents an attractive candidate for future study. We found that mug14* was a second meiotic gene that exhibited Php4-dependent changes at the transcriptional level. This gene encodes a methylthioribulose-1-phosphatase dehydratase-like protein that is the third enzyme involved in the methionine salvage pathway present in numerous organisms (Pirkov et al. 2008; Albers 2009; Mary et al. 2012). This pathway requires iron and involves six enzymes, including an iron-requiring aicreductone dioxygenase (Adi1 in yeast), which performs the fifth step of the pathway. In response to iron starvation, meiotic S. pombe represses Mug14 expression and that may trigger arrest (at step 3) of the meiotic salvage pathway. If this were the case, this block in the salvage pathway would prevent the superfluous and futile demand of downstream proteins such as iron-consuming Adi1 (at step 5). This situation would therefore contribute to limit cellular iron utilization under iron deficiency.

Out of the 137 genes found to be up-regulated by both iron repletion and a php4Δ disruption, 119 (87%) of these genes contained one or more copies of the 5'-CCAAT-3' consensus sequence within their promoters (Table 2). In the cases of hry1* and mug14*, a ChIP approach was used to validate that Php4 associated with hry1* and mug14* promoters in vivo. In the case of genes (13%) lacking the CCAAT consensus sequence, the possibility exists that a noncanonical sequence may act as a functional DNA binding site of the Php2/3/4/5 complex. Alternatively, an up-regulation of gene expression in the absence of Php4 may be indirect. For example, it is possible that Php4 represses a gene encoding a repressor, which would downregulate expression of a subset of Php4 target genes.

Comparison of the mRNA expression profile of php4+/php4Δ diploid cells synchronously induced into meiosis under low-iron conditions with cells incubated under iron-replete conditions led to the identification of 57 genes up-regulated after 7 hr of meiotic induction. Some of these genes encoded for known components involved in iron acquisition from inorganic iron and heme (Table S2) (Labbé et al. 2013; Mourer et al. 2015). We also found several uncharacterized genes, including some that are meiosis-specific (e.g., spo5*, cum1*, and mei3*) for which a putative iron starvation-dependent function remains unclear.

Despite the fact that there was a reduction of php4* transcripts in iron-replete fep1+/fep1* cells, the presence of weak steady-state levels of php4* mRNA was still detected, revealing an incomplete repression of the transcription of php4* mRNA. Based on these observations, we expected to detect weak levels of Php4 protein in iron-replete wild-type cells. However, TAP-Php4 steady-state levels were undetectable in iron-replete cells using immunoblot assays. Fluorescent microscopy analysis showed that, 20 min after initiation of the meiotic program in iron-replete cells, GFP-Php4-associated fluorescence disappeared and was not observed during the duration of the meiotic program. mRNA and protein steady-state levels of Php4 exhibited strikingly distinct expression profiles under elevated levels of iron, suggesting the existence of a meiotic posttranslational mechanism that eliminates...
highly resistant and are adapted for effi-

gens (Botts and Hull 2010; Oiartzabal-Arano

poor environment (Becker and Skaar 2014; Ganz and Nemeth 2015),

mation for these observations is the fact that host organisms offer an iron-

et al.

here for sporulation under low-iron conditions. Because there are sev-

complete the entire meiotic process that depends on Php4, as shown

reason explaining why these proteins are essential for infection.

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