Phosphorylation of the transcription factor Atf1 at multiple sites by the MAP kinase Sty1 controls homologous recombination and transcription

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Running Title: Activation of Atf1 by phosphorylation at multiple sites

ABSTRACT

Transcription factors are often the downstream effectors of signaling cascades. In fission yeast, the transcription factor Atf1 is phosphorylated by the MAP kinase Sty1 under several environmental stressors to promote transcription initiation of stress genes. However, Sty1 and Atf1 have also been involved in other cellular processes such as homologous recombination at hotspots, *ste11* gene expression during mating and meiosis, or regulation of *fbp1* gene transcription under glucose starvation conditions. Using different phospho-mutants of Atf1, we have investigated the role of Atf1 phosphorylation by Sty1 in those biological processes. An Atf1 mutant lacking the canonical MAP kinase phosphorylation sites cannot activate *fbp1* transcription when glucose is depleted, but it is still able to induce recombination at *ade6.M26* and to induce *ste11* after nitrogen depletion; in these last cases, Sty1 is still required, suggesting that additional non-canonical sites are activating the transcription factor. In all cases, an Atf1 phosphomimetic mutant bypasses the requirement of the Sty1 kinase in these diverse biological processes, highlighting the essential role of the DNA binding factor Atf1 on chromatin remodeling and cell adaptation to nutritional changes. We propose that post-translational modifications of Atf1 by Sty1, either at canonical or non-canonical sites, are sufficient to activate some of the functions of Atf1, those involving chromatin remodelling and transcription initiation. However, in the case of *fbp1* where Atf1 acts synergistically with other transcription factors, elimination of the canonical sites is sufficient to hamper some of the interactions required in this complex scenario and to impair transcription initiation.

Keywords: transcription regulation; *Schizosaccharomyces pombe*; Sty1; Atf1; homologous recombination; phosphorylation; *fbp1*; *ste11*

Abbreviations used:

TF, Transcription Factor; MAP kinase, Mitogen-Activated Protein Kinase; S/TP, serine/threonine-proline; bZIP, basic zipper; Pol II, RNA polymerase II; CRE, cAMP-response element; STRE, Stress-Response Element; UAS, Upstream Activation Sequence; mlonRNA, metabolic long non coding RNA; TSS, transcriptional start site; ChIP, chromatin immuno-precipitation; MNase, Micrococcal nuclease; SAGA, Spt-Ada-Gcn5 acetyltransferase; SD, standard deviation.

INTRODUCTION

The response to environmental cues in all eukaryotic organisms is triggered by mitogen-activated protein MAP kinase pathways that up-regulate stress-dependent gene expression programs to allow cell adaptation [1]. After stress imposition, phosphorylation of MAP kinase promotes its translocation to the nucleus and triggers phosphorylation of transcription factors (TFs). Activated TFs adapt the complex RNA polymerase II (Pol II) transcriptional machinery into particular sets of genes to mediate specific changes in the gene expression program in order to turn extracellular signals into specific cellular responses [2].

The fission yeast *Schizosaccharomyces pombe* responds to environmental stressors through the Sty1/Spc1 MAP kinase pathway, which is activated by phosphorylation events in response to several stress conditions and elicits a wide change of the gene expression program at the transcriptional level [3-7]. Upon stress, Sty1 is phosphorylated and accumulates in the nucleus to promote transcriptional activation or repression of genes [7-10], and these transcriptional changes are totally or partially dependent on the Atf1 TF [6, 11]. Atf1 is a basic zipper (bZIP)-containing TF that form heterodimers with Pcr1, another bZIP protein. Upon stress, this TF is phosphorylated by Sty1, and it is worth to mention that nuclear accumulation of the kinase is not sufficient to induce phosphorylation of Atf1: Sty1 phosphorylation is also required to activate its kinase activity and phosphorylate the TF [12, 13].

In response to extracellular hydrogen peroxide (H_2O_2) at sub-lethal concentration, more than 500 genes are up-regulated more than 2-fold in a Sty1-Atf1 dependent manner [5, 6]. The role of the signal-induced phosphorylation of Atf1 by Sty1 in transcription activation was not clear: it had been proposed to be relevant only as a protein stabilization factor [14]. However, we recently described that a phosphomimetic Atf1 mutant is able to engage the anti-stress transcriptional program in a Sty1-independent manner. Phosphorylation of the TF *per se* does not largely contribute to Atf1 recruitment to DNA, but rather promotes transcription initiation. The main function of Sty1 in transcription activation is to promote Atf1 phosphorylation, since expression of phosphomimetic Atf1 mutants bypasses the requirement of the MAP kinase [11]. Interestingly, cells expressing Atf1 mutants lacking the canonical MAP kinase phospho sites (serine/threonine-proline S/TP sites) are sensitive to oxidative stress due to the lack of transcriptional induction of a subset of stress genes, including *ctt1*

and *srx1* [11]; these genes are co-synergistically regulated by Atf1 and another TF, Pap1. On the contrary, genes such as *gpd1* and *hsp9*, only dependent on active Atf1, are not largely affected by expression of a hypo-phosphorylation mutant of Atf1, even though this induction is still dependent on the Sty1 kinase [11, 15]. We proposed that phosphorylation of Atf1 by Sty1 establishes a platform for interactions with the basal transcriptional machinery (either Pol II itself, chromatin remodelers or other TFs) to facilitate transcription initiation [11].

Apart from their essential role in the activation of gene transcription under stress conditions, Sty1-Atf1-Pcr1 have also been implicated in other cellular processes such as meiotic homologous recombination at the mutated chromosomal locus *ade6-M26*. The binding of the bZIP-containing proteins to this hotspot is due to a single base pair substitution at the *M26* allele of the *ade6* gene, which generates a cAMP-response element (CRE)-like DNA site 5'-ATGACGT-3' [16]. TF binding promotes meiotic recombination at this particular hotspot [and at other naturally occurring Atf1 binding sites [17, 18]] through formation of double-strand DNA breaks around it by topoisomerase II-like protein Rec12/Spo11 [19], although this process seems to be independent of Atf1 phosphorylation [20].

The Sty1-Atf1 pathway has also an important role in the response to nutrient starvation, with nitrogen or glucose deprivation triggering the activation of the cascade. Sty1 is involved in the initiation of the mating and meiosis program through regulation of *ste11* levels, a master regulator of this process [21-23]. Accumulation of the *ste11* transcripts upon nitrogen depletion is triggered by activation of the Sty1-Atf1-Pcr1 pathway and inactivation of the cAMP-dependent Pka1 kinase by Cgs1 [9, 24]. When Pka1 is inactivated, it can no longer phosphorylate the TF Rst2, substrate of Pka1, which becomes active after its hyper-phosphorylation via other kinases, and suffers a cytoplasm-to-nucleus translocation [25]. However, CRE sites are not present at the *ste11* promoter, and neither Atf1, nor Pcr1 or Sty1 have been found at this gene in a genome-wide study [26]. Indeed, up-regulation of *ste11* by the Sty1-Atf1 pathway is an indirect process: Sty1-Atf1 regulates *cgs2* transcription; it codes for the phosphodiesterase Cgs2, a negative regulator of the cAMP-dependent protein kinase (protein kinase A) pathway [27].

Regarding glucose starvation, it has been described that Sty1 and Atf1 are also involved in the response to glucose starvation by transcriptional regulation of the fructose-1,6-bisphosphatase coding gene (*fbp1*), a key enzyme in the gluconeogenic pathway [28]. Upon glucose depletion, Atf1 and

Rst2 TFs participate in the progressive modification of chromatin configuration in the *fbp1* promoter region to an open state [29, 30]. The binding sites for Atf1 and Rst2 at the *fbp1* promoter region have been named upstream activation sequences UAS1 and UAS2, respectively [31], and are more than 600 base pairs apart. UAS1 contains the conserved CRE site to which Atf1 has high affinity, while UAS2 contains the stress-response element (STRE site) which is the target sequence for Rst2 [31]. In the current model, the chromatin of *fbp1* promoter region remains in a closed configuration. When glucose is depleted, Atf1 is recruited at UAS1 site promoting the transcription of a cascade of *metabolic long non coding RNAs (mlonRNAs)* that induce the opening of chromatin around UAS1 and UAS2 [32, 33]. Likewise, *mlonRNAs* transcription also facilitates promoter association of Atf1, enhancing *fbp1* gene expression [34]. The binding of Atf1 to UAS1 is stabilized by Rst2, probably through the formation of a chromatin loop which spatially brings both UAS close together [30]. UAS2 site also serves as a binding site for another C₂H₂ Zn finger TF, the Scr1 repressor. Rst2 and Scr1 reciprocally regulate the induction of *fbp1* in low glucose conditions [35]. Thus, chromatin immunoprecipitation assays revealed a switching of Scr1 to Rst2 bound at UAS2 during glucose derepression. The high glucose-dependent repressive role of Scr1 has been characterized in depth in the genes coding for the high-affinity glucose transporter Ght5 [36] and for the TF Rsv1 [37]. After glucose starvation, Scr1 is quickly exported from the nucleus to the cytoplasm, at which time Rst2 is imported into the nucleus.

In all the above scenarios, Atf1-dependent transcription or recombination initiation has been associated with chromatin remodeling [27, 38, 39], but only in the case of *fbp1* additional TFs participate in the process. We aimed at deciphering the role of the signal-dependent phosphorylation of Atf1 by Sty1 in the previously described biological processes. To this end, we have expressed HA-tagged wild-type Atf1 from a constitutive promoter, as well as hypo-phosphorylated and phosphomimetic mutants, and studied their behavior in homologous recombination and in the activation of the *ste11* and *fbp1* genes upon nitrogen or glucose depletion, respectively. We demonstrate that Atf1 phosphorylation is not required for the regulation of recombination at *ade6.M26* and the induction of *ste11* after nitrogen starvation, but it is critical to activate *fbp1* gene transcription when glucose is depleted. Importantly, Atf1 phosphomimetic mutant bypasses the requirement of the Sty1 kinase in all those biological processes, revealing the essential role of the Atf1 TF and its phosphorylation status on chromatin remodeling and cell adaptation to stress.

RESULTS

Atf1 phospho-mutants and chronological aging

We designed and constitutively expressed two types of Atf1 phosphorylation mutants: HA-Atf1.10M has ten out of eleven putative MAP kinase phosphorylation sites mutated to alanine or isoleucine, and therefore is a hypo-phosphorylation mutant, while the HA-Atf1.10D, with substitutions to acidic residues, is a phosphomimetic one (Figure 1(a)). The 11th SP site at position 438-439 lies close to the bZIP domain and has been shown before to have no impact on Atf1 activity as a TF [11]. These mutants allowed us to identify two subsets of stress genes, so that cells expressing HA-Atf1.10M are unable to activate one group (i.e. *ctt1* or *srx1*) but can still activate the other one in a Sty1-dependent manner (Figure 1(b)). Expression of HA-Atf1.10D is sufficient to trigger the expression of both sets of genes in a Sty1-independent manner, either in response to peroxides (*ctt1*, *srx1*) or constitutively (*gpd1*, *hsp9*).

Sty1 and Atf1 were previously reported to participate in the regulation of lifespan [40, 41]. Thus, cells lacking Sty1 display reduced viability during stationary phase, while strain $\Delta pyp1$, encoding a negative regulator of the cascade, is a long-lived mutant [40]. As shown in Figure 1(c) and previously published [40], glucose-rich cultures of wild-type and $\Delta sty1$ cells are not viable five days after reaching stationary phase. Similar to what has been reported for $\Delta pyp1$ [40], cells expressing HA-Atf1.10D are long-lived, even in the absence of Sty1 (Figure 1(c)).

Cells expressing HA-Atf1.10M are still able to activate *ste11* after nitrogen starvation

Sty1 and Atf1 are also involved in the transcriptional activation of the *ste11* or *fbp1* genes, and they also trigger meiotic recombination (Figure 1(d)) [17, 18, 21-23, 28]. We first confirmed that $\Delta atf1$ cells expressing HA-Atf1 display a wild type pattern of activation of *ste11* and its downstream gene, *mei2* [22] (Figure S1A). As indicated in the Introduction, Sty1 and Atf1 do not regulate *ste11* directly in response to nitrogen depletion, but rather activate transcription of the *cgs2* gene, coding for a negative regulator of the Pka1 pathway [27]. We confirmed that *ste11* and *mei2* transcription is totally abolished in $\Delta cgs2$ cells expressing any of the HA-Atf1 mutant derivatives, even HA-Atf1.10D (Figure S1B). In order to study the role of Atf1 phosphorylation in the up-regulation of the *cgs2-ste11-me2* cascade (Figure 2(a)), we expressed HA-Atf1 and its mutant derivatives in $\Delta atf1$ cells, and measured

cgs2, *ste11* and *mei2* transcripts upon nitrogen starvation by quantitative PCR. As shown in Figure 2(bcd), expression of either HA-Atf1 or its two mutant derivatives are capable of activating these genes. However, only cells expressing HA-Atf1.10D were able to induce *ste11* (Figure 2(e)) or *mei2* (Figure S1C) transcription after nitrogen starvation in cells lacking Sty1.

Because *ste11* induction and cell cycle G1 phase arrest are both essential to initiate the mating and meiosis program [42], we also studied the role of Atf1 phosphorylation in the G1 phase arrest after nitrogen depletion. As described before [42], ~75% of wild-type cells accumulate in G1 16 hours after nitrogen starvation according to flow cytometry analysis (Figure 2(f), left panel), while G1 arrest is significantly deficient in cells lacking Atf1. Expression of HA-Atf1 or both mutant derivatives can suppress this effect. Again, only HA-Atf1.10D phosphomimetic mutant is able to bypass the absence of Sty1 (Figure 2(f), bottom right panel).

In conclusion, upon nitrogen starvation both Atf1 and Sty1 are required for *ste11* induction and G1 arrest [42], and HA-Atf1.10M mutant is able to complement the lack of Atf1 but not the absence of Sty1. This suggests that other non-canonical MAP kinase phosphorylation sites in Atf1 are required for full activation of the TF at either the *cgs2* or the *ste11* promoters, as described before for the stress genes *gpd1* and *hsp9* [11]. As reported for these genes, expression of the phosphomimetic mutant HA-Atf1.10D is able to activate *ste11* in the absence of Sty1. In an attempt to genetically trace those additional phosphorylation sites, we first confirmed that the Atf1.11M mutant, lacking the last MAP kinase site in Atf1, is still capable of inducing *ste11* and *mei2* (Figure S2A). We then introduced four and six mutations, based on phospho-proteomic and genetic reports [42-44] (our unpublished results), to generate the HA-Atf1.15M and HA-Atf1.17M, but both are still able to promote *ste11* and *mei2* transcription (Figure S2B).

HA-Atf1.10M is able to induce recombination at the *ade6-M26* hotspot, but only when Sty1 is present

To determine the role of Atf1 phosphorylation in homologous recombination at *ade6-M26*, we determined whether the hotspot is active by measuring the frequency of meiotic recombination between cells expressing the different Atf1 phospho-mutants (HA-Atf1, HA-Atf1.10M and HA-Atf1.10D) and harboring two sets of *ade6* alleles (Figure 3(a)). The *ade6-M26* allele contains a DNA binding site for the Atf1-Pcr1 heterodimer, whereas the *ade6-M375* and *ade6-M210* alleles do not

(Figure 3(b)). As reported before [20], crosses between strains harboring the *ade6-M375* and *ade6-M210* alleles revealed basal (control) recombination levels, while crosses between strains harboring the *ade6-M26* and *ade6-M210* alleles displayed elevated recombination levels, which depend on the presence of Atf1.

In $\Delta atf1$ cells expressing wild-type HA-Atf1, crosses harboring *ade6-M26* allele displayed a frequency of *ade6+* recombinants significantly higher than crosses harboring the control (*M375*) allele, which demonstrates the activation of hotspot (Figure 3(c)) [18]. The frequency of recombinants from control (*M375*) crosses in $\Delta atf1$ and cells expressing wild-type Atf1 protein mutants was similar. However, *M26* DNA site-dependent recombination increase was abolished in $\Delta atf1$ mutant. So, Atf1 is not required to maintain basal recombination but is essential to activate the *ade6-M26* meiotic recombination hotspot [45]. Figure 3(c) shows similar levels of recombination at the hotspot in $\Delta atf1$ cells expressing HA-Atf1 and both phospho-mutants, demonstrating that HA-Atf1.10M is sufficient to induce meiotic homologous recombination at *ade6-M26* hotspot, as previously shown [20]. Nevertheless, when we delete Sty1, levels of *ade6-M26* recombination in HA-Atf1 and HA-Atf1.10M mutants were drastically reduced to basal levels (*M375*), while HA-Atf1.10D retains its recombination activity (Figure 3(c)). These results suggest that both Atf1 and Sty1 are required to trigger homologous recombination at the *ade6-M26* hotspot [17], and probably Sty1 can phosphorylate Atf1 at non-canonical sites to promote its activation as a recombination factor.

In response to low glucose, Atf1 phosphorylation is essential for the recruitment of Atf1 to the *fbp1* promoter

Sty1 and Atf1 also respond to glucose deprivation to trigger transcription of genes such as *fbp1* (Figure 4(a)) [28]. Figure 4(b) illustrates the *fbp1* promoter based on numerous reports (see Introduction). The binding sites for Atf1 (UAS1) and Rst2 (UAS2) relative to the translation origin (+1) are indicated, although both TFs can be detected in both sites [30]. The relative nucleosome positions, extracted from a genome-wide study [46], are also indicated, with arbitrary numbers -1 to -7 starting upstream of the transcriptional start site (TSS). To study the role of Atf1 phosphorylation in the response to glucose starvation, we analyzed *fbp1* transcription in cells expressing the Atf1 phospho-mutants. Northern blot analysis revealed that neither $\Delta atf1$ mutant nor cells expressing the hypo-phosphorylation mutant HA-Atf1.10M are able to trigger *fbp1* transcription 1 hour after a shift to

low glucose medium (Figure 4(c), upper panel), while cells expressing the phosphomimetic HA-Atf1.10D show similar levels of *fbp1* transcripts than wild type cells (Figure 4(c) upper panel). Again, expression of the phosphomimetic HA-Atf1.10D bypasses the requirement of Sty1 in the transcriptional response to glucose depletion (Figure 4(c) lower panel).

In order to check whether Atf1 phosphorylation affects its recruitment to the *fbp1* promoter, we performed chromatin immuno-precipitation (ChIP) analysis. We analyzed the binding of Atf1 at UAS1 and UAS2 sites in glucose rich conditions and after different time points of glucose starvation. As shown in Figure 4(d), HA-Atf1 and HA-Atf1.10D are recruited at both UAS sites after glucose depletion, while the HA-Atf1.10M binding is largely suppressed. Therefore, Atf1 phosphorylation is important for its recruitment to the *fbp1* promoter after glucose deprivation.

Since Rst2 recruitment to the *fbp1* promoter region in glucose starved cells is Atf1-dependent [47], the next step was to analyze Rst2 binding at UAS1 and UAS2 sites in cells expressing HA-Atf1 and mutant derivatives. In cells lacking Atf1 or expressing HA-Atf1.10M we did not detect Rst2-FLAG recruitment at UAS1 and UAS2 sites after glucose starvation, while HA-Atf1 and Atf1.10D promoted its binding at both sites (Figure 4(e)). In cells lacking Rst2, both recruitment to DNA of HA-Atf1, HA-Atf1.10M and HA-Atf1.10D (Figure 4(f)) and *fbp1* transcription (Figure 4(g)) upon glucose depletion are fully abolished. Therefore, the HA-Atf1.10D is not able to overcome the absence of Rst2 in promoting *fbp1* transcription; similarly, it cannot promote *ctt1* and *srx1* expression in cells lacking Pap1 [11]. In conclusion, these results demonstrate that Atf1 has to be phosphorylated by Sty1 in order to ensure the recruitment of both Atf1 and Rst2 TFs to the *fbp1* promoter.

Nucleosome eviction from the *fbp1* promoter after glucose starvation is dependent on Atf1 phosphorylation

Given the importance of Atf1 phosphorylation for its binding to the *fbp1* promoter after glucose starvation, and taking into account that the TF is essential to promote chromatin remodeling in this region [47], we decided to study the effect of the Atf1 phospho-mutants in nucleosome organization at the *fbp1* promoter by nucleosome scanning. Briefly, we treated chromatin from cells expressing wild type HA-Atf1, $\Delta atf1$ and HA-Atf1.10M cells with micrococcal nuclease (MNase), isolated mononucleosomes and PCR-amplified them with pairs of overlapping primers encompassing more than 1 kb, from -100 to -1250 base pairs relative to the translation initiation site, as described

elsewhere [48]. The nucleosome pattern obtained in HA-Atf1-expressing cells in basal conditions (Figure 5(a)) was very similar to the previously described genome-wide analysis for wild-type cells [46], with nucleosomes stably positioned in a region spanning the whole *fbp1* promoter.

Nucleosomes -1, -2, -4 and -6 are fully evicted in cells expressing HA-Atf1 1 hour after glucose starvation (Figure 5(a)), while nucleosomes -3 and -5 are partially maintained (circled in blue in Figure 5(a)), as will be more evident hereafter in other strain backgrounds. Nucleosome eviction is completely absent in the same conditions both in $\Delta atf1$ cells (Figure 5(b)) [47] and in cells expressing HA-Atf1.10M (Figure 5(c)). Remodeling of the *fbp1* promoter by nucleosome eviction is probably essential to allow recruitment of the large RNA polymerase II (Pol II) machinery. As shown in Figure S3, recruitment of the Pol II subunit Rpb3-HA upon glucose starvation is clear in cells expressing wild-type Atf1 or a phosphomimetic Atf1 mutant (untagged Atf1.7D, to combine it with Rpb3-HA), but not in cells expressing a hypo-phosphorylation mutant (Atf1.7M). We conclude that Atf1 phosphorylation has a key role in nucleosome eviction at the *fbp1* promoter and Pol II loading after glucose starvation, since the hypo-phosphorylation HA-Atf1.10M mutant cannot induce chromatin remodeling in this ~1 kb area.

Constitutive phosphorylation of Atf1 accelerates chromatin remodeling of *fbp1* promoter to an open state

We noticed that HA-Atf1.10D enrichment at the *fbp1* promoter 30 minutes after a shift to glucose-poor medium was higher compared to HA-Atf1 (Figure 4(d)), suggesting that this could affect the kinetics of *fbp1* induction in speed or in magnitude. Indeed, the levels of *fbp1* mRNA at longer times upon glucose starvation are more sustained in cells expressing the phosphomimetic HA-Atf1.10D (Figure 6(a)).

This observation led us to hypothesize that the phosphorylation of Atf1 previous to glucose deprivation could accelerate the opening of chromatin and this way affect the magnitude or duration of the transcriptional response. We analyzed the nucleosome eviction dynamics in cells expressing HA-Atf1 and HA-Atf1.10D. In cells expressing wild-type HA-Atf1, the first nucleosome evicted 30 min after stress imposition is nucleosome -6, the closest one to the Atf1-binding site, UAS1, while nucleosome -1, downstream of UAS2, is partially evicted at this time point (Figure 6(b), 30 min, circled in red). 60 minutes after glucose depletion most nucleosomes are gone, with -3 and -5 still weakly

positioned (Figure 6(b)). Interestingly, in glucose-rich conditions nucleosome -6 is missing in cells expressing HA-Atf1.10D strain (Figure 6(c)), even though we could not detect Atf1.10D binding to UAS1 by ChIP under basal conditions (Figure 4(d)). Only 30 minutes after glucose starvation we observed an intermediate chromatin profile, with nucleosomes -1 and -2 (surrounding UAS2) evicted, in cells expressing HA-Atf1.10D (Figure 6(c), center panel). So, these evidences suggest that constitutive phosphorylation of Atf1 promotes the early opening of chromatin surrounding *fbp1* promoter region, inducing a faster and more sustained response to glucose starvation.

Since the sequential transcription of three overlapping *mlonRNA* species (-a, -b and -c) has been reported to be essential for the opening of chromatin [33, 39], we hypothesized that the faster remodeling in HA-Atf1.10D cells may be due to *mlonRNAs* transcription deregulation in cells expressing this phosphomimetic mutant. As reported [33], we detected induction of *mlonRNAs* -b and -c after 30 minutes of glucose starvation only in cells expressing HA-Atf1, whereas this induction was absent in cells lacking Atf1 or expressing HA-Atf1.10M, as expected. Surprisingly, induction of *mlonRNAs* b and c was absent in cells expressing the transcriptionally active HA-Atf1.10D mutant (Figure S4). We conclude that expression of the phosphomimetic mutant HA-Atf1.10D is able to promote *fbp1* activation after glucose deprivation in the absence of *mlonRNAs* production, maybe due to the constitutive eviction of nucleosome -6.

Complete nucleosome eviction at the *fbp1* promoter is necessary but not sufficient for maximal transcription activation

As shown above, Atf1 phosphorylation is essential to initiate chromatin remodeling at the *fbp1* promoter: the HA-Atf1.10M, dead in transcription, is unable to induce nucleosome eviction, while the HA-Atf1.10D mutant, which promotes a faster chromatin remodeling than wild-type Atf1, also displays enhanced transcriptional levels. However, Atf1 phosphorylation cannot be the only factor involved, since the phosphomimetic HA-Atf1.10D mutant does not display constitutive *fbp1* transcription. As explained above, the Pka1 pathway-dependent Rst2 TF is also required for *fbp1* transcription (Figure 4) [29, 30]. In glucose rich conditions, Pka1 kinase phosphorylates Rst2, keeping it excluded from the nucleus and in an inactive form. When glucose is depleted, Pka1 kinase is inactivated by Cgs1 and Rst2 is translocated to the nucleus [25]. Lack of nuclear Rst2 could be sufficient to explain the absence of constitutive *fbp1* activation in cells expressing HA-Atf1.10D. We analyzed the response to

glucose starvation in cells lacking Pka1, in which Rst2 is active also in glucose rich conditions. As previously reported [28], *fbp1* expression is constitutive in strain $\Delta pka1$, even though further induction is accomplished in low glucose medium (Figure 7(a)). Importantly, both basal and induced *fbp1* expression in cells lacking Pka1 is fully dependent on the presence of Atf1 or Sty1 [28]. The fact that strain $\Delta pka1$ displays constitutive *fbp1* transcription in glucose-rich conditions can only be explained if both pathways, Sty1- and Pka1-driven, are inter-connected, as previously suggested [40, 45], so that Atf1 is partially activated in this strain background.

fbp1 is also negatively regulated by the negative TF Scr1, which has to be released from UAS2 site to allow the binding of Rst2 [35]. We tested whether the lack of Scr1 alone, or in combination with the constitutively active HA-Atf1.10D, would cause a full de-repression of *fbp1* in glucose-rich medium. As shown in Figure 7(b), basal levels of *fbp1* transcripts are 4-5 times higher in $\Delta scr1$ than in wild-type cells when combined with the constitutive-promoter expressed HA-Atf1, and expression of HA-Atf1.10D increases those levels two-fold. This result indicates that expression of HA-Atf1.10D in cells lacking Scr1 causes a significant increase in basal *fbp1* transcription.

We tested the nucleosome architecture of the *fbp1* promoter in these two backgrounds, $\Delta pka1$ and $\Delta scr1$. Figure 7(c) shows the relaxed configuration of chromatin around the *fbp1* promoter before and after glucose starvation in $\Delta pka1$ cells, which only display nucleosomes -3 and -5 stably positioned [compare it to the wild-type strain in Figure 5(a)]. Deletion of Scr1 is not sufficient to cause the same effect in glucose-rich medium, and only nucleosome -1, downstream of the Scr1/Rst2 binding site (UAS2) is clearly evicted prior to glucose deprivation (Figure 7(d), circled in red). $\Delta scr1$ cells expressing HA-Atf1.10D display complete opening of chromatin before and after glucose deprivation (Figure 7(e)), even though transcription levels are only moderately de-repressed in this background (Figure 7(b)). From these experiments we conclude that nucleosome eviction in the *fbp1* promoter, ruled by Scr1 inactivation and phosphorylated Atf1, is an essential step in transcription initiation, but the Rst2 TF, which is not activated in $\Delta scr1$ cells expressing HA-Atf1.10D, is essential to facilitate promoter initiation by the complex Pol II machinery and fully activate *fbp1* transcription.

DISCUSSION

We and others have previously shown that the main role of active Sty1 in the regulation of a wide anti-stress gene expression program is phosphorylating Atf1 [10, 11]. Specifically, in response to H₂O₂, Atf1 phosphorylation promotes transcription initiation, but TF recruitment to stress promoters (1.5-3-fold) is not quantitatively sufficient to explain transcription activation (2-35-fold) [11]. We showed that the effect of the Atf1 phosphorylation mutants was different depending on two subsets of stress genes, with Atf1.10M mutant being able to activate transcription in one of them (Figure 1(b)) [11]. Since Sty1 and Atf1 have been described to participate in processes other than activation of stress genes, here we have studied the role of TF phosphorylation in homologous recombination at *ade6-M26* hotspot, initiation of mating and meiosis program by regulation of *ste11*, and *fbp1* transcription in response to glucose starvation. As will be discussed hereafter, we demonstrate that Atf1 phosphorylation by Sty1 is required in all cases. Phosphorylation at either canonical or non-canonical sites seems to be sufficient to trigger chromatin remodelling in homologous recombination and in *cgs2-ste11* transcription, so that HA-Atf1.10M can initiate these processes. However, when Atf1 has to work synergistically with other TFs, such as in the case of the *fbp1* promoter, not only non-canonical but also the canonical Ser/ThrP sites are required to establish all the interactions required in this complex context.

The effect of the hypo-phosphorylation mutant in *cgs2-ste11-mei2* activation and *ade6-M26* recombination resembles that on the *gpd1* and *hsp9* stress genes [11]. Atf1.10M, lacking the canonical Sty1 phosphorylation sites, is capable of activating *cgs2* and its downstream *ste11* and *mei2* genes after nitrogen starvation (Figure 2(bcd)) and of inducing homologous recombination at *ade6-M26* locus (Figure 3(c)). However, the presence of Sty1 is essential for the activity of Atf1.10M, suggesting that other non-canonical sites in Atf1 may be sufficient to promote these transcription and recombination events. The phosphomimetic Atf1.10D mutant is able to induce *ste11* transcription (Figure 2(e)) and homologous recombination (Figure 3(c)) even in the absence of Sty1. This fact further supports the claim that phosphorylation of Atf1 by Sty1, either at the canonical S/TP sites or at so far unidentified residues, is the key molecular event causing activation of the TF. In all these genes/scenarios, Atf1 seems to be the only TF promoting transcription or recombination, and we propose that phosphorylation at canonical or non-canonical sites is sufficient to trigger a TF-mediated

opening of the chromatin at those specific chromosomal locations, maybe by recruiting the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex which has been demonstrated to regulate stress genes [48], *ste11* [49] and recombination at *ade6-M26* [38, 50]. Another possibility, the Sty1-dependent modification of Atf1 by a mechanism other than phosphorylation, could also explain why Sty1 is required for the activity of Atf1.10M; nevertheless, enzymatic properties other than kinase have never been attributed to this family of enzymes. Finally, hetero-dimerization with the TF Pcr1 cannot explain the differences reported here, since Pcr1 is required for homologous recombination, *ste11* and *fbp1* transcription [51, 52].

The situation is different at *fbp1*. It is worth mentioning here that we have analyzed at least three cascades and downstream TFs mediating activation of *fbp1*: (i) the Scr1 repressor is bound to UAS2 prior to stress, and it has to be excluded from the nucleus upon glucose depletion; (ii) simultaneously, the Rst2 TF is activated upon Pka1 inactivation by low glucose, and binds to UAS2; (iii) in parallel, Atf1 has to be phosphorylated by Sty1 when glucose is depleted. In cells expressing HA-Atf1.10M, the activation of *fbp1* after glucose depletion is totally suppressed (Figure 4(c), upper panel), resembling the lack of activity of this mutant at the *srx1* and *ctt1* stress genes (Figure 1(b)) [11]. Also common to the effect at those genes, the HA-Atf1.10D phosphomimetic mutant cannot constitutively trigger *fbp1* expression, but has a slightly higher activity than wild-type Atf1 (Figure 4(c), lower panel). Quantitatively speaking, the recruitment of HA-Atf1 to DNA does not justify the fold-transcriptional activation, but is worth to point out that the enrichment of the TF at the *fbp1* promoter region after 60 minutes of glucose starvation is abolished in cells expressing HA-Atf1.10M (Figure 4(d)).

Contrary to the nucleosome-depleted promoters of stress genes [48], the *fbp1* promoter is full of nucleosomes stably positioned prior to glucose depletion [46, 53] (Figure 5(a)). We show here that most of them are completely evicted 60 minutes after glucose deprivation in wild-type cells, and nucleosome eviction is fully abolished in cells lacking Atf1 or expressing HA-Atf1.10M. Therefore, phosphorylation of Atf1 has an essential role in promoting the chromatin rearrangements that the Pol II machinery requires at the *fbp1* promoter. This hypothesis is further supported by the fact that nucleosome -6, the closest to the Atf1 binding site UAS1, is the first one evicted upon glucose depletion in wild-type cells (Figure 6(b), 30 min), and it is not even positioned in glucose-rich medium in cells expressing HA-Atf1.10D (Figure 6(c), basal). Moreover, the absence of *mlonRNAs b* and *c* in

cells expressing the phosphomimic HA-Atf1.10D could be related with the Tup co-repressors: it has been reported that cells lacking Tup11 and Tup12 do not display expression of *mlonRNAs* [39], and binding of the HA-Atf1.10D to UAS1 may participate in the eviction of nucleosome -6 by releasing the Tup11/Tup12 co-repressors, so that synthesis of the *mlonRNAs* would be dispensable. In conclusion, we propose that Atf1 phosphorylation by Sty1 facilitates its recruitment to UAS1, from where it opens the chromatin to allow the entrance of the transcriptional machinery to trigger *fbp1* transcription.

Several experimental evidences suggest that recruitment of Atf1 to UAS1 has to be done synergistically with Rst2 binding to UAS2, causing DNA looping. First, neither wild-type Atf1 nor the phosphomimetic HA-Atf1.10D mutant are able to bind to DNA nor activate *fbp1* transcription in cells lacking Rst2 (Figure 4(fg)). Second, the *fbp1* gene is fully repressed in glucose-rich medium, since Rst2 is not active (Figure 4(c)). Third, expression of HA-Atf1.10D in cells lacking the repressor Scr1 only display a moderate (but not full) basal de-repression of *fbp1* transcription (Figure 7(b)), even though the nucleosomes at the promoter are fully evicted in basal conditions (Figure 7(e)). Forth, the only background in which Rst2 is active in basal conditions, Δ *pka1*, displays (almost) full de-repression in basal conditions (Figure 7(a)); we propose that in this background the Sty1-Atf1 pathway is partially engaged in the absence of stress [40], and therefore the *fbp1* promoter is constitutively free of nucleosomes (Figure 7(c)). As explained in the Introduction, it has been previously demonstrated that binding of Atf1 to UAS1 and of Rst2 to UAS2 favors the formation of a chromatin loop which spatially brings both UAS close together [30]. We propose that activated Atf1 and Rst2 associate and recognize both sites synergistically distorting the DNA between both sites through looping; the main role of Atf1 is to modify chromatin, while Rst2 probably interacts directly with Pol II or with general Pol II TFs. Additional studies will be necessary to demonstrate whether Atf1-Rst2 association occurs prior to DNA binding and DNA looping, using wild-type and mutant forms of Atf1. Regarding DNA distortion, it is worth mentioning that a recent study has demonstrated by ChIP the presence of Rst2, Atf1 and Scr1 at both the promoter and 3' region of the *rsv1* gene [37]. *rsv1* and *fbp1* genes share many common regulatory features (involvement of Sty1/Atf1 and PKA pathway, activated by Atf1 and Rst2, repressed by Scr1 under glucose-rich conditions...), and therefore additional DNA looping events linking UAS1, UAS2 and the 3' gene regions could be ruling these glucose-responding genes.

In summary, we demonstrate that Atf1 phosphorylation by Sty1 at canonical or non-canonical sites is essential for its biological roles, and the complexity of transcriptional regulation by this activated TF increases when it has to work synergistically with other TFs such as Rst2. Working alone or in combination, chromatin remodeling seems to be Atf1's main role, together with associating and promoting DNA binding of the other TFs, like Rst2 in the case of *fbp1* transcription. Whether this specific TF-TF complex is assembled prior to DNA binding or after UAS interaction will have to be analyzed. These results highlight the diverse mechanisms employed by TFs to modulate chromatin remodeling, gene expression and cell adaptation depending on the biological process regulated and on the chromatin context.

MATERIAL AND METHODS

Yeast strains, plasmids and growth conditions

Cells were grown in rich medium (YE) or synthetic minimal medium (MM) as described previously [54]. Origins and genotypes of strains used in this study are outlined in S1 Table. We constructed the EP48 strain ($\Delta pyp1$) by crossing the wild-type strain (975) with strain EP33 ($\Delta pyp1$), in which the entire *pyp1* open reading frame has been substituted by an antibiotic resistance cassette. To delete the *atf1* genes in strains harbouring the different *ade6* alleles, we transformed the strains JA123 (*ade6-M26*), JA126 (*ade6-M375*) and JA381 (*ade6-M210*) with linear fragments containing *ORF::kanMX6*, obtained by PCR amplification using ORF-specific primers and plasmid *pFA6a-kanMX6* as a template, as described previously [55, 56], obtaining strains LS9 (*ade6-M26*), LS10 (*ade6-M375*) and LS13 ($\Delta atf1 ade6-M210$). The resulting strains were crossed out to wild-type strains to eliminate auxotrophies (except *ade*), yielding strains LS29, LS20 and LS50. LS9, LS10 and LS13 strains also were transformed with integrative plasmids p428' [57] and p428' mutant derivatives [11] to express HA-Atf1 and HA-Atf1 phospho-mutants under the control of the constitutive *sty1* promoter. These plasmids were inserted at the *leu1-32* loci yielding the strains sets expressing HA-Atf1 or mutant derivatives LS11 (*ade6-M26*), LS12 (*ade6-M375*) and LS14 (*ade6-M210*). To delete *sty1* in these backgrounds, we crossed strains JA123 (*ade6-M26*), JA126 (*ade6-M375*) and JA381 (*ade6-M210*) with EP303 ($\Delta atf1 \Delta sty1$ HA-Atf1), EP303.10M ($\Delta atf1 \Delta sty1$ HA-Atf1.10M) and EP303.10D ($\Delta atf1 \Delta sty1$ HA-Atf1.10D) to obtain the $\Delta atf1 \Delta sty1$ strains sets expressing HA-Atf1 or mutant derivatives LS24 (*ade6-M26*), LS28 (*ade6-M375*) and LS56 (*ade6-M210*). We obtained the $\Delta atf1 \Delta sty1$ strains LS53 (*ade6-M26*), LS52 (*ade6-M375*) and LS59 (*ade6-M210*), used as negative controls, by crossing strains JA123 (*ade6-M26*), JA126 (*ade6-M375*) and JA381 (*ade6-M210*) with EP288 ($\Delta atf1 \Delta sty1$). 22G4 ($\Delta cgs2$) from the Bioneer deletion collection [58] was mated with wild-type strain (972) to generate MS110 strain ($h^- \Delta cgs2$), which was mated with wild-type strain (975) to change the mating type, yielding LS40 ($h^+ \Delta cgs2$) strain. By mating strain LS40 with EP193 ($\Delta atf1$) strain [11], we obtained the strain LS41 ($\Delta atf1 \Delta cgs2$). To construct LS85 ($\Delta atf1 \Delta rst2$) strain, EP201 ($\Delta atf1$) was mated with strain JX233 from the Yamamoto laboratory [25]. Then, the plasmids p428' and its mutant variants were also inserted at the *leu1-32* loci of different strain backgrounds [LS41 ($\Delta atf1 \Delta cgs2$) and LS85 ($\Delta atf1 \Delta rst2$)], to obtain the strain series LS46 ($\Delta atf1 \Delta cgs2$) and RF22 ($\Delta atf1 \Delta rst2$), both of them expressing HA-Atf1, HA-Atf1.10M or HA-Atf1.10D. To

generate AZ75 strain, we crossed the wild-type strain (972) with 25B1 strain ($\Delta pka1$) from the Bioneer deletion collection [58]. We also used the strain 25B1 ($\Delta pka1$) to delete *sty1* gene with a cassette containing *ura4⁺* gene, yielding strain AZ61 ($\Delta pka1 \Delta sty1$). We constructed strains RF94 ($\Delta atf1 \Delta pka1$ HA-Atf1) and RF95 ($\Delta atf1 \Delta pka1$) by crossing of strain AZ75 ($\Delta pka1$) with EP203 ($\Delta atf1$ HA-Atf1) and with EP201 ($\Delta atf1$), respectively. Strains LS105 ($\Delta atf1 \Delta scr1$ HA-Atf1) and RF103.10D ($\Delta atf1 \Delta scr1$ HA-Atf1) were generated by transformation of EP203 ($\Delta atf1$ HA-Atf1) and EP203.10D ($\Delta atf1$ HA-Atf1.10D) with linear fragments containing *scr1::kanMX6*, obtained by PCR amplification using ORF-specific primers and plasmid *pFA6a-kanMX6*. To tag *rst2* with *FLAG*, we transformed EP203 series ($\Delta atf1$ expressing HA-Atf1 or mutant derivatives) and EP201 ($\Delta atf1$) with a linear fragment containing the 3' end of *rst2* fused to *FLAG::kanMX6*, obtained by PCR amplification using *rst2* specific primers and the plasmid *pFA6a-5FLAG::kanMX6*, yielding the strain set LS106 ($\Delta atf1$ Rst2-FLAG) expressing HA-Atf1 or mutant versions, and the negative control strain LS116 ($\Delta atf1$ Rst2-FLAG), respectively.

Stationary phase conditions and survival assays

Strains were grown at 30°C in YE medium until they reached stationary phase, at an approximate OD₆₀₀ of 5–9, depending on the strains and growth conditions. The same number of cells (10⁵–10¹) in 3 µl was spotted on YE agar plates from cultures at the logarithmic phase (OD₆₀₀ of 0.5) or several days after reaching the stationary phase. The spots were allowed to dry and the plates were incubated at 30°C for 2–4 days.

Glucose- and nitrogen- deprivation growth conditions

To induce glucose starvation conditions, cultures of the indicated strains growing in YE with a 6% of glucose were grown until an approximate OD₆₀₀ of 0.5. The cultures were filtered and transferred to YE medium containing 0.1% of glucose and 3% of glycerol. 50 ml samples were used for each time point. For nitrogen deprivation, cells growing in minimal medium until an approximate OD₆₀₀ of 0.3 were shifted to the same medium lacking ammonium chloride by filtration. 50 ml samples were collected at indicated times.

RNA analysis by Northern Blot

Total RNA was extracted from cultures of cells at OD₆₀₀ of 0.5 by standard hot-phenol method, as described before [13]. Extracted RNA was loaded on formaldehyde agarose gels and transferred to membranes, which were hybridized with the [α -³²P]-dCTP labeled *fbp1*, containing the complete ORF.

RNA analysis by quantitative PCR

Reverse transcription was performed with purified RNA treated with DNase I using Reverse Transcription System of Applied Biosystems (Thermo Fisher Scientific), following manufacturer's instructions. The cDNA was quantified by real-time qPCR on Light Cycler II using Light Cycler 480 SYBR Green I Master (Roche). The error bars (SD) were calculated from at least three biological replicates, unless indicated otherwise, and *act1* gene was used as a control for normalization. Fold induction was calculated comparing the value of each strain and condition to that of the wild type strain during exponential growth, to which we assigned a value of 1. Primers are listed in S2 Table.

Flow cytometry

Cells were fixed on 70% ethanol, washed with 50 mM of sodium citrate, resuspended in 50 mM sodium citrate and incubated with RNase A at 37°C overnight. About 50 mM sodium citrate containing propidium iodide (final concentration 2.5 g/ml) was added to cells, which were sonicated. To determine the DNA content, the fluorescence intensities of stained cells were measured and analyzed on a FACSCalibur flow cytometer using CellQuest software.

Analysis of meiotic recombinant frequencies

Induction of mating and meiosis, as well as preparation and plating of ascospores were performed as described [54] with minor modifications. After cells mating on plates of minimal medium lacking nitrogen, the mating mixtures were resuspended and washed with 1% glucose, instead of H₂O. After treatment of cells mated with glucuronidase, the spore suspensions were treated with a final concentration of ethanol of 15%. Spores obtained were plated immediately on YE medium in the presence and absence of adenine, and meiotic recombinant frequencies were determined by counting of total viable titer and *ade6+* recombinant titer, respectively. For each cross, experimental replicate and plating condition, we counted at least 100 colonies to determine the respective titers. The

recombinant frequency was calculated as adenine+ titer/viable titer, and data are represented as mean value and standard deviation (SD), calculated from three biological replicates.

Chromatin immunoprecipitation (ChIP)

Cells were grown in YE medium and chromatin isolation and immunoprecipitation were carried out as previously described [48], with minor modifications. Briefly, cells from 50 ml of culture were cross-linked with 1% formaldehyde for 15 min. After stopping cross-linking with 125 mM glycine, pellets were lysed with a bead beater, and lysates were sonicated, yielding chromatin fragments of ≈ 400 bp average size. Chromatin isolated was immuno-precipitated with specific antibodies [5 μ l of anti-HA antiserum (12CA5; house-made), 1 μ l of anti-FLAG (F7425, Sigma), or 1 μ l anti-phospho Ser2 CTD of Pol II (Ab5095 Abcam)] overnight at 4°C rotating. Beads were washed, DNA was eluted and formaldehyde cross-linking was reversed. After protein digestion and chromatin extraction, DNA was amplified by quantitative real-time qPCR using Light Cycler 480 SYBR Green I Master (Roche). The error bars (SD) were calculated from at least three biological triplicates, unless indicated otherwise and *act1* gene was used as a negative control. The primers used are listed in S2 Table.

Nucleosome-scanning analysis

Mononucleosomes were obtained as described previously [48] and the resulting DNA was analyzed by RT-qPCR as described before. Briefly, cells growing in 250 ml of YE medium to an OD₆₀₀ of 0.5 were cross-linked with 0.5% formaldehyde for 20 min at 25°C. Spheroplasts were obtained by digestion with zymolyase 20T (Amsbio) and treated with increasing concentrations of micrococcal nuclease (MNase; Sigma). After DNA purification and electrophoretic separation, samples displaying 80–90% mononucleosomal DNA without subnucleosomal fragments (faster migration than mononucleosomes in the electrophoresis) were further analysed by RT-qPCR with a set of overlapping primer pairs (see S2 Table). For each primer pair, numbers in Y-axis correspond to the relative value to the input, which was obtained using as a template DNA from cells not treated with MNase, and received a value of 1.

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AUTHOR CONTRIBUTIONS

All authors designed and analyzed the data. L.S.-M. and R.F. conducted the experiments. E.H. and J.A. supervised the work, and L.S.-M. and E.H. wrote the manuscript.

APPENDIX A: SUPPLEMENTARY DATA

Supplementary data to this article can be found online

Declarations of interest: none

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FIGURES

Figure 1. Atf1 phosphorylation by MAPK Sty1 is essential for its activation as a transcription factor. (a) Scheme depicting the putative MAPK phosphorylation sites, and their modifications to imitate non-phosphorylated status (10M) and constitutively phosphorylated (10D) respectively. (b) MAP kinase module of the Sty1/Atf1 pathway, including the redundant triple MAP kinases Wak1 and Win1, the MAPK kinase Wis1, MAP kinase Sty1 and the main TF, Atf1. Groups of differently regulated genes, one of them further regulated by the TF Pap1, and their biological outcome are also shown. (c) Serial dilutions of the strains 972 (WT), AV18 ($\Delta sty1$), EP48 ($\Delta pyp1$), EP303.10M ($\Delta atf1 \Delta sty1$ + HA-Atf1.10M) and EP303.10D ($\Delta atf1 \Delta sty1$ + HA-Atf1.10D), growing in YE liquid medium at the logarithmic phase or 5 days after reaching stationary phase, were spotted in YE plates. (d) Scheme proposing the participation of Sty1-mediated phosphorylation of Atf1 on different biological processes, including mating and meiosis, recombination and gluconeogenesis.

Figure 2. Cells expressing HA-Atf1.10M are still able to activate *ste11* after nitrogen starvation, but in a Sty1-dependent manner. (a) Scheme of nitrogen depletion-, Sty1- and Atf1-dependent regulation of *cgs2*, *ste11* and *mei2* transcription. (b, c and d) Expression of *cgs2*, *ste11* and *mei2* was analyzed by RT-qPCR in the strains EP201 ($\Delta atf1$ + empty plasmid), EP203 ($\Delta atf1$ + WT HA-Atf1), EP203.10M ($\Delta atf1$ + HA-Atf1.10M) and EP203.10D ($\Delta atf1$ + HA-Atf1.10D). Amplification with *act1* primers was used as a control for normalization. Each column represents the mean value and SD, calculated from three biological replicates. *, $p < 0.05$; **, $p < 0.01$ (Student's t test). (e) Expression of *ste11* was analyzed by RT-qPCR as in B, C and D, from strains EP288 ($\Delta sty1 \Delta atf1$ + empty plasmid), EP303 ($\Delta sty1 \Delta atf1$ + WT HA-Atf1), EP303.10M ($\Delta sty1 \Delta atf1$ + HA-Atf1.10M), EP303.10D ($\Delta sty1 \Delta atf1$ + HA-Atf1.10D). (f) Accumulation in G1 phase was studied by analysis of the DNA content by flow cytometry from minimal medium cultures of 972 (WT) and strains as in (b, c), before and after 4 and 16 h of a shift to nitrogen depleted medium assays. Histograms represent the percentage of cells with DNA content 1C or 2C.

Figure 3. HA-Atf1.10M is able to induce recombination at the *ade6-M26* hotspot, but only when Sty1 is present. (a and b) Atf1 and the Sty1 MAPK pathway are key factors in meiotic recombination. The *ade6-M26* allele contains a DNA binding site for the Atf1-Pcr1 heterodimer, whereas the *ade6-M375* and *ade6-M210* alleles do not. (c) In order to analyze the effect of Atf1 phosphorylation in recombination, we used the following strains: LS19 (*h⁺ Δatf1 ade6-M26*), LS50 (*h⁻ Δatf1 ade6-M210*), LS11 (*h⁺ Δatf1 ade6-M26 + HA-Atf1*), LS11.10M (*h⁺ Δatf1 ade6-M26 + HA-Atf1.10M*), LS11.10D (*h⁺ Δatf1 ade6-M26 + HA-Atf1.10D*), LS12 (*h⁺ Δatf1 ade6-M375 + HA-Atf1*), LS12.10M (*h⁺ Δatf1 ade6-M375 + HA-Atf1.10M*), LS12.10D (*h⁺ Δatf1 ade6-M375 + HA-Atf1.10D*), LS14 (*h⁻ Δatf1 ade6-M210 + HA-Atf1*), LS14.10M (*h⁻ Δatf1 ade6-M210 + HA-Atf1.10M*), LS14.10D (*h⁻ Δatf1 ade6-M210 + HA-Atf1.10D*), LS53 (*h⁺ Δsty1 Δatf1 ade6-M26 + empty plasmid*), LS59 (*h⁻ Δsty1 Δatf1 ade6-M210 + empty plasmid*), LS24 (*h⁺ Δsty1 Δatf1 ade6-M26 + HA-Atf1*), LS24.10M (*h⁺ Δsty1 Δatf1 ade6-M26 + HA-Atf1.10M*), LS24.10D (*h⁺ Δsty1 Δatf1 ade6-M26 + HA-Atf1.10D*), LS28 (*h⁺ Δsty1 Δatf1 ade6-M375 + HA-Atf1*), LS28.10M (*h⁺ Δsty1 Δatf1 ade6-M375 + HA-Atf1.10M*), LS28.10D (*h⁺ Δsty1 Δatf1 ade6-M375 + HA-Atf1.10D*), LS56 (*h⁻ Δsty1 Δatf1 ade6-M210 + HA-Atf1*), LS56.10M (*h⁻ Δsty1 Δatf1 ade6-M210 + HA-Atf1.10M*), LS56.10D (*h⁻ Δsty1 Δatf1 ade6-M210 + HA-Atf1.10D*). Mating between cells containing *M375* and *M210* sites serves as a control of basal recombination, while crosses involving strains with a mutation in *M26* serve as a site-dependent recombination reporter. Data are mean $\pm$ SD from three independent experiments.

Figure 4. Atf1 phosphorylation is essential for *fbp1* induction. (a) Scheme of glucose depletion-, Sty1- and Atf1-dependent regulation of *fbp1* transcription. (b) Scheme depicting the *fbp1* promoter and open reading frame regions, including the relative localization of the nucleosomes, the binding sites of the TFs Atf1 (UAS1) and Rst2 (UAS2), the transcription start site (TSS) and the TATA box. Numbers refer to the translation start site. (c) Total RNA from cultures of the strains 972 (wild type), EP201 ($\Delta atf1$ + empty plasmid), EP203 ($\Delta atf1$ + WT HA-Atf1), EP203.10M ($\Delta atf1$ + HA-Atf1.10M), EP203.10D ($\Delta atf1$ + HA-Atf1.10D), EP288 ($\Delta sty1 \Delta atf1$ + empty plasmid), EP303 ($\Delta sty1 \Delta atf1$ + WT HA-Atf1), EP303.10M ($\Delta sty1 \Delta atf1$ + HA-Atf1.10M), EP303.10D ($\Delta sty1 \Delta atf1$ + HA-Atf1.10D) was obtained, either in exponential growth in YE with 6% glucose or after 60 minutes of growth in YE with

0.08% glucose, was analyzed by Northern blot with labeled *fbp1* probe. rRNA was used as loading control. (d) ChIP experiment using anti-HA antibody to localize HA-Atf1 mutants binding to UAS1 and UAS2 sites in the *fbp1* promoter after glucose depletion. Samples from strains EP203 (Δ atf1 + WT HA-Atf1), EP203.10M (Δ atf1 + HA-Atf1.10M) and EP203.10D (Δ atf1 + HA-Atf1.10D) were taken in exponential growth, or 30, 60 or 120 min after switching the cultures to low-glucose medium. Primers covering the UAS1 and UAS2 were used. Data are presented as mean $\pm$ SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (Student's *t* test). (e) ChIP experiment using anti-FLAG antibody to localize Rst2-FLAG. Samples were obtained from cultures of the strains LS116 (Δ atf1 *rst2*-FLAG with empty plasmid), LS106 (Δ atf1 *rst2*-FLAG + WT HA-Atf1), LS106.10M (Δ atf1 *rst2*-FLAG + HA-Atf1.10M) and LS106.10D (Δ atf1 *rst2*-FLAG + HA-atf1.10D) and LS116 (Δ atf1 *rst2*-FLAG with empty plasmid), either from exponential cultures or after 60 minutes of glucose deprivation. Data are presented as mean $\pm$ SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (Student's *t* test). (f) ChIP experiment as performed in D, from strains EP203 (Δ atf1 + HA-Atf1), RF22 (Δ rst2 Δ atf1 + WT HA-Atf1), RF22.10M (Δ rst2 Δ atf1 + HA-Atf1.10M) and RF22.10D (Δ rst2 Δ atf1 + HA-Atf1.10D). *act1* gene was used as negative control. Data are presented as mean $\pm$ SD; ****, $p < 0.0001$ (Student's *t* test). (g) *fbp1* expression from the strains and conditions as in f was analyzed by RT-qPCR.

Figure 5. Nucleosome eviction from the *fbp1* promoter after glucose starvation depends on Atf1 phosphorylation. (a-c) Mononucleosomes of strains EP203 ($\Delta atf1$ + HA-Atf1) (a), EP201 ($\Delta atf1$ + empty plasmid) (b) and EP203.10M ($\Delta atf1$ + HA-Atf1.10M) (c) were isolated from logarithmic cultures or 60 minutes after switching to low glucose medium. RT-qPCR was performed using 16 pairs of primers covering 1.2 kb of the *fbp1* promoter, including TSS, UAS1 and UAS2 as seen in the upper scheme. The values of nucleosome occupancy for each RT-qPCR reaction (Y-axis) is plotted against the gene position of the PCR-amplified fragment for each primers pair, relative to the first nucleotide of the ORF, with a value of 0 (X-axis). Mean of three biological replicates is shown, with error bars reflecting SD. Vertical dotted lines correlate to nucleosome peaks based on a genome-wide study [46]. Blue circles indicate nucleosomes -3 and -5, which are not evicted as efficiently as the other nucleosomes.

Figure 6. Constitutive phosphorylation of Atf1 accelerates the chromatin remodeling at *fbp1* to an open state. (a) RNA from cultures of the following strains was obtained during exponential growth and 30, 60, 120 and 180 minutes after switching the culture to 0.08% glucose medium: EP201 ($\Delta atf1$ + empty plasmid), EP203 ($\Delta atf1$ + WT HA-Atf1), EP203.10M ($\Delta atf1$ + HA-Atf1.10M) and EP203.10D ($\Delta atf1$ + HA-Atf1.10D). RNA was extracted and analyzed with *fbp1* primers, and normalized with *act1*. Fold induction levels were calculated by assigning a value of 1 to *fbp1* levels in a wild type strain during exponential growth. (b and c) Time course nucleosome scanning experiments. Nucleosomes from cultures of strains EP203 ($\Delta atf1$ + WT HA-Atf1) (b) and EP203.10D ($\Delta atf1$ + HA-Atf1.10D) (c), grown at exponential phase or 30 and 60 minutes after depletion of glucose, were isolated and analyzed as in Figure 5. Dotted red circles indicate nucleosomes -6 and -1, the closer nucleosomes to UAS1 and UAS2 respectively, and the first ones evicted after nucleosome remodeling at 30 min in wild-cells (b), or earlier in HA-Atf1.10D expressing cells (c).

Figure 7. Role of Pka1 kinase and Scr1 repressor in *fbp1* promoter chromatin remodeling. (a and b) Expression of *fbp1* was analyzed by RT-qPCR in strains EP203 ($\Delta atf1$ + HA-Atf1), EP201 ($\Delta atf1$ + empty plasmid), AZ75 ($\Delta pka1$), AZ61 ($\Delta pka1 \Delta sty1$), RF94 ($\Delta pka1 \Delta atf1$ + HA-Atf1), RF95 ($\Delta pka1 \Delta atf1$ + empty plasmid), EP203.10D ($\Delta atf1$ + HA-Atf1.10D), LS105 ($\Delta atf1 \Delta scr1$ + HA-Atf1) and RF103.10D ($\Delta atf1 \Delta scr1$ + HA-Atf1.10D). Samples were obtained from exponential 6% glucose YE cultures or 60 minutes after switching them to 0.08% glucose YE medium. We show the mean and SD from three biological replicates. Fold induction was calculated compared to *fbp1* levels in a EP203 strain during exponential growth. (c-e) Nucleosome positioning experiments in cells lacking Pka1 or Scr1. Strains RF94 ($\Delta atf1 \Delta pka1$ + HA-Atf1) (c), LS105 ($\Delta atf1 \Delta scr1$ + HA-Atf1) (d) and RF103.10D ($\Delta atf1 \Delta scr1$ + HA-Atf1.10D) (e) were grown and nucleosome positioning determined as described in Figure 5. Dotted red circle in (D) indicates nucleosome -1, constitutively evicted in cells lacking the repressor Scr1.

Figure S1. Phosphodiesterase Cgs2 is needed for the regulation of meiosis genes regardless of Atf1 phosphorylation. (A) Samples of the strains 972 (wild type) and EP203 ($\Delta atf1$ + HA-Atf1) were obtained during exponential growth and 4 hours after switching the culture to low nitrogen media. RNA was extracted and analyzed with *ste11* and *mei2* primers, and normalized with *act1*. Fold induction levels were calculated by assigning a value of 1 to the levels in a wild type strain during exponential growth. (B) Expression of *ste11* and *mei2* was analyzed as in A, using strains EP203 ($\Delta atf1$ + HA-Atf1), LS46 ($\Delta atf1 \Delta cgs2$ + HA-Atf1), LS46.10M ($\Delta atf1 \Delta cgs2$ + HA-Atf1.10M) and LS46.10D ($\Delta atf1 \Delta cgs2$ + HA-Atf1.10D). (C) Expression of *mei2* was analyzed as in A, using strains EP288 ($\Delta sty1 \Delta atf1$ + empty plasmid), EP303 ($\Delta sty1 \Delta atf1$ + WT HA-Atf1), EP303.10M ($\Delta sty1 \Delta atf1$ + HA-Atf1.10M), EP303.10D ($\Delta sty1 \Delta atf1$ + HA-Atf1.10D).

Figure S2. Activation of *ste11* and *mei2* is still functional in cells expressing the Atf1.11M, Atf1.15M and 17M mutants. (A) Samples of the strains EP203 (Δ atf1 + HA-Atf1), EP203.10M (Δ atf1 + HA-Atf1.10M) and EP203.11M (Δ atf1 + HA-Atf1.11M) were obtained during exponential growth and 4 hours after switching the cultures to medium lacking nitrogen. RNA was extracted and analyzed with *ste11* and *mei2* primers, and normalized with *act1*. Fold induction levels were calculated by assigning a value of 1 to the levels in a wild type strain during exponential growth. (B) Scheme depicting the non-canonical MAPK phosphorylation sites in HA-Atf1.15M and HA-Atf1.17M mutants. Samples of the strains EP203 (Δ atf1 + HA-Atf1), EP203.15M (Δ atf1 + HA-Atf1.15M) and EP203.17M (Δ atf1 + HA-Atf1.17M) were obtained during exponential growth and 4 hours after switching the culture to media lacking nitrogen. RNA was extracted and analyzed with *ste11* and *mei2* primers, and normalized with *act1*. Fold induction levels were calculated by assigning a value of 1 to the levels in a wild type strain during exponential growth.

Figure S3. Recruitment of Pol II to *fbp1* depends on Atf1 phosphorylation. ChIP experiment using anti-HA antibody to localize Pol II subunit Rpb3 tagged with HA. Samples from strains JF5 (*rpb3-HA*), CS81.7M (*rpb3-HA atf1.7M*) and CS81.7D (*rpb3-HA atf1.7D*) were taken in exponential growth, and 60 minutes after switching the culture to low-glucose media. Data are presented as mean $\pm$ SD; **, $p < 0.01$; ****, $p < 0.0001$ (Student's *t* test) from biological triplicates. Primers designed to bind *act1* ORF were used as control. ($\Delta atf1$ + HA-Atf1.10D) were taken during exponential growth and after 60 minutes of glucose deprivation.

Figure S4. Metabolic long non-coding RNAs (*mlonRNA*) b and c transcription rates do not change in phosphomutants Atf1.10M and Atf1.10D. Samples of the strains EP203 ($\Delta atf1$ + HA-Atf1), EP201 ($\Delta atf1$), EP203.10M ($\Delta atf1$ + HA-Atf1.10M) and EP203.10D ($\Delta atf1$ + HA-Atf1.10D) were obtained during exponential growth and every 15 minutes after switching the culture to low glucose media. RNA was extracted and analyzed with specific primers for *mlonRNA b* (left panel) and c (right panel). Fold induction levels were calculated by assigning a value of 1 to the levels in a wild type strain during exponential growth.

Table S1. Strains used in this study

Strain	Genotype	Origin
972	<i>h⁻</i>	[1]
975	<i>h⁺</i>	[1]
EP201	<i>h⁻ atf1::natMX6 sty1::HA::leu1 leu1-32</i>	[2]
EP203	<i>h⁻ atf1::natMX6 sty1::HA-atf1::leu1 leu1-32</i>	[3]
EP203.10M	<i>h⁻ atf1::natMX6 sty1::HA-atf1.10M::leu1 leu1-32</i>	[2]
EP203.10D	<i>h⁻ atf1::natMX6 sty1::HA-atf1.10D::leu1 leu1-32</i>	[2]
EP288	<i>h⁻ atf1::natMX6 sty1::ura4 sty1::HA::leu1 leu1-32 ura4.D18</i>	[2]
EP303	<i>h⁻ atf1::natMX6 sty1::ura4 sty1::HA-atf1::leu1 leu1-32 ura4.D18</i>	[2]
EP303.10M	<i>h⁻ atf1::natMX6 sty1::ura4 sty1::HA-atf1.10M::leu1 leu1-32 ura4.D18</i>	[2]
EP303.10D	<i>h⁻ atf1::natMX6 sty1::ura4 sty1::HA-atf1.10D::leu1 leu1-32 ura4.D18</i>	[2]
AV18	<i>h⁺sty1::kanMX6</i>	[4]
EP33	<i>h⁻ pyp1::natMX6 leu1-32 ura4-D18</i>	This work
EP48	<i>h⁻ pyp1::natMX6</i>	This work
JA123	<i>h⁺ ade6-M26 leu1-32</i>	Lab stock
JA126	<i>h⁺ ade6-M375 leu1-32</i>	Lab stock
JA381	<i>h⁻ ade6-M210 leu1-32</i>	Lab stock
LS9	<i>h⁺ atf1::natMX6 ade6-M26 leu1-32</i>	This work
LS10	<i>h⁺ atf1::natMX6 ade6-M375 leu1-32</i>	This work
LS13	<i>h⁻ atf1::natMX6ade6-M210 leu1-32</i>	This work
LS19	<i>h⁺ atf1::natMX6 ade6-M26</i>	This work
LS20	<i>h⁺ atf1::natMX6 ade6-M375</i>	This work
LS50	<i>h⁻ atf1::natMX6 ade6-M210</i>	This work
LS11	<i>h⁺ atf1::natMX6 sty1::HA-atf1::leu1+ ade6-M26 leu1-32</i>	This work
LS11.10M	<i>h⁺ atf1::natMX6 sty1::HA-atf1.10M::leu1+ ade6-M26 leu1-32</i>	This work
LS11.10D	<i>h⁺ atf1::natMX6 sty1::HA-atf1.10D::leu1+ ade6-M26 leu1-32</i>	This work
LS12	<i>h⁺ atf1::natMX6 sty1::HA-atf1::leu1+ ade6-M375 leu1-32</i>	This work
LS12.10M	<i>h⁺ atf1::natMX6 sty1::HA-atf1.10M::leu1+ ade6-M375 leu1-32</i>	This work
LS12.10D	<i>h⁺ atf1::natMX6 sty1::HA-atf1.10D::leu1+ ade6-M375 leu1-32</i>	This work
LS14	<i>h⁻ atf1::natMX6 sty1::HA-atf1::leu1+ ade6-M210 leu1-32</i>	This work
LS14.10M	<i>h⁻ atf1::natMX6 sty1::HA-atf1.10M::leu1+ ade6-M210 leu1-32</i>	This work
LS14.10D	<i>h⁻ atf1::natMX6 sty1::HA-atf1.10D::leu1+ ade6-M210 leu1-32</i>	This work
LS53	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA::leu1+ ade6-M26 leu1-32 ura4-D18</i>	This work
LS52	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA::leu1+ ade6-M375 leu1-32 ura4-D18</i>	This work
LS59	<i>h⁻ sty1::ura atf1::natMX6 sty1::HA::leu1+ ade6-M210 leu1-32 ura4-D18</i>	This work
LS24	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA-atf1::leu1+ ade6-M26 leu1-32 ura4-D18</i>	This work
LS24.10M	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA-atf1.10M::leu1+ ade6-M26 leu1-32 ura4-D18</i>	This work
LS24.10D	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA-atf1.10D::leu1+ ade6-M26 leu1-32 ura4-D18</i>	This work
LS28	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA-atf1::leu1+ ade6-M375 leu1-32 ura4-D18</i>	This work

LS28.10M	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA-atf1.10M::leu1+ ade6-M375 leu1-32 ura4-D18</i>	This work
LS28.10D	<i>h⁺ sty1::ura atf1::natMX6 sty1::HA-atf1.10D::leu1+ ade6-M375 leu1-32 ura4-D18</i>	This work
LS56	<i>h⁻ sty1::ura atf1::natMX6 sty1::HA-atf1::leu1+ ade6-M210 leu1-32 ura4-D18</i>	This work
LS56.10M	<i>h⁻ sty1::ura atf1::natMX6 sty1::HA-atf1.10M::leu1+ ade6-M210 leu1-32 ura4-D18</i>	This work
LS56.10D	<i>h⁻ sty1::ura atf1::natMX6 sty1::HA-atf1.10D::leu1+ ade6-M210 leu1-32 ura4-D18</i>	This work
22G4	<i>h⁺ cgs2::kanMX4 leu1-32 ura4-D18 ade6-M210</i>	[5]
MS110	<i>h⁻ cgs2::kanMX4</i>	This work
LS40	<i>h⁺ cgs2::kanMX4</i>	This work
EP193	<i>h⁻ atf1::natMX6 leu1-32</i>	[2]
LS41	<i>h⁺ atf1::natMX6 cgs2::kanMX6 leu1-32</i>	This work
LS46	<i>h⁺ atf1::natMX6 cgs2::kanMX6 sty1::HA-atf1::leu1 leu1-32</i>	This work
LS46.10M	<i>h⁺ atf1::natMX6 cgs2::kanMX6 sty1::HA-atf1.10M::leu1 leu1-32</i>	This work
LS46.10D	<i>h⁺ atf1::natMX6 cgs2::kanMX6 sty1::HA-atf1.10D::leu1 leu1-32</i>	This work
LS106	<i>h⁻ atf1::natMX6 sty1::HA-atf1::leu1 rst2-FLAG::kanMX6 leu1-32</i>	This work
LS106.10M	<i>h⁻ atf1::natMX6 sty1::HA-atf1.10M::leu1 rst2-FLAG::kanMX6 leu1-32</i>	This work
LS106.10D	<i>h⁻ atf1::natMX6 sty1::HA-atf1.10D::leu1 rst2-FLAG::kanMX6 leu1-32</i>	This work
LS116	<i>h⁻ atf1::natMX6 sty1::HA::leu1 rst2-FLAG::kanMX6 leu1-32</i>	This work
JX233	<i>h90 rst2::ura4 ade6-M216 leu1 ura4-D18</i>	[6]
LS85	<i>h⁻ atf1::natMX6 rst2::ura4 leu1-32 ura4.D18</i>	This work
RF22	<i>h⁻ atf1::natMX6 rst2::ura4 sty1::HA-atf1::leu1 leu1-32 ura4.D18</i>	This work
RF22.10M	<i>h⁻ atf1::natMX6 rst2::ura4 sty1::HA-atf1.10M::leu1 leu1-32 ura4.D18</i>	This work
RF22.10D	<i>h⁻ atf1::natMX6 rst2::ura4 sty1::HA-atf1.10D::leu1 leu1-32 ura4.D18</i>	This work
RF94	<i>h⁻ atf1::natMX6 pka1::kanMX6 sty1::HA-atf1::leu1 leu1-32</i>	This work
RF95	<i>h⁻ atf1::natMX6 pka1::kanMX6 sty1::HA::leu1 leu1-32</i>	This work
25B1	<i>h⁺ pka1::kanMX4 leu1-32 ura4-D18 ade6-M210</i>	[5]
AZ75	<i>pka1::kanMX6 leu1-32</i>	This work
AZ61	<i>pka1::kanMX6 sty1::ura</i>	This work
LS105	<i>h⁻ atf1::natMX6 scr1::kanMX6 sty1::HA-atf1::leu1 leu1-32</i>	This work
RF103.10D	<i>h⁻ atf1::natMX6 scr1::kanMX6 sty1::HA-atf1.10D::leu1 leu1-32</i>	This work
JF5	<i>h⁻ rpb3-HA::natMX6</i>	(3)
CS81.7M	<i>h⁻ rpb3-HA::natMX6 atf1.7M</i>	(3)
CS81.7D	<i>h⁻ rpb3-HA::natMX6 atf1.7D</i>	(3)

Table S2. Primers used in this study for RT-qPCR, ChIP and nucleosome scanning experiments

Primer	Sequence	Gene ^a	Application
OLEH-N7	5'-ACACTAGACGAAGAGACGCATGTAT-3'	<i>ste11</i> F	RT-qPCR
OLEH-N8	5'-ACCGAACTTTTATCTACGCCTTATT-3'	<i>ste11</i> R	RT-qPCR
OLEH-N9	5'-TTTGTAAAGCCATGGACCTAATAGA-3'	<i>mei2</i> F	RT-qPCR
OLEH-N10	5'-TATTTGTTGGGCACTAGCAATAGAT-3'	<i>mei2</i> R	RT-qPCR
OLEH-S41	5'-AAAACGTACAACACTATACCAAAGCA-3'	<i>mlon</i> RNA <i>b</i> F	RT-qPCR
OLEH-S42	5'-GTATGATTGATCGCCATTGTGTAG-3'	<i>mlon</i> RNA <i>b</i> R	RT-qPCR
OLEH-S43	5'-AGTCACGTAAACCTCGATACGATAC-3'	<i>mlon</i> RNA <i>c</i> F	RT-qPCR
OLEH-S44	5'-GACTCATTCCACCCTATTCATCTTT-3'	<i>mlon</i> RNA <i>c</i> R	RT-qPCR
OLEH-S77	5'-AAAACCATACTCTGCTCGATACATT-3'	ORF <i>fbp1</i> F	RT-qPCR/ ChIP
OLEH-S78	5'-TTATTTCTTTTGTAGCATGGATAAG-3'	ORF <i>fbp1</i> R	RT-qPCR/ ChIP
OLEH-H48	5'-TGCCCCTAGAGCTGTATTCC-3'	<i>act1</i> F	RT-qPCR/ ChIP
OLEH-H49	5'-TTTGAGCTTCATCACCAACG-3'-	<i>act1</i> R	RT-qPCR/ ChIP
OLEH-R76	5'-CTAACTACTCCATCCACCCA-3'	UAS1 <i>fbp1</i> F	ChIP
OLEH-R77	5'-AAGCAAAATCAGAAATTGTG-3'	UAS1 <i>fbp1</i> R	ChIP
OLEH-R78	5'-TGTTTAGCAGGCTGAAACAG-3'	UAS2 <i>fbp1</i> F	ChIP
OLEH-R79	5'-CCGCTTAATTAATAATGCAT-3'	UAS2 <i>fbp1</i> R	ChIP
OLEH-S45	5'-GTTGTTTACATGGGAAATACATGG-3'	TSS <i>fbp1</i> F	ChIP
OLEH-S46	5'-CTAACAAACGTTTCACCTTGACTG-3'	TSS <i>fbp1</i> R	ChIP
OLEH-T69	5'-TCCCTCTCTTTAATTACCTGGATGAC-3'	<i>fbp1</i> -1307 bp	Nucl. Scann.
OLEH-T70	5'-AGTAGTTAGTAGGAGGAGAAATGAACC-3'	<i>fbp1</i> -1205 bp	Nucl. Scann.
OLEH-T71	5'-TCTCCTCCTACTAACTACTCCATCC-3'	<i>fbp1</i> -1224 bp	Nucl. Scann.
OLEH-T72	5'-CAGAAATTGTGAAATTCGATGATGAGT-3'	<i>fbp1</i> -1122bp	Nucl. Scann.
OLEH-T73	5'-CGAATTTACAAATTTCTGATTTTCG-3'	<i>fbp1</i> -1140 bp	Nucl. Scann.
OLEH-T74	5'-CCTGTGTTAACTCAGAATCAACATA-3'	<i>fbp1</i> -1039 bp	Nucl. Scann.
OLEH-T75	5'-AGCCATGTTATGTTGATTCTGAGTT-3'	<i>fbp1</i> -1072 bp	Nucl. Scann.
OLEH-T76	5'-TAGTGTTGTACGTTTTAGAGCACAC-3'	<i>fbp1</i> -971 bp	Nucl. Scann.
OLEH-T79	5'-TACACAATGGCGATCAATCATACAC-3'	<i>fbp1</i> -912 bp	Nucl. Scann.
OLEH-T80	5'-ACCGACCTCAATTGCATACCCACAG-3'	<i>fbp1</i> -812 bp	Nucl. Scann.
OLEH-T81	5'-TCGATCTTATGTAATTGCTGTGGGT-3'	<i>fbp1</i> -854 bp	Nucl. Scann.
OLEH-T82	5'-TGGCACTCCCTTAATACATTCGTAA-3'	<i>fbp1</i> -752 bp	Nucl. Scann.
OLEH-T83	5'-TTTACGAATGTATTAAGGGAGTGCC-3'	<i>fbp1</i> -778 bp	Nucl. Scann.
OLEH-T84	5'-GTAAATACGGGATGGGAAGGTTAGA-3'	<i>fbp1</i> -676 bp	Nucl. Scann.
OLEH-T85	5'-ATACAAGCTCTAACCTTCCCATCCC-3'	<i>fbp1</i> -709 bp	Nucl. Scann.
OLEH-T86	5'-GGCTTACCAAACGATTGCTAGAGCG-3'	<i>fbp1</i> -608 bp	Nucl. Scann.
OLEH-T87	5'-GAGTCCGCTCTAGCAATCGTTTGGT-3'	<i>fbp1</i> -637 bp	Nucl. Scann.
OLEH-T88	5'-CTTGCAATGAAAACACAGGGCAAT-3'	<i>fbp1</i> -534 bp	Nucl. Scann.
OLEH-T89	5'-TGCCCTGTAGTTTTATTGCAAG-3'	<i>fbp1</i> -557 bp	Nucl. Scann.
OLEH-T90	5'-CTATTTTCATACGCCACCTGAGT-3'	<i>fbp1</i> -455bp	Nucl. Scann.
OLEH-T91	5'-ACTCAGGTGGGCGTATGAAATAG-3'	<i>fbp1</i> -478bp	Nucl. Scann.
OLEH-T92	5'-GCTATGTTTAGTTCCGCGAATCATA-3'	<i>fbp1</i> -380bp	Nucl. Scann.
OLEH-T93	5'-TATGATTGCGGAACTAAACATAGCG-3'	<i>fbp1</i> -405 bp	Nucl. Scann.
OLEH-T94	5'-ACAAAAATCAACGAGCCATGATGGA-3'	<i>fbp1</i> -303 bp	Nucl. Scann.

OLEH-T95	5'-TCCATCATGGCTCGTTGATTTTGT-3'	<i>fbp1</i> -328 bp	Nucl. Scann.
OLEH-T96	5'-GTCTCTCTCCAGTGTAACAAAA-3'	<i>fbp1</i> -226 bp	Nucl. Scann.
OLEH-V29	5'-ACTGGAGAGAGACAACAACACTC-3'	<i>fbp1</i> -239 bp	Nucl. Scann.
OLEH-V30	5'-AAGTTGGGCTAGAAACCGAGTG-3'	<i>fbp1</i> -138 bp	Nucl. Scann.
OLEH-V31	5'-CCACTCGGTTTCTAGCCCAACTTAA-3'	<i>fbp1</i> -160 bp	Nucl. Scann.
OLEH-V32	5'-GGGGAATGAAGTTGAATAAGCTGGC-3'	<i>fbp1</i> -59 bp	Nucl. Scann.

^aWhen included, the number indicates the position of the 5' end of the primer relative to the translation start point

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