**SPO24 Is a Transcriptionally Dynamic, Small ORF-Encoding Locus Required for Efficient Sporulation in Saccharomyces cerevisiae**

Sara Hurtado, Karen S. Kim Guisbert, Erik J. Sontheimer

Department of Molecular Biosciences, Northwestern University, Evanston, Illinois, United States of America

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**Abstract**

In *Saccharomyces cerevisiae*, meiosis and sporulation are highly regulated responses that are driven in part by changes in RNA expression. Alternative mRNA forms with extended 5' UTRs are atypical in *S. cerevisiae*, and 5' extensions with upstream open reading frames (uORFs) are even more unusual. Here we characterize the gene YPR036W-A, now renamed *SPO24*, which encodes a very small (67-amino-acid) protein. This gene gives rise to two mRNA forms: a shorter form throughout meiosis and a longer, 5'-extended form in mid-late meiosis. The latter form includes a uORF for a 14-amino-acid peptide (Spo24u14). Deletion of the downstream ORF (dORF) leads to sporulation defects and the appearance of pseudohyphae-like projections. Experiments with luciferase reporters indicate that the uORF does not downregulate dORF translation. The protein encoded by the dORF (Spo24d67) localizes to the prospore membrane and is differentially phosphorylated during meiosis. Transcription of the 5'-extended mRNA in mid-meiosis depends upon the presence of two middle sporulation elements (MSEs). Removal of the MSEs severely inhibits the mid-meiotic appearance of the 5'-extended mRNA and limits the ability of plasmid-borne *SPO24* to rescue the sporulation defect of a spo24Δ mutant, suggesting that the 5'-extended mRNA is functionally important. These results reveal Spo24d67 as a sporulation-related factor that is encoded by a transcriptionally dynamic, uORF-containing locus.

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**Introduction**

Meiosis enables the formation of haploid gametes from a diploid progenitor. In the budding yeast *Saccharomyces cerevisiae*, meiosis is followed by spore formation and depends upon a highly ordered transcriptional cascade that is triggered by specific nutritional and genetic conditions [1–4]. Budding yeast meiosis is driven in part by three transcriptional phases [5]. The early phase includes genes important for the response to nutrient stress and initiation of meiosis I (MI), which includes DNA replication and recombination. The middle phase includes the end of MI as well as meiosis II (MII). During the late transcriptional phase, synthesis of the spore wall occurs and the mature ascus is generated. Checkpoints monitor the success of critical steps within the pathway, enforcing correct completion before allowing the cell to progress [3].

Several transcription factors are involved in turning on the early, middle and late genes of sporulation. Early genes are activated at their URS1 (Upstream Regulatory Sequence) 1 site by Ume6 [6], which is converted from a negative regulator to a positive regulator by Ime1 [2,6,7]. The majority of midsporulation genes are regulated by a small sequence called the midsporulation element (MSE) [8], to which the transcription factor Ndt80 binds to activate the associated genes. These Ndt80-regulated genes are involved in a variety of processes related to progression through meiotic divisions, formation of the anaphase-promoting complex, and sporulation [3,5,9–11]. Ndt80, in collaboration with other factors, also helps to regulate the smaller subset of late genes induced after MII [12].

During and after the late phase of expression, the four haploid nuclei are encapsulated into spores [13]. Spore formation begins with recruitment of vesicles to the cytoplasmic sides of the four spindle pole bodies of the haploid nuclei [14,15]. These vesicles flatten to form the prospore membrane [15]. Throughout MII, the four prospore membranes expand to fully engulf the nuclear lobes that are anchored to the spindle pole bodies [16]. At the end of nuclear division, the encapsulation is complete and the daughter nuclei are each contained within an individual cytoplasm, creating four separate prosproes with double membranes [13]. Maturation of the spores occurs through synthesis of the spore wall [17] and the breakdown of the outer membrane [18,19]. After formation of the spore wall, the anucleate mother cell remodels to form the encapsulating ascus [20–22].

Many meiosis- and sporulation-defective mutants have been identified, initially through a classical genetic screen [23] and more recently through the analysis of genome-wide collections of deletions at long (>80–100 codons) open reading frames (ORFs)
coding and noncoding RNAs in both log-phase and sporulating S. cerevisiae cells from the SK1 strain background [29]. We noted the existence of numerous transcripts with extended 5′ UTRs that appear during meiosis. Included in this set was the gene YPR036W-A (Figure 1A), which exhibits a sharp shift to a longer transcript at mid-meiosis (Figure 1B). This longer transcript, which adds 100±5 nts to the 5′ UTR (relative to the mRNA that is expressed in early meiosis), persists through the remainder of meiosis and sporulation. The extended mRNA includes a single fifteen-codon (including the stop codon) uORF that is not detectably expressed during early meiosis or log phase. The annotated dORF encoded by YPR036W-A is small (68 codons), and the function of the encoded protein is unknown. Because the YPR036W-A dORF is less than 80–100 codons, it was excluded from ORF deletion and GFP fusion collections – and therefore from genome-wide screens and analyses using these collections – reported to date. YPR036W-A was originally identified in 2001 in a screen for transcripts regulated by the transcription factor Pdr1 [37], but very little else is known about its expression and function. Because it was not included in previous genome-wide screens for ORF deletions with meiotic-specific phenotypes, its roles during meiosis and sporulation, if any, are unknown.

We confirmed the expression of a 5′-extended meiotic form of the YPR036W-A mRNA using qRT-PCR (normalized to PRP8 mRNA, Figure 1C), primer extension (Figure S1A) and 5′ RACE analysis (Figure S1B). The qRT-PCR and 5′ RACE analyses using a reverse primer ("R" in Figure 1B) within the dORF revealed that the 5′-extended signal detected by our microarray analysis (Figure 1B) reflects a transcript that is contiguous into the dORF (Figures 1C and S1B). qRT-PCR experiments with RNA samples taken every two hours during a twelve-hour meiotic time course, and using forward and reverse primers from within the YPR036W-A dORF, showed that dORF-encoding mRNAs (regardless of 5′ UTR length) are expressed throughout meiosis (Figure 1C, light bars). In contrast, when we performed qRT-PCR on the same samples using a forward primer specific for the 5′-extended region, the longer transcript was detected only at the mid-meiotic time point (6 hours) and thereafter (Figure 1C, dark bars), in agreement with the microarray data. The induction of the long form in mid-meiosis is not accompanied by a loss of the shorter form, as both transcripts can be detected later in meiosis by primer extension and 5′ RACE (Figures S1A and S1B). We conclude that YPR036W-A encodes two mRNA forms: a short form that is expressed throughout meiosis, and a 5′-extended, uORF-containing form that is restricted to middle and late meiosis.

The YPR036W-A dORF Is Required for Efficient Sporulation

To address the function of YPR036W-A, we used homologous recombination in the SK1-derived strain DKB98 to replace the dORF with a selectable marker (NAT), ypr036w-aΔ haploid spores readily formed colonies on solid media, indicating that the dORF is not essential for germination or viability. Furthermore, we observed no growth defect of the ypr036w-aΔ haploid cells (relative to DKB98) in liquid media (Figure S2). However, ypr036w-aΔ homozogous diploid cells were partially sporulation-defective, with 15%±11.5% (n = 600) of cells giving rise to tetrads after twelve hours of sporulation, compared to 68.5%±8.2% (n = 600) for the isogenic wildtype control strain (p<0.01, Student’s t-test) (Figure 2A, C and G). Of the ypr036w-aΔ cells that did not sporulate, the majority (64%, n = 100) were tetranucleate (Figure S3) and an additional 16% were binaucleate, as indicated by DAPI staining. This suggests that most ypr036w-a-
Figure 1. YPR036W-A expresses two mRNA forms. (A) Map of the YPR036W-A locus in S. cerevisiae. The region upstream of the 204-nt YPR036W-A dORF includes a 45-nt uORF that is present in a longer form of expressed mRNA that is induced in mid-meiosis. Further upstream are two consensus Ndt80 transcription factor binding sites (middle sporulation elements, or MSEs). The line denotes the boundaries of the genomic fragment included in the rescue plasmid pSH101. Black arrows mark the approximate transcription start sites, as mapped through the tiling array signal and confirmed by 5' RACE. (B) Heat map of tiling array data showing YPR036W-A expression during meiosis. Genomic coordinates along the YPR036W-A locus correspond to the horizontal axis. The array signals (from three separate cultures for each time point) are stacked vertically with the beginning of meiosis at the top, and with sporulation times indicated to the right of the heat map. The bottom six layers are from log-phase haploid and diploid cells. The positions of the uORF and dORF are given by the upper arrows. After ~6 hours of sporulation a meiosis-specific RNA is induced. Small
arrows underneath represent primer-binding sites for reverse (R), forward (F), and upstream forward (uF) primers. (C) qRT-PCR analyses using the primers depicted in (B) show expression patterns consistent with the array data, confirming the presence of a longer RNA. Moreover, the analysis indicates that the extended signal detected on the arrays is a longer RNA that is contiguous into the dORF, and does not simply reflect expression of a distinct, neighboring transcript that abuts a shorter YPR036W-A mRNA. Error bars represent the standard error of the mean (SEM) from three biological replicates. 

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a diploid cells successfully initiate meiosis, and that most of those complete MI and MI but not sporulation. Interestingly, the ypr036w-a-aΔ/+ heterozygote (Figure 2B) also exhibited a sporulation defect [22.5% ± 2.1% (n = 600) of the cells yielded asci with four spores after twelve hours], suggesting a gene dosage dependency. Transformation of a complementing CEN plasmid (pSH101, Figure 1A) partially rescues the sporulation efficiency defect, with 42.2% ± 3.5% (p < 0.05) and 49.6% ± 1.8% (p < 0.01) of plasmid-bearing cells successfully completing sporulation in the ypr036w-a-aΔ heterozygous and homozygous backgrounds, respectively (Figure 2E–G). Overexpression of YPR036W-A [via introduction of pSH101 into wild-type cells (Figure 2D and G)] did not significantly inhibit sporulation (63.5% ± 2.8% tetrad) (Figure 2A and G). Based on the sporulation efficiency defects in the ypr036w-a-aΔ mutants, we have renamed this gene SPO24.

Sporulation of wild-type diploid cells usually yields four discrete spores, with the mother cell having collapsed to form the mature ascus (Figure 2A). When one copy of the SPO24 dORF is deleted, the asci no longer appear fully mature and collapsed, but rather appear to have a void volume between the ascus wall and the spores (Figure 2B). Morphological changes that include a “pseudohyphae-like,” “extended” cell shape are also apparent in the asci generated by spo24Δ/+ heterozygous diploids (Figure 2B), and this phenotype is even more pronounced with the spo24Δ homozygotes (Figure 2C). Rescue with pSH101 ameliorated both the sporulation efficiency and pseudohyphae-like phenotypes (Figure 2E–F). Intriguingly, the spo24Δ diploid strain (and, to a lesser extent, the spo24Δ/+ heterozygous strain) also shows signs of an elongated, pseudohyphae-like morphology during vegetative growth (Figure S4), consistent with the detectable levels of SPO24 expression (Figure 1B and Figure S1A).

The SPO24 uORF Modestly Increases Translational Efficiency of the SPO24 dORF

The gene dosage-dependent effect of the SPO24 dORF on sporulation efficiency suggests that Spo2467 expression or activity is tightly controlled during meiosis. We hypothesized that the presence of the uORF in the 5’-extended mRNA that is induced in mid-meiosis could contribute to this control, since uORFs sometimes (but not always) limit translation of dORFs by preventing scanning translation initiation complexes from reaching the dORF initiation codon [36]. To test this hypothesis, we inserted 95 nts from the SPO24 5’-extended region (including the uORF, along with 43 and 7 nts of 5’- and 3’-flanking sequence, respectively) into the yeast-specific luciferase reporter construct yCP22FL1 [38] to generate yCP22FL1-uORF (Figure 3A). We also generated a control construct (yCP22FL1-uORFMUT) that was identical except for an AUG→CUG mutation in the uORF initiation codon (Figure 3A). We also used the empty plasmid yCP22FL’ (i.e., containing no uORF upstream of luciferase) for standardization of luciferase expression and activity. In all cases, reporter expression was driven by the TEF1 promoter. Plasmids were transformed into wildtype W303A yeast, and following growth and expression, cells were lysed and assayed for mRNA levels as well as luciferase activity. Normalized to yCP22FL’, yCP22FL1-uORF had a relative luciferase expression of 1.3 ± 0.2 (Figure 3B), suggesting that the SPO24 uORF can have at most a mild effect on expression. Mutation of the uORF’s AUG initiation codon in yCP22FL1-uORFMUT led to relative expression (again normalized to that of yCP22FL’) of 1.6 ± 0.4 (Figure 3B), which is marginally different from that of the unmutated plasmid (p = 0.37, Student’s t-test).

Luciferase expression is a function of both RNA accumulation as well as translation. It therefore remained possible that the uORF insert affected both in opposing manners, masking any translation-specific effect. We therefore assayed reporter transcript levels by qRT-PCR, normalizing to mRNAs expressed from a control housekeeping gene (ACT1). We found that mutation of the uORF start codon led to an ~2-fold increase in steady-state mRNA levels (Figure 3C) relative to the unmutated uORF-containing construct, perhaps by limiting the ability of the uORF to direct the reporter mRNA into the nonsense-mediated-decay pathway [39]. The translation efficiency, defined as the ratio of protein expression (as reflected in luciferase activity) to mRNA levels (see Methods), decreased slightly when the start codon was abolished (Figure 3D), indicating that the intact uORF has a modest stimulatory effect on translation. We conclude that the SPO24 uORF is unlikely to play an inhibitory role in Spo2467 translation. These experiments, which for technical reasons were done with vegetatively growing cultures, do not by themselves exclude the possibility that the SPO24 uORF does inhibit Spo2467 translation specifically during meiosis, when the 5’-extended form of SPO24 mRNA is transcribed. Nonetheless, recent ribosome profiling experiments in staged meiotic cultures have revealed numerous cases in which increased uORF translation is positively correlated with ribosome density on the associated dORF, and the SPO24 uORF and dORF were in this category [30].

Two SPO24 uORF Modifiers of Translational Efficiency are Required for Efficient Sporulation

To further understand the regulatory basis for Spo2467 expression, we examined the sequences upstream of the SPO24 transcribed regions and identified two candidate middle sporulation elements (MSEs), which in many other genes serve as binding sites for the meiosis-specific transcription factor Ndt80. This DNA-binding protein is the major transcription factor driving the expression of genes that, like the 5′-extended form of SPO24, are activated in mid-meiosis. To examine the role of the MSEs, we used site-directed mutagenesis to create deletion mutations in both putative MSEs in the SPO24 rescue plasmid (Figure 1A), generating pSH110(MSEΔ). We carried out qRT-PCR analyses of RNA samples from a meiotic time course, using the reverse and upstream forward primers (Figure 1B) that detect expression of the 5′-extended SPO24 mRNA. When spo24Δ cells were complemented with the SPO24 plasmid pSH101, the 5′-extended mRNA was readily detected six hours into meiosis and thereafter (Figure S5A), consistent with our previous results (Figure 1C). In contrast, removal of the MSEs from the rescue plasmid led to a drastic reduction in levels of the 5′-extended mRNA (Figure S5A). Surprisingly, overall levels of dORF-containing transcripts were unchanged or slightly higher (up to ~2.5-fold, depending on the time point) with the pSH110(MSEΔ) rescue plasmid (Figure S5B), suggesting that the loss of the 5′-extended transcripts is
compensated for by other transcriptional or post-transcriptional effects on mRNA accumulation. These results indicate that the MSEs are indeed important for induction of the 5'-extended SPO24 mRNA during meiosis.

We next tested the requirement for the MSEs during meiosis by comparing sporulation efficiencies of the spo24Δ/Δ heterozygous deletion derivative (B), and the spo24Δ homozygous deletion derivative (C), respectively. The spo24Δ/+ heterozygote exhibits decreased sporulation efficiency and a more elongated morphology (B). Both the sporulation defect and the pseudohyphae-like morphology are exacerbated in the spo24Δ homozygote (C), suggesting a gene dosage effect on the phenotypes. (D–F) As in A–C, respectively, but with cells that harbor the SPO24-containing, CEN rescue plasmid pSH101 (see Figure 1A). The rescue plasmid abolishes the sporulation efficiency defect as well as the elongated cellular morphology. (G) Quantification of sporulation efficiency, as measured by the number of spores per ascus (n = 200 for each of three biological replicates) after 40 hours on solid sporulation medium. Complementation of the spo24 deletion with the pSH101 plasmid results in partial rescue the sporulation defect.

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Figure 2. YPR036W-A (SPO24) is required for efficient sporulation. (A–C) Differential interference contrast (DIC) images of sporulated wildtype (DKB98) yeast cells (A), the spo24Δ/+ heterozygous deletion derivative (B), and the spo24Δ homozygous deletion derivative (C), respectively. The spo24Δ/+ heterozygote exhibits decreased sporulation efficiency and a more elongated morphology (B). Both the sporulation defect and the pseudohyphae-like morphology are exacerbated in the spo24Δ homozygote (C), suggesting a gene dosage effect on the phenotypes. (D–F) As in A–C, respectively, but with cells that harbor the SPO24-containing, CEN rescue plasmid pSH101 (see Figure 1A). The rescue plasmid abolishes the sporulation efficiency defect as well as the elongated cellular morphology. (G) Quantification of sporulation efficiency, as measured by the number of spores per ascus (n = 200 for each of three biological replicates) after 40 hours on solid sporulation medium. Complementation of the spo24 deletion with the pSH101 plasmid results in partial rescue the sporulation defect.

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compensated for by other transcriptional or post-transcriptional effects on mRNA accumulation. These results indicate that the MSEs are indeed important for induction of the 5'-extended SPO24 mRNA during meiosis.

We next tested the requirement for the MSEs during meiosis by comparing sporulation efficiencies of the spo24Δ mutant strain carrying pSH110(MSEΔ) to those of the same strain carrying either the wildtype rescue plasmid (pSH101) or no rescue plasmid, as well as the parental SPO24 strain. In this experiment, after 41 hours on sporulation plates, 55%±7.1% of wildtype DKB98 cells had formed tetrads (Figure 4). When analyzed in parallel, only 18.3%±1.8% of spo24Δ cells completed sporulation (p = 0.065). Introduction of the complementing SPO24 plasmid pSH101 into the spo24Δ cells rescues sporulation back to wildtype levels (58.2%±8.3%) (Figure 4). Complementation is mostly lost when the MSEs are deleted, with only 26%±7.0% of the pSH110(MSEΔ)-transformed spo24Δ cells forming tetrads (p = 0.02) (Figure 4). These results indicate that the MSEs are important for full SPO24 function during meiosis, despite the fact that their deletion does not affect overall dORF-containing mRNA accumulation (see discussion). Although pSH110(MSEΔ) failed to restore efficient tetrad formation to spo24Δ cells, we did observe an increase in dyads (asci containing two spores), with dyad frequencies rising from 6.5%±2.8% to 27.8%±12% in the spo24Δ and pSH110(MSEΔ) strains, respectively (Figure 4). The low frequency of dyad formation in the spo24Δ cells may be due in part to a defect in meiotic entry or early meiosis. The increase in dyad but not tetrad frequency upon introduction of pSH110(MSEΔ) may reflect a rescue of Spo24d67 function early in the pathway, but then a failure to rescue later Spo24 d67 functions due to the loss of the 5'-extended transcript. Interestingly, pSH110(MSEΔ)-rescued, sporulated spo24Δ cells did not exhibit the pseudohyphae-like phenotype (Figure S3C), suggesting that the 5'-extended mRNA does not play a role in the morphological aspects of SPO24 function.
SPO24 Encodes a Phosphoprotein that is Conserved in Other Yeasts

Protein expression from the SPO24 locus has not been well characterized. To address this issue, we used homologous recombination to introduce a 9xMyc tag to the C-terminus of the SPO24 ORF. Unlike deletion of the SPO24 ORF, the addition of the tag had no effect on sporulation efficiency (Figure S6), suggesting that the tag does not compromise SPO24 function. Protein samples from a meiotic time course were analyzed by probing western blots with anti-myc antibody, and a protein of the predicted size (21.5 kDa) was detected throughout meiosis (Figure 5). This protein was not detected in the untagged wildtype cells (Figure S7), indicating that the detection was specific.

The western blots revealed not only the expected 21.5 kDa protein, but also a slightly slower-migrating band that was most pronounced during mid-meiosis (Figure 5). The slightly retarded mobility of the upper band suggested that it may be a post-translationally modified (e.g., phosphorylated) form of the Spo24d67 protein. To test whether the upper band was a phosphoprotein, we subjected protein samples from the epitope-tagged strain to lambda phosphatase digestion, in parallel with a mock-digested control. The treated samples were then analyzed by anti-myc western blots (Figure 6B). Lambda phosphatase treatment collapsed the doublet to single band, indicating that the upper band of the doublet is a phosphorylated form of the Spo24d67 protein (Figure 6B). The Spo24d67 amino acid sequence includes several consensus phosphorylation sites (Figure S8), and inspection of publicly available S. cerevisiae phosphoproteomic datasets confirms these sequences as sites of phosphorylation [40–43], consistent with our findings. We conclude that the Spo24d67 protein is expressed and differentially phosphorylated during meiosis. Aside from the apparent phosphorylation sites, the Spo24d67 amino acid sequence and conservation reveals no known domains that would suggest particular cellular or biochemical functions.

The Spo24d67 Protein Localizes to the Prospore

The subcellular localization of a protein can provide important insight into its function. To examine Spo24d67 localization, we used gap repair to fuse the coding sequence for green fluorescent protein (GFP) to the C-terminus of the SPO24 ORF in the
pSH101 rescue plasmid. Sporulation assays demonstrated that the GFP tag had no detrimental effect on the rescue of the spo24Δ sporulation defect, indicating that the GFP-tagged protein is functional (Figure S6).

We next used fluorescence microscopy to examine the subcellular distribution of GFP-tagged Spo24d67 protein in sporulated cultures. Ring-like structures were evident in spo24Δ cells carrying the Spo24d67-GFP-expressing plasmid (Figure 6A). We observed these rings in cells that contained no spores that were discernable by differential interference contrast microscopy, as well as those with visible spores. This suggests that the Spo24d67 localization pattern is established early in sporulation, such as during prospore membrane formation [18]. We obtained a plasmid expressing a red fluorescent protein (RFP)-tagged prospore marker (Spo2051–91) [44] and modified it by the insertion of our SPO24-GFP construct. We introduced this plasmid into wildtype DKB98, and the transformed cells were imaged for RFP and GFP fluorescence. We observed clear co-localization of Spo24d67-GFP with the RFP-Spo2051–91 [44] prospore marker (Figure 6B), confirming that the Spo24d67-GFP rings reflect localization to the prospore membrane.

**Discussion**

We report the characterization of SPO24 (previously YPR036W-A), a gene that encodes a novel and unusually small (67 amino acids) sporulation factor and that exhibits a dynamic expression pattern during meiosis. As noted previously by us [29] and others [30], a SPO24 5’-extended mRNA is induced at mid-meiosis, and the 5’-extension includes a uORF with the potential to encode a 14-amino-acid peptide. Ribosome profiling data indicate that this uORF is translated during meiosis [30], but the regulation and roles (if any) of the SPO24 5’-extended mRNA, the uORF, and the protein encoded by the dORF (Spo24d67) are poorly understood.

Our analyses reveal that in the absence of the dORF, sporulation efficiency decreases significantly, in keeping with the strong induction of this gene during meiosis. Furthermore, the morphology of the sporulated cells is perturbed, with the asci exhibiting an extended shape almost as if the mother cell had begun to enter a “pseudohyphal-like” state during meiosis and sporulation. Intriguingly, the defects are also observed during sporulation of the spo24Δ/+ heterozygous strain (though they are less severe than in the homozygous deletion strain), suggesting partial haploinsufficiency. Although the elongated structures are reminiscent of partially pseudohyphal cells, we do not know if this appearance bears any relationship with true pseudohyphal growth. Nonetheless, the relationship between sporulation and pseudohyphal growth is suggestive. Sporulation is induced in yeast through two nutritional conditions: limitation of certain required nutrients, like nitrogen, and the absence of a fermentable carbon source [4,45–48]. When nitrogen is limited in the presence of a fermentable carbon source like glucose, the yeast cells do not sporulate, but rather form pseudohyphae to scavenge further for...
Western analysis of epitope-tagged Spo24<sup>d67</sup> protein. (A) Protein samples from a sporulation time course were subjected to Western analysis with antibodies against a 9-Myc tag fused to the C-terminus of Spo24<sup>d67</sup>. The blots reveal a protein of the expected molecular weight (21.5 kDa), indicating that the Spo24<sup>d67</sup> protein is expressed throughout meiosis. Nap1 was also analyzed as a loading control. The protein appears as a doublet at multiple time points, suggesting the existence of a posttranslationally modified form. (B) The upper band of the Spo24<sup>d67</sup> doublet is susceptible to λ phoshatase treatment. Both Spo24 bands persist after mock digestion, whereas the upper band is abolished by λ phosphatase, indicating that the upper band is a phosphorylated isofrom.

Figure 6. Spo24<sup>d67</sup> localizes to the prospore cortex. (A) spo24<sup>Δ</sup> cells carrying a plasmid (pSH112) expressing Spo24-GFP were sporulated on SPM plates for 24 hours. GFP fluorescence microscopy (right panel) revealed ring-like structures that appear to correspond with spore locations in the DIC image (left panel). (B) As in (A), except that the cells carrying a plasmid (pRS426_RFP_Spo20<sup>51–91</sup> Spo24_GFP) expressing both RFP-Spo20<sup>51–91</sup> (a prospore marker) and Spo24-GFP. The GFP ring-like structures co-localize with the prospore marker protein (in red, merge represented by yellow), suggesting that Spo24 is associated with the prospore cortex.

Intriguingly, the 5′-extended form of the SPO24 mRNA expression from a plasmid (accomplished by MSE deletion) renders that plasmid less effective at rescuing the sporulation defect caused by a spo24 deletion. These results can be reconciled if the uORF serves a regulatory function, perhaps by modulating Spo24<sup>d67</sup> translation. Loss of the 5′-extended form via MSE deletion does not reduce the overall accumulation of dORF-containing mRNA, suggesting that the mid-meiotic induction does not serve simply to increase overall SPO24 mRNA expression levels above a functionally important threshold. Although uORFs are commonly thought to inhibit translation of dORFs by reducing access of scanning ribosomes to the dORF’s initiation codon, our results with luciferase reporter constructs, as well as ribosome profiling data, support a modestly positive role for the Spo24 uORF in promoting dORF translation, which could account for the phenotypic effects of MSE deletion. At present we cannot rule out the possibility that the Spo24 peptide itself exerts a biochemical function, though future studies will be required to address this issue. The ribosome profiling work found many uORFs whose translation is positively correlated with dORF translation, though the mechanisms involved are unknown. Our results support the inclusion of the SPO24 uORF as a member of this category.

In summary, we have identified SPO24 (formerly YPR036W-A) as a previously unrecognized sporulation gene that encodes a 67-amino-acid phosphoprotein that can localize to the prospore cortex. Loss of Spo24<sup>d67</sup> function leads to defects in sporulation efficiency as well as ascus morphology. Further delineation of this protein’s roles in sporulating cells promises to reveal novel aspects of the meiotic program in yeast.

Materials and Methods

RNA Isolation and Real-time Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

mRNA levels were determined using SYBR Green-based quantitative PCR. RNA was extracted using hot phenol chloroform extraction. Briefly, cells were resuspended in AE buffer (50 mM NaOAc, 10 mM EDTA, pH 8.0) with 1.7% SDS. An equal volume of acid phenol:chloroform (5:1) was added along with zircon/silica beads, vortexed in the cold for 2 minutes, and then alternated between heating at 65°C for 2 minutes and vortexing at 4°C for 2 minutes for ten cycles. The lysate was spun in a microfuge at full speed for 15 minutes and the supernatant was extracted again without heat. A chloroform clean-up was

Figure 5. Western analysis of epitope-tagged Spo24<sup>d67</sup> protein. (A) Protein samples from a sporulation time course were subjected to Western analysis with antibodies against a 9-Myc tag fused to the C-terminus of Spo24<sup>d67</sup>. The blots reveal a protein of the expected molecular weight (21.5 kDa), indicating that the Spo24<sup>d67</sup> protein is expressed throughout meiosis. Nap1 was also analyzed as a loading control. The protein appears as a doublet at multiple time points, suggesting the existence of a posttranslationally modified form. (B) The upper band of the Spo24<sup>d67</sup> doublet is susceptible to λ phosphatase treatment. Both Spo24 bands persist after mock digestion, whereas the upper band is abolished by λ phosphatase, indicating that the upper band is a phosphorylated isofrom.

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performed, and RNA was then precipitated with isopropl alcohol and sodium acetate, washed with 70% ethanol, and resuspended in water.

For cDNA synthesis we used Superscript III (Invitrogen) with 1 µg of RNA and gene-specific reverse primers, according to the supplier’s protocol. Real-time PCR was performed using gene-specific primers (Table S1) and SYBR Green. Samples were run in triplicate with a genomic DNA control used in the PCR step. For miotic time course analyses, primers specific for the PRP8 mRNA were used for normalization.

**Strains, Media and Plasmids**

The yeast SK1 strain DRB83 (MATα, ura3−, lys2−, his3−, leu2−:hisG1−, his4X::LEU2/ his4B::LEU2, arg4−:NspI/ arg4−:BglII) was graciously provided by Doug Bishop (University of Chicago) and used for sporulation and diploid analysis. The haploid sister strain DRB83 (MATα, lys2−, ura3−, leu2−:hisG1−, his4X::LEU2/ his4B::LEU2, arg4−:NspI) was used for haploid analysis. W303-1A (MATα, leu2−:3-112 trpl−1 can1−100 ura3−1 ade2−1 his3−11,15) was used for luciferase experiments. Yeast strains (Table S2) were constructed by standard genetic crosses or by LiAc transformation [52]. Deletions and chromosomal tags were created using homologous recombination with PCR products created through methods described in Janke et al. and Longtine et al. [33,34].

Yeast media including YPD, antibiotic selection plates and synthetic drop-out plates were all made according to standard recipes. Mciosis was induced by shaking cells at 30°C in 50 mL SPM+1/5 COM liquid sporulation medium for 18–48 hours as noted. In the cases where time courses were used, cells were synchronized using pre-sporulation medium containing 10% KOAc following the Bishop lab protocol (http://bishops.bsd.uchicago.edu/protocols/SporulationProtocol.pdf) and described previously [29]. Briefly, yeast were grown overnight at 30°C in 5 mL YPD liquid and then diluted into 25 mL of SPS. Cells were shaken for approximately 5 hours, and then diluted for overnight growth to obtain the proper concentration (OD600 between 0.5 to 1.4; most cultures were within our preferred range of 0.7–1.0) by the next morning. If cells grew properly they were switched to SPM+1/5 COM medium to begin sporulation. For quantification of sporulation (Figure S2), 5 mL of YPD was inoculated with one colony of each yeast strain to be tested. Three biological replicates of each were cultured. Yeast were shaken at 30°C in YPD overnight then washed with water and then transferred to SPM and shaken again at 30°C for 40 hours. 200 asci from each sample were examined for the number of spores in each, and the average numbers and standard deviations were calculated. DAPI stains were also performed on cells prepared in the same manner, and the numbers of mononucleate, binucleate, and tetranucleate cells were counted out of 100 unsporulated cells total.

All newly constructed plasmids (Table S3) were confirmed by DNA sequencing. Rescue plasmids were created by first TopoTA cloning (Invitrogen) a PCR amplicon of SPO24 from genomic DNA preparations of SK1, including approximately 700 nucleotides (nt) upstream and 400 nt downstream of the annotated ORF. The TA plasmid was then digested with EcoRI, and the gel-purified insert was ligated into the EcoRI site of the pRS316 vector to yield pSH101. The GFP-tagged derivative, pSH112, was generated by gap repair with a GFP PCR amplicon made with primers that included the appropriate SPO24 sequences. The PCR product was co-transformed into W303-1A along with BrG1-digested pSH101, and then selected on -Ura plates to yield plasmid pSH112. The plasmid was then extracted and propagated in E. coli before transformation into SK1. pSH110 (a pSH101 derivative carrying deletions of the two MSEs) was created through site-directed mutagenesis.

**Western Blotting and Phosphatase Assay**

Protein was extracted from the samples through a TCA protein preparation. Briefly, cell pellets from 5 mL of sporulated culture were resuspended in 300µL of 10% cold TCA, chilled for 10 min on ice, re-centrifuged, and washed with 1 mL of acetone. The pellet was dried in a laminar flow hood for 2 hours and then resuspended in 150 µL protein breaking buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 2.7 mM DTT]. The sample was vortexed with glass beads in the cold room for 15 minutes with one-minute pauses on ice every 2 minutes. Concentrations were determined using the BioRad Protein Assay. 25 µg of protein was loaded for each sample onto a 15% SDS polyacrylamide gel. A 1:5000 dilution of anti-myc antibody 9E10 (Santa Cruz Biotechnology) and a 1:15000 dilution of donkey anti-rabbit IR680 (1:20,000; LI-COR Biosciences) were used to visualize myc-tagged Sp024GFP. Anti-Nap1 loading control antibody (1:2000) was generously provided by Doug Kellogg (University of California, Santa Cruz). Donkey anti-rabbit IR680 (1:20,000; LI-COR Biosciences) was used as the secondary antibody for Nap1 detection. Blots were imaged on an Odyssey instrument.

Phosphatase treatment consisted of addition of lambda phostphatase and the supplied buffer (New England Biolabs) to 25 µg of protein from the previous extraction. After a one hour incubation at 37°C, samples were loaded onto a 15% SDS polyacrylamide gel and blotted/imagined as above. Mock treatments containing only protein and buffer were done in parallel.

**Luciferase Assays of Translation Efficiency**

FL′ and yCP22FL1 plasmids [38] kindly provided by John McCarthy (University of Manchester) and their derivatives, yCP22FL1 uORF and yCP22FL1 uORFmut, were transformed into W303A and selected on -Trp plates. Positive colonies were grown overnight in 5 mL of -Trp media. The following day 1 mL of overnight culture was diluted into 25 mL -Trp media and were grown exponentially until A600 = 0.8 to 1.0. 5 mL of cells were then harvested for preparation of protein extracts for luciferase analysis using the Promega reporter system. Another 0.1mL was also harvested for total RNA extraction and qRT-PCR as described above, except that ACT1 mRNA was used for normalization. All analyses were done with three technical replicates. In each case, translational efficiency was calculated as the ratio of translation (represented by luciferase expression) to RNA levels (obtained by qRT-PCR).

**Microscopy and Image Analysis**

Fluorescence and differential interference contrast microscopy was performed using a Zeiss Axiovert 200 M microscope equipped with a Cascade II-512B camera (Photometrics, Inc.). Images were taken with a 100×/1.45-numerical aperture oil immersion objective. Images were acquired using the Openlab software (v5.5.0; Improvision).
Supporting Information

Figure S1 Confirmation of a 5′-extended YPR036W-A mRNA.

Figure S2 Growth is not inhibited in the ypr036w-a strain.

Figure S3 DAPI staining of ypr036w-aA strain reveals that the majority of unsporulated cells are tetranucleate.

Figure S4 Growth in YPD of WT, spo24Δ+/– heterozygous strain and spo24Δ diploid strain shows signs of an elongated, pseudohypha-like morphology during vegetative growth, consistent with the detectable levels of SPO24 expression (Figure 1B and Figure S1A).

Figure S5 Figure S5: A and B. Deletion of the MSEs decreases expression of the longer (5′-extended) mRNA form.

Figure S6 C-terminal Spo24d67 fusions support wild-type sporulation efficiencies.

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