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Antagonistic Controls of Chromatin and mRNA Start Site Selection by Tup Family Corepressors and the CCAAT-Binding Factor

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The Tup family corepressors contribute to critical cellular responses, such as the stress response and differentiation, presumably by inducing repressive chromatin, though the precise repression mechanism remains to be elucidated. The *Schizosaccharomyces pombe* fission yeast Tup family corepressors Tup11 and Tup12 (Tup11/12), which are orthologs of Tup1 in *Saccharomyces cerevisiae* budding yeast and Groucho in *Drosophila*, negatively control chromatin and the transcriptional activity of some stress-responsive genes. Here, we demonstrate that Tup11/12 repress transcription of a gluconeogenesis gene, *fbp1*$^+$, by three distinct mechanisms. First, Tup11/12 inhibit chromatin remodeling in the *fbp1*$^+$ promoter region where the Atf1 and Rst2 transcriptional activators bind. Second, they repress the formation of an open chromatin configuration at the *fbp1*$^+$ TATA box. Third, they repress mRNA transcription *per se* by regulating basic transcription factors. These inhibitory actions of Tup11/12 are antagonized by three different types of transcriptional activators: CREB/ATF-type Atf1, C_2H_2 zinc finger-type Rst2, and CBF/NF-Y-type Php5 proteins. We also found that impaired chromatin remodeling and *fbp1*$^+$ mRNA transcription in *php5*Δ strains are rescued by the double deletions of *tup11*$^+$ and *tup12*$^+$, although the distribution of the transcription start sites becomes broader than that in wild-type cells. These data reveal a new mechanism of precise determination of the mRNA start site by Tup family corepressors and CBF/NF-Y proteins.

Eukaryotic chromosomal DNA is packaged in a highly organized and condensed chromatin structure. Many DNA-associated reactions, including DNA damage repair, replication, recombination, and transcription, are regulated by the chromatin structure (1, 2). The chromatin structure is modulated by two distinct classes of regulators, histone modification enzymes and ATP-dependent chromatin remodeling factors (3, 4). Such regulatory components are recruited by two types of *cis*-acting regulatory factors, transcriptional activators and repressors. Transcriptional activators and repressors bind to *cis*-acting elements to activate and repress transcription, respectively, by affecting the chromatin structure and regulating RNA polymerase II accumulation in the promoter region (5–7). These transcriptional regulators also interact with coactivators and corepressors to regulate gene expression (8, 9). The Tup family transcriptional corepressors are conserved between yeast and humans and regulate gene expression during the stress response and cellular differentiation (10, 11). *Saccharomyces cerevisiae* Tup1 represses some genes regulated by cell type, glucose, oxidative stress, DNA damage, and other cellular stress responses (12, 13). Tup1 represses the expression of genes via distinct mechanisms: by establishing a repressive chromatin structure around the target gene promoter, by recruiting histone deacetylases, and by directly interfering with the general transcription machinery (14–18). Two Tup1 orthologs in *Schizosaccharomyces pombe*, Tup11 and Tup12 (Tup11/12), regulate multiple stress-responsive genes, including the *fbp1*$^+$ and *cta3*$^+$ genes, to provide stress specificity (19, 20). However, the precise molecular mechanisms of Tup1 family proteins in gene repression have not been fully uncovered.

The *fbp1*$^+$ gene encodes fructose-1,6-bisphosphatase and is robustly induced upon glucose starvation (21, 22). *fbp1*$^+$ expression is strictly repressed by Tup11/12 and activated by the transcriptional activators Atf1, Rst2, and Php5 (23–26). Atf1, a bZIP protein, is regulated through phosphorylation by the mitogen-activated protein kinase pathway (27–29), while Rst2, a C_2H_2 Zn finger-type protein, is under the regulation of the protein kinase A pathway (23, 30). Php5, a component of the *S. pombe* CCAAT-binding factor (CBF; also known as NF-Y) that possesses a histone hold domain, forms a complex with Php2/Php3 and contributes to *cyc1*$^+$ transcription (31, 32). In addition, two *cis*-acting elements required for *fbp1*$^+$ transcription have been identified (33). Upstream activation sequence 1 (UAS1) contains a cyclic AMP response element (CRE) and is the binding site for Atf1 and its binding partner, Pcr1 (34), while UAS2 resembles the *S. cerevisiae* stress response element (STRE) and serves as the binding site for Rst2 (24) (Fig. 1A). The binding site for the CBF complex has not yet been identified (26).

We identified a cascade of transcription initiation of long noncoding RNA (lncRNA) in the *fbp1*$^+$ promoter region which is pivotal for chromatin remodeling and the binding of transcription activators at the *fbp1*$^+$ promoter (35) and involves at least three distinct lncRNA initiation sites (Fig. 1A, sites a to c). Such lncRNA transcription-mediated chromatin remodeling was also identified at a meiotic recombination hot spot, *ade6-M26*, under

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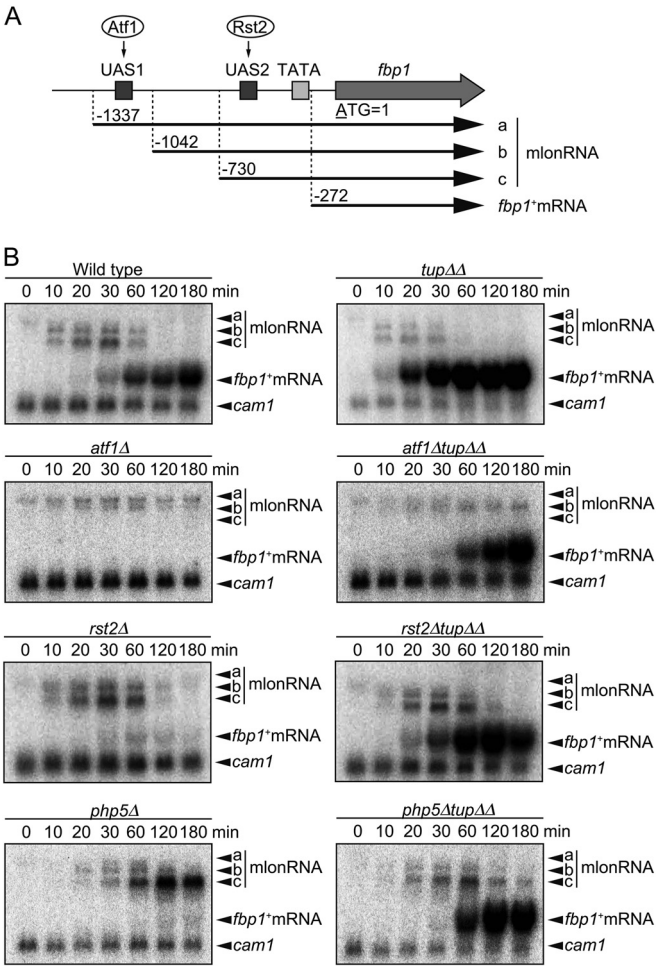

FIG 1 Three transcription activators, Atf1, Rst2, and Php5, induce *fbp1*$^+$ transcription by counteracting Tup11/12-mediated repression. (A) Schematic drawing of distinct *fbp1*$^+$ transcript mlonRNAs. The transcripts labeled a, b, and c initiate from the 5′ region of *fbp1*$^+$. *fbp1*$^+$ mRNA, corresponding to the full-length *fbp1*$^+$ transcript, initiates from its canonical TSS. The numbers indicate the TSS position of each transcript (in base pairs) from the A residue of the first ATG in the *fbp1*$^+$ gene. UAS1 and UAS2 represent the binding sites of Atf1 and Rst2, respectively (24, 33). (B) Northern analysis to detect *fbp1*$^+$ transcripts. Cells were grown to 2.0×10^7 cells/ml in YER medium containing 6% glucose and transferred to YED medium containing 0.1% glucose and 3% glycerol. Cells were harvested at the times indicated above the lanes. The *cam1* transcript was used as an internal control (52).

nitrogen-starved conditions (in meiosis) (36). We previously referred to this lncRNA as metabolic stress-induced long noncoding RNA (mlonRNA) and hypothesized that other lncRNAs are involved in similar gene regulation processes (the mlonRNA hypothesis) (37, 38).

In this study, we describe the three distinct repression mechanisms carried out by Tup11/12 in *fbp1*$^+$ gene regulation. In the first mechanism, Tup11/12 establish repressive chromatin at transcriptional activator binding sites, which is counteracted by Atf1. Second, Tup11/12 repress chromatin remodeling at the TATA box, which leads to the loss of mRNA transcription start site (TSS) establishment and is antagonized by Php5. Third, Tup11/12 repress transcriptional activation after chromatin remodeling by interfering with the actions of the transcription machinery, which is counteracted by Rst2. These results provide new insights into the roles of global corepressors and CBF/NF-Y proteins in eukaryotic gene regulation.

MATERIALS AND METHODS

Fission yeast strains, genetic methods, and media. General genetic procedures were carried out as described previously (39). Strain construction was carried out by mating haploids on sporulation agarose medium (SPA), followed by tetrad dissection. The standard rich yeast extract liquid medium (with 2% glucose) was used to culture cells. Yeast extract repression (YER) medium (containing 6% glucose) and yeast extract derepression (YED) medium (containing 0.1% glucose plus 3% glycerol) were used for glucose repression and derepression, respectively (25). Transformation was performed using the lithium acetate method, as previously described (40). To select kanamycin-resistant colonies, culture suspensions were inoculated onto YE plates, incubated for 16 h, and then replica plated onto YE plates containing 100 μg/ml G-418 sulfate (Nakalai). For the construction of strains expressing proteins with epitope tags, we followed a standard integration method using integration vectors int15 and int16, which were derived from int1 and int2, respectively (40), replacing the green fluorescent protein open reading frame with a 3×Flag tag. We confirmed that the resultant strains (the *rst2-3flag*, *php2-3flag*, and *tbp1-3flag* strains) express the *fbp1*$^+$ gene similarly to a wild-type strain, indicating that the fusion proteins are functional. The *S. pombe* strains used in this study are listed in Table 1.

Deletion of the *php5* gene. The locus containing the *php5*$^+$ gene was amplified from *S. pombe* using primer set CTGGATTGAAGTCAAT TACT and CAACTGATAGTTTTAGCAAC and cloned into the pCR-BluntII-TOPO vector (Invitrogen). The AccI-HpaI fragment (1 kb) was eliminated from the clone around the *php5* gene sequence and replaced by a kanamycin resistance gene prepared from plasmid pFA6a-KanMX6

TABLE 1 Fission yeast strains used in this study

Strain	Genotype[a]
K16	*h*$^+$ *ura4-D18*
K131	*h*$^-$ *ade6-M26 leu1-32*
SPH1	*h*$^-$ *leu1-32*
SPH13	*h*$^-$ *ade6-M26 leu1-32 ura4-D18 tup11::ura4 tup12::ura4*$^+$
SPH18	*h*$^+$ *ade6-M26 ura4-D18 his3-D1 atf1::ura4*$^+$
SPH19	*h*$^-$ *ade6-M26 leu1-32 rst2::kanMX6*
SPH20	*h*$^-$ *ade6-M26 rst2-3flag≪kanMX6 leu1-32*
SPH113	*h*$^+$ *ade6-M216 ura4::fbp1-lacZ leu1-32 his7-366 atf1::ura4*$^+$ *tup11::ura4*$^+$ *tup12::ura4*$^+$
SPH117	*h*$^+$ *ura4-D18 php5::kanMX6*
SPH141	*h*$^-$ *ade6-M26 leu1-32 ura4-D18 rst2::kanMX6 tup11::ura4*$^+$ *tup12::ura4*$^+$
SPH156	*h*$^+$ *ade6-M26 ura4-D18 php5::kanMX6 tup11::ura4*$^+$ *tup12::ura4*$^+$
SPH157	*h*$^+$ *ade6-M26 leu1-32 ura4-D18 php5::kanMX6 rst2-3flag≪kanMX6*
SPH164	*h*$^+$ *ade6-M26 ura4-D18 his3-D1 atf1::ura4*$^+$ *rst2-3flag≪kanMX6*
SPH166	*h*$^+$ *ade6-M26 ura4-D18 his3-D1 atf1::ura4*$^+$ *php2-3flag≪kanMX6*
SPH167	*h*$^-$ *ade6-M26 leu1-32 rst2::kanMX6 php2-3flag≪LEU2*
SPH168	*h*$^-$ *leu1-32 ura4-D18 php2-3flag≪LEU2*
SPH197	*h*$^-$ *ade6-M26 leu1-32 tbp1-3flag≪LEU2*
SPH198	*h*$^-$ *ade6-M26 leu1-32 tbp1-3flag≪LEU2 rst2::kanMX6*
SPH216	*h*$^-$ *ade6-M26 leu1-32 ura4-D18 atf1::ura4 tup11::ura4 tup12::ura4 rst2-3flag≪kanMX6*
SPH217	*h*$^-$ *ade6-M26 ura4-D18 atf1::ura4 tup11::ura4 tup12::ura4 php2-3flag≪kanMX6*

[a] Marker genes used to integrate the epitope tag are represented by ≪*LEU2* and ≪*kanMX6*.

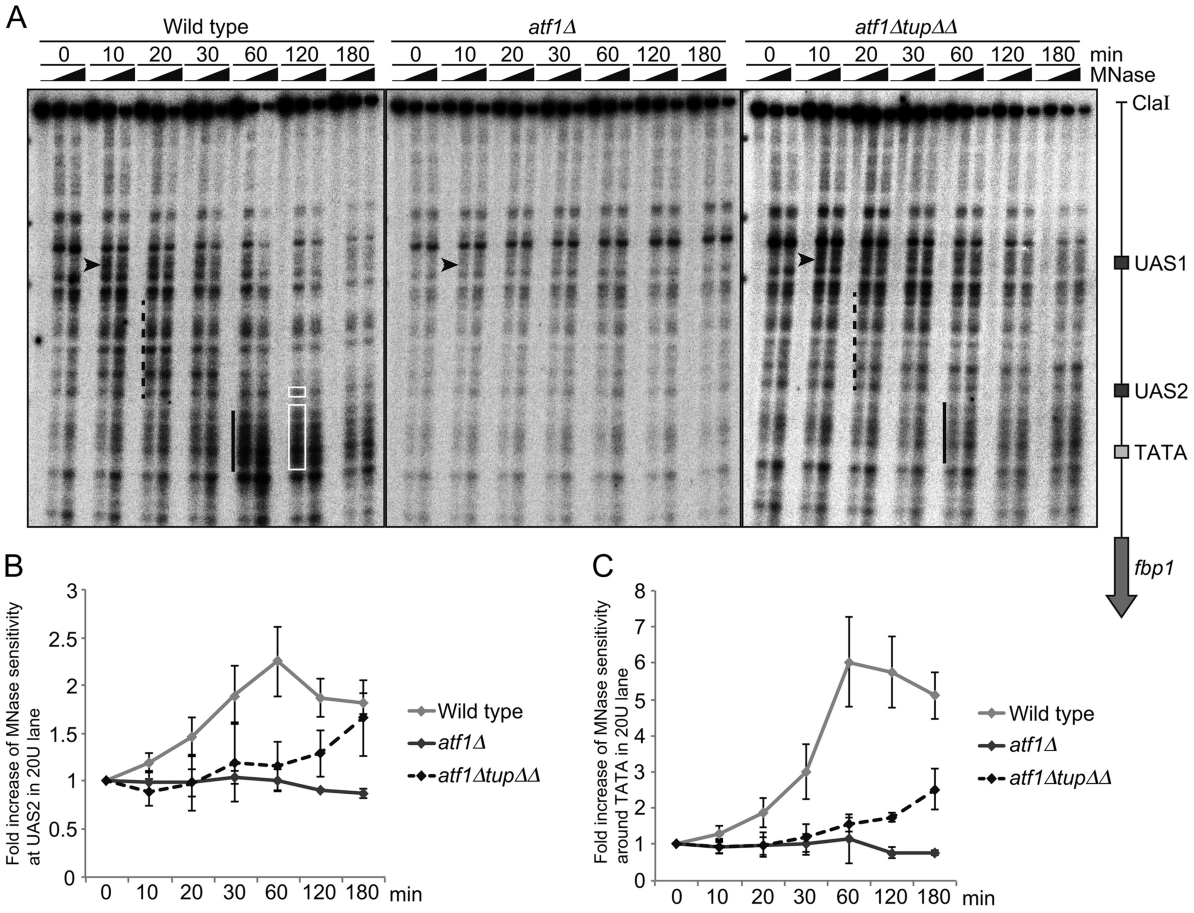

FIG 2 Relationship between Tup11/12 and Atf1 in chromatin regulation at the *fbp1* promoter during glucose starvation. (A) Chromatin structures around the *fbp1*$^+$ promoter in wild-type, *atf1*Δ, and *atf1*Δ *tup*ΔΔ cells. Lanes contain chromatin from cells cultured in YED medium for the times indicated at the top. The isolated chromatin was digested with 0, 20, or 50 U/ml of MNase at 37°C for 5 min. Purified DNA was digested with ClaI and analyzed by Southern blotting. Arrowheads, regions with MNase-sensitive sites at UAS1 (positions −1566 to −1573 from the first A residue of the *fbp1*$^+$ open reading frame); dashed lines, MNase-sensitive sites around UAS1 to UAS2 (positions −926 to −938); solid lines, MNase-sensitive sites around the TATA box. (B, C) Quantification of MNase-sensitive sites around UAS2 (B) and the TATA box (C). The intensities of the bands corresponding to MNase digestion in the UAS2 and TATA regions (enclosed by white boxes in panel A) were quantified by use of an FLA 7000 fluorescent image analyzer (Fuji Film, Japan), and the ratios of the band intensity around the TATA box to the entire signal for each lane were calculated. The relative increase in the ratio at the indicated time after glucose starvation is indicated. The error bars show the standard deviations from at least two independent experiments. 20U lane, the lane with 20 U of MNase per ml.

(41). The SnaBI fragment carrying *php5*::*kanMX6* was transformed into a wild-type strain.

Northern blot, chromatin, and ChIP analyses. Northern blot, chromatin, and chromatin immunoprecipitation (ChIP) analyses were performed as described previously (24). In the ChIP analysis, anti-Atf1 antibody (42) and anti-Flag M2 antibody (Sigma) were used.

Quantification of ChIP DNA. DNA concentrations were quantified using a thermal cycler Dice real-time system (TP800; TaKaRa), a Thunderbird SYBR quantitative PCR (qPCR) mix (TOYOBO), and the following primer sets: fbp1-1 (ACGATCTAACGAAACAGGAA and CCCTTTG TGGACATTTAGAC), fbp1-2 (GAAAATTCCACGGGACATTAG and CCCTTCCTATTAGCAATAAGG), fbp1-3 (GGGATGAAAACAATCAA CCTC and GGAATGCAGCAACGAAAATC), fbp1-4 (GATTTTCGTTG CTGCATTCC and CCTATGATTTGATGTCTAGC), fbp1-5 (GCTAGA CATCAAATCATAGG and CATTCCACCCTATTCATC), fbp1-6 (GGG TGGAATGAGTCCGC and GTTCCGCGAATCATAAGCC), fbp1-7 (CG CGGAACTAAACATAGCG and GCTAGAAACCGAGTGGTG), fbp1-8 (GCCCAACTTAACTCAGCTC and GCTTCTGATTGTATCGGCG), fbp1-9 (CGCCGATACAATCAGAAGC and CGATGAGTTTGCAGCAT CC), and prp3 (GCACAGTCGTTGTACAAATTCGTATTCCC and ACG ATTCTAAACGCCTCTTGTTACGATCC). fbp1-3, fbp1-6, and fbp1-7 represent the UAS1, UAS2, and TATA primer sets, respectively.

RACE. 5′ rapid amplification of cDNA ends (RACE) was carried out using a SMARTer RACE cDNA amplification kit (Clontech). The 5′ ends of the transcripts were amplified by PCR using the universal primer mix included in the kit and the gene-specific primer CACCGGCGTCAATGT TGGAAGAGCCATC. PCR products were gel purified (QIAquick; Qiagen) and cloned into pCR-BluntII-TOPO (Invitrogen). The sequences were determined using the M13 primer.

RESULTS

Three types of transcription activators antagonize Tup11/12-mediated *fbp1*$^+$ transcriptional repression. To study the function of Tup family repressors, we analyzed *fbp1*$^+$ transcription in the absence of Tup11/12 in combination with deletions of three types of transcription activators involved in *fbp1*$^+$ gene activation under conditions of glucose starvation (Fig. 1).

Figure 1A illustrates four distinct *fbp1*$^+$ transcripts, including mlonRNA (transcripts a to c). After glucose starvation, a cascade

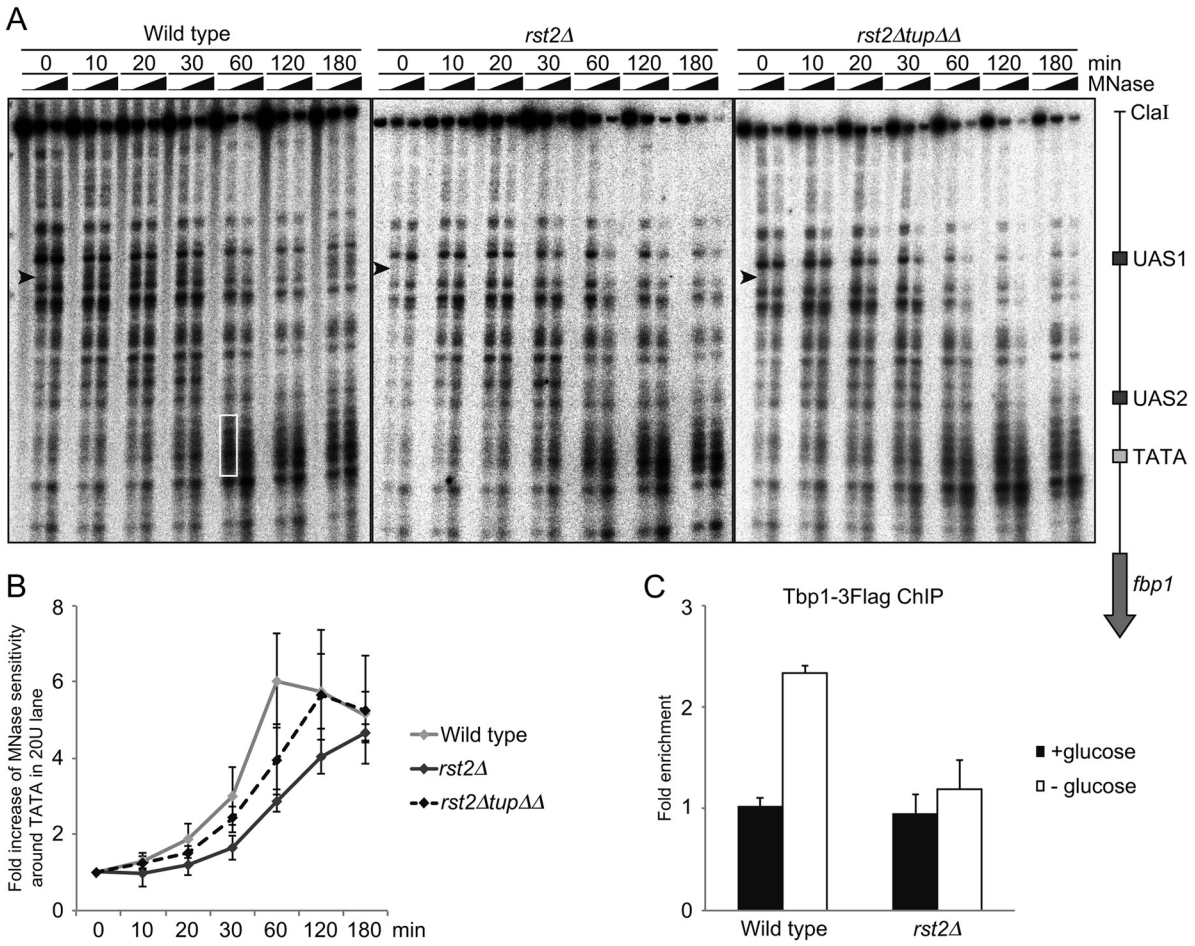

FIG 3 Tup11/12 repress loading of the TATA-binding protein at the *fbp1*$^+$ TATA box, which is antagonized by Rst2. (A) Chromatin structure at the *fbp1*$^+$ promoter in wild-type, *rst2*Δ, and *rst2*Δ *tup*ΔΔ cells. Cell culture and chromatin analyses were performed as described in the legend to Fig. 2A. Arrowheads are as described in the legend to Fig. 2A. (B) Quantification of MNase-sensitive sites at the TATA box. The intensity of bands corresponding to the TATA box (enclosed by a white box in panel A) digested by MNase was quantified as described in the legend to Fig. 2. The relative increase in the ratio at the indicated times after glucose starvation is indicated. The error bars show the standard deviations from at least two independent experiments. (C) Cells expressing Tbp1-3Flag were cultured in YER medium to mid-log phase and then transferred to YED medium. Cells were cross-linked at 60 min after glucose starvation. Coprecipitated DNA was quantified using primers corresponding to the TATA box region of *fbp1*$^+$ and *prp3* (as a control). The ChIP efficiency of Tbp1 in the TATA box of *fbp1*$^+$ was normalized to that of the control *prp3* amplification. The error bars show the standard deviations from three independent experiments.

of transcriptional initiation of mlonRNA from the 5′ region of the *fbp1*$^+$ promoter was detected (Fig. 1B, wild type, transcripts a, b, and c). At 60 min after glucose starvation, a massive transcriptional initiation of mRNA from the TATA box (Fig. 1A, *fbp1*$^+$ mRNA) was induced (Fig. 1B, wild type, *fbp1*$^+$ mRNA). In the absence of the CREB/ATF-type transcription activator (*atf1*Δ), transcript c and *fbp1*$^+$ mRNA were not expressed, even at 180 min after glucose starvation, indicating the critical role played by Atf1 in *fbp1*$^+$ mRNA expression. We further examined a deletion mutant of the Rst2 C_2H_2 Zn finger-type transcription activator (*rst2*Δ). The *fbp1*$^+$ mRNA was weakly detected at 60 to 180 min after glucose starvation, demonstrating that Rst2 is required for the robust induction of *fbp1*$^+$ mRNA. In a mutant of the third type of transcriptional activator, CBF/NF-Y (*php5*Δ), transcript c was highly induced, while *fbp1*$^+$ mRNA was not. These results indicate the distinct and pivotal roles of the three types of transcriptional activators in *fbp1*$^+$ regulation.

We then examined genetic interactions between the three transcription activators and the Tup11/12 corepressors. We disrupted the *tup11* and *tup12* genes (*tup*ΔΔ) in combination with *atf1*Δ, *rst2*Δ, and *php5*Δ mutations. The loss of Tup11/12 restored *fbp1*$^+$ gene activation in *atf1*Δ, *rst2*Δ, and *php5*Δ cells. This indicates that the three types of transcription activators activate the *fbp1*$^+$ gene by antagonizing the repressive functions of the Tup11/12 corepressors.

Tup11/12 establish repressive chromatin at the activator binding site in the *fbp1*$^+$ promoter, and Atf1 counteracts this repression. The pivotal roles of the three types of transcriptional activators and their antagonistic roles against the Tup11/12 corepressors led us to study the impact of these regulators on chromatin control, since *fbp1*$^+$ derepression via stepwise chromatin remodeling at the *fbp1*$^+$ promoter is mediated by mlonRNA transcription (35). Therefore, we employed an indirect end-labeling analysis involving the partial digestion of chromatin DNA with micrococcal nuclease (MNase) to map the positions of individual nucleosomes and nucleosome-free, hypersensitive sites.

Wild-type, *atf1*Δ, and *atf1*Δ *tup11*Δ *tup12*Δ (*atf1*Δ *tup*ΔΔ) cells were cultured in YER medium (containing 6% glucose) to

2.0×10^7/ml cells and transferred to YED medium (containing 0.1% glucose). Cells were harvested at the time points indicated in Fig. 2A. In wild-type cells, an MNase-sensitive site appeared at 10 min after glucose starvation, probably due to nucleosome eviction (Fig. 2A, arrowheads). Another MNase-sensitive region appeared around UAS1-UAS2 at 20 to 30 min after glucose starvation (Fig. 2A and B, dashed lines). Finally, intense MNase-sensitive sites appeared around the TATA box at 60 to 180 min after glucose starvation (Fig. 2A, solid lines), when massive amounts of *fbp1*$^+$ mRNA transcription occurred. These observations indicate that the chromatin in the *fbp1*$^+$ promoter progressively converts into an open configuration. In the *atf1*Δ strain, no alteration of the MNase digestion pattern was observed after glucose starvation (Fig. 2A), indicating that Atf1 is required for chromatin remodeling at the UAS1 and UAS2 activator binding sites and for later chromatin alteration at the TATA box. More importantly, the loss of Tup11/12 completely rescued the chromatin conversion defect at UAS1 in *atf1*Δ cells (Fig. 2A, arrowhead). In addition, the chromatin remodeling around UAS2 and the TATA box was partly restored in *atf1*Δ *tup*ΔΔ cells (Fig. 2A and B). These results indicate that the Tup11/12 corepressors establish repressive chromatin at activator binding sites and thereby repress later chromatin alteration.

Tup11/12 repress *fbp1*$^+$ transcription after TATA box chromatin converts into an open configuration. We next examined the role of Rst2 in chromatin alteration. In *rst2*Δ cells, intense MNase-sensitive sites around the TATA box appeared at 60 to 180 min after glucose starvation, and the chromatin configuration pattern in this region was indistinguishable between wild-type and *rst2*Δ cells (Fig. 3A and B). On the other hand, chromatin remodeling at UAS1 was significantly attenuated in *rst2*Δ cells (Fig. 3A, arrowheads). Moreover, the defect in chromatin remodeling at UAS1 was not restored by the loss of Tup11/12. Considering that the *fbp1*$^+$ mRNA transcription defect in *rst2*Δ cells was completely restored in the *rst2*Δ *tup*ΔΔ cells, it seems unlikely that attenuated UAS1 remodeling is the cause of the *fbp1*$^+$ transcription defects in *rst2*Δ cells. Normal levels of chromatin conversion around the TATA box suggest that the Tup11/12 corepressors repress mRNA transcription *per se* after chromatin remodeling, presumably by affecting the basic transcription machinery. This was indeed the case, because the accumulation of the TATA-binding protein (Tbp1) at the TATA box was significantly reduced in *rst2*Δ cells (Fig. 3C). Consistently, depletion of Rst2 also reduces RNA polymerase II accumulation at the TATA box (35).

Tup11/12 repress the formation of open chromatin at the TATA box, and Php5 counteracts this mechanism. We next analyzed the role of Php5 in the regulation of the chromatin structure. In *php5*Δ cells, chromatin remodeling at UAS1 was normally detected at 10 min after glucose starvation (Fig. 4A, arrowheads; see also Fig. S1 in the supplemental material). At 60 to 180 min after glucose starvation, only a few MNase-sensitive sites appeared at the TATA box (Fig. 4A, solid lines), while massive MNase-sensitive regions appeared at UAS1-UAS2 (Fig. 4A and B, dashed lines). The loss of Tup11/12 partly restored the chromatin conversion defect at the TATA box in *php5*Δ cells (Fig. 4A, solid lines). To address the role played by Php5 in the regulation of the local chromatin configuration, we analyzed the intensity of an MNase-sensitive band corresponding to one nucleosome at the TATA box (Fig. 4A, white box). The results in Fig. 4C support the conclusion that the Tup11/12 corepressors repress the formation of an open chromatin configuration at the TATA box, which is antagonized by the CBF complex.

Atf1 is required for Php2 and Rst2 binding to *cis*-acting elements in the *fbp1*$^+$ promoter. To investigate the interplay of Tup corepressors and three types of transcription activators (Atf1, Php5, and Rst2), we further examined the interdependence of their binding to sites within the *fbp1*$^+$ promoter. Atf1 and Rst2 are known to bind at UAS1 and UAS2, respectively (24, 33), while the Php5 binding site at *fbp1*$^+$ has not been identified. Thus, we first searched for the binding site of the CBF complex in the *fbp1*$^+$ promoter. Since Flag-tagged Php5 is not efficiently recognized by anti-Flag antibody (data not shown), we constructed a Flag-tagged version of Php2, another component protein of the CBF complex. The loss of Php2 or Php5 has similar effects on *fbp1*$^+$ activation (26), consistent with data showing that the homologous proteins Hap2 and Hap5, together with Hap3, form the CBF complex in *S. cerevisiae* (32). Then, we divided the *fbp1*$^+$ promoter region into ~250-bp segments (Fig. 5A), and the primer sets for each region were used for qPCR analysis to measure the ChIP efficiency of each segment. Flag-tagged Php2 was enriched in the UAS1-UAS2 region and peaked at the UAS2 region in glucose-starved cells (Fig. 5A), a distribution pattern which is very similar to that of Tup11/12 (24). We thus considered the UAS2 segment to be a CBF binding site in a later analysis.

We then compared the binding of Atf1 in wild-type, *php5*Δ, and *rst2*Δ cells. Atf1 occupancy at UAS1 in wild-type cells was significantly increased upon glucose starvation. The ChIP efficiency of Atf1 increased in *php5*Δ cells but was reduced in *rst2*Δ cells. These results are consistent with the attenuated chromatin remodeling at UAS1 observed in *rst2*Δ cells (Fig. 3). However, the reduction in Atf1 occupancy at UAS1 in *rst2*Δ cells (~30% of that in wild-type cells) is not sufficient to explain the much greater decrease in the amount of the *fbp1*$^+$ transcript, because chromatin remodeling around the UAS2-TATA box is normally induced in the absence of Rst2.

We further examined the binding of Php2-3Flag in wild-type, *atf1*Δ, and *rst2*Δ cells. Occupancy of the Php2-3Flag at UAS2 was totally absent in *atf1*Δ cells but not in *rst2*Δ cells. We analyzed the binding of the Rst2-3Flag at UAS2 in wild-type, *atf1*Δ, and *php5*Δ cells and found that Rst2 binding at UAS2 was absent in *atf1*Δ cells but not in *php5*Δ cells. These results suggest that Rst2 and Php2 independently bind to the *fbp1*$^+$ promoter. More importantly, these results indicate that Atf1 is required for Php2 and Rst2 binding to the *fbp1*$^+$ promoter. This idea is consistent with the essential role of Atf1 in chromatin opening at UAS1 and UAS2. To explore the role of Atf1 in Php2 and Rst2 binding to the *fbp1*$^+$ promoter, we examined the status of Php2 and Rst2 binding in *atf1*Δ *tup*ΔΔ cells, in which chromatin opening defects were partly restored (Fig. 2B). In *atf1*Δ *tup*ΔΔ cells, Rst2 binding was partly restored, while Php2 binding was not restored (Fig. 5C). These results indicate that Rst2 binding is regulated through the chromatin configuration, but Atf1 itself is required for CBF complex binding.

Determination of TSS through modulation of the local chromatin configuration by Tup corepressors and the CBF complex. Lastly, we examined the biological significance of the CBF complex in *fbp1*$^+$ gene activation. We have shown that CBF plays a role in the formation of accessible chromatin at the TATA box, contributing greatly to *fbp1*$^+$ induction from its genuine TSS (Fig. 1 and 4). This activation is mediated by the suppression of the repressive Tup function. In *php5*Δ cells, transcript c but not the

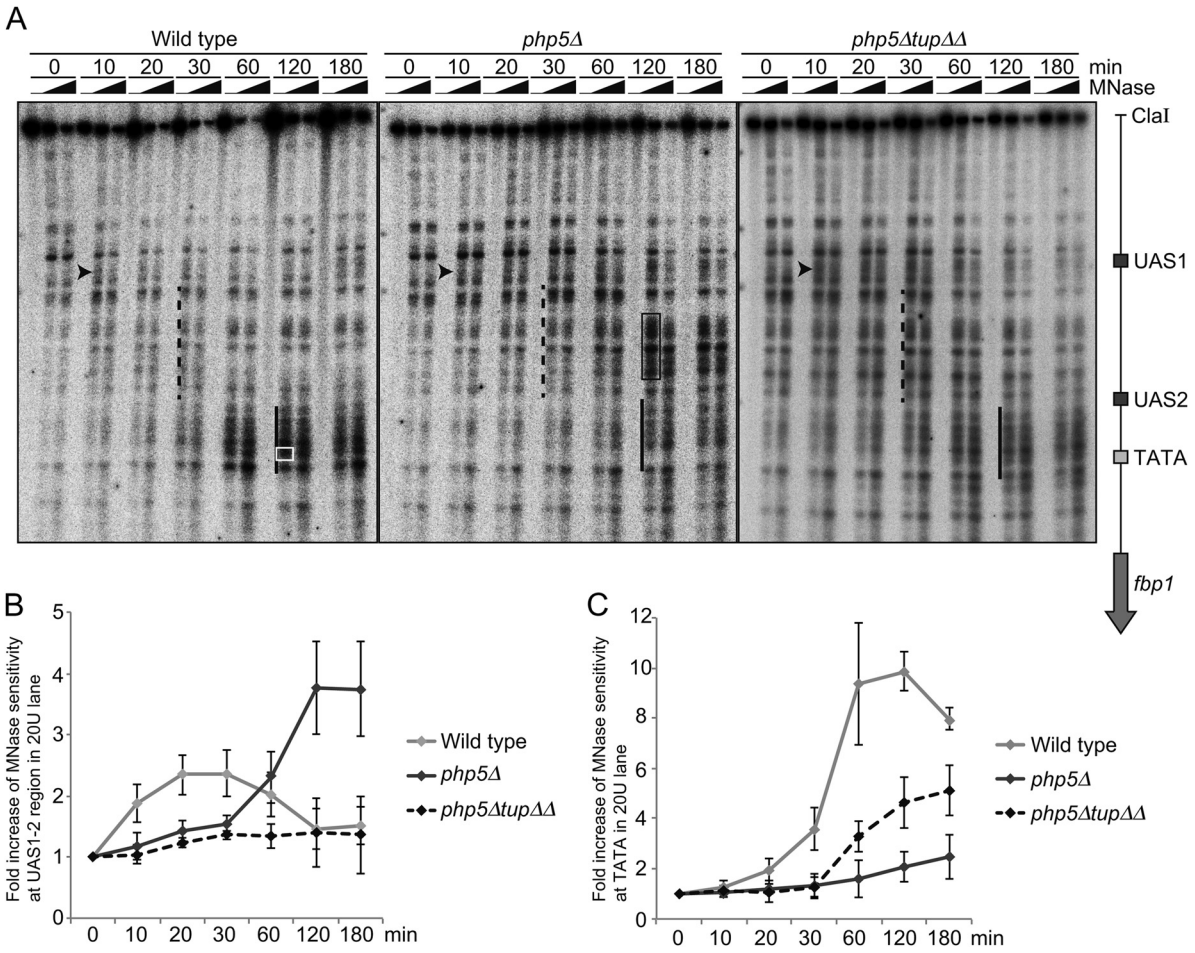

FIG 4 Relationship between Tup11/12 and Php5 in chromatin regulation in the *fbp1* promoter region during glucose starvation. (A) Chromatin structure at the *fbp1*$^+$ promoter in wild-type, *php5*Δ, and *php5*Δ *tup*ΔΔ cells. Cell culture and chromatin analyses were performed as described in the legend to Fig. 2A. Arrowheads, dashed lines, and solid lines are as described in the legend to Fig. 2A. (B) Quantification of MNase-sensitive sites at UAS1-UAS2. The intensity of bands digested by MNase between UAS1 and UAS2 (enclosed by the black box in the *php5*Δ lanes in panel A) was quantified as described in the legend to Fig. 2. (C) Quantification of MNase-sensitive sites at the TATA box. The intensity of bands corresponding to the TATA box digested by MNase was quantified as described in the legend to Fig. 2. The relative increase in the ratio at the indicated times after glucose starvation is indicated. To distinguish between remodeling at UAS2 and the TATA box, quantitation for TATA box remodeling was restricted to a single nucleosome, as shown by the white box in panel A. The error bars show the standard deviations from three independent experiments.

genuine *fbp1*$^+$ mRNA was induced, while the loss of Tup11/12 restored the transcription of *fbp1*$^+$ mRNA (Fig. 1). We also observed that the band corresponding to the *fbp1*$^+$ mRNA in *php5*Δ *tup*ΔΔ cells was composed of at least two transcripts of different lengths (Fig. 6A). This suggests that the CBF complex plays a critical role in the determination of the canonical TSS for *fbp1*$^+$ transcription. To examine this possibility, we examined TSS in wild-type, *tup*ΔΔ, and *php5*Δ *tup*ΔΔ cells by 5′ RACE. The majority of TSSs detected in the wild-type and *tup*ΔΔ cells were distributed within ~10 bp of the TATA box, while the TSSs in the *php5*Δ *tup*ΔΔ cells were distributed over a range of ~250 bp (Fig. 6B). This result indicates that the regulation of the local chromatin configuration by Tup corepressors and the CBF complex plays a pivotal role in the determination of the genuine TSS.

DISCUSSION

In this study, we have uncovered unappreciated functions and regulatory mechanisms of the Tup family corepressor using the fission yeast *fbp1*$^+$ locus as a model system. We found that fission yeast Tup corepressors repress *fbp1*$^+$ transcription via three distinct mechanisms. First, they establish repressive chromatin at activator binding sites. Atf1, a bZIP transcription activator antagonizes this repression and thereby forms an open chromatin structure at activator binding sites (Fig. 2). Second, they repress the formation of an open chromatin configuration at the TATA box, which is a prerequisite to mRNA start site determination. Php5, a CBF component, is required for the suppression of this second mechanism (Fig. 4). Third, they repress transcription itself after chromatin disassembly at the TATA box. Rst2, a C_2H_2 zinc finger transcription activator, counteracts this third mechanism (Fig. 3).

These three distinct repression mechanisms and their suppression by the three activators may proceed in a stepwise fashion. Under glucose-rich conditions, the Tup corepressors mask activator binding sites and thereby establish repressive chromatin at activator binding sites (Fig. 7i), similar to the action of Tup1p in *S.*

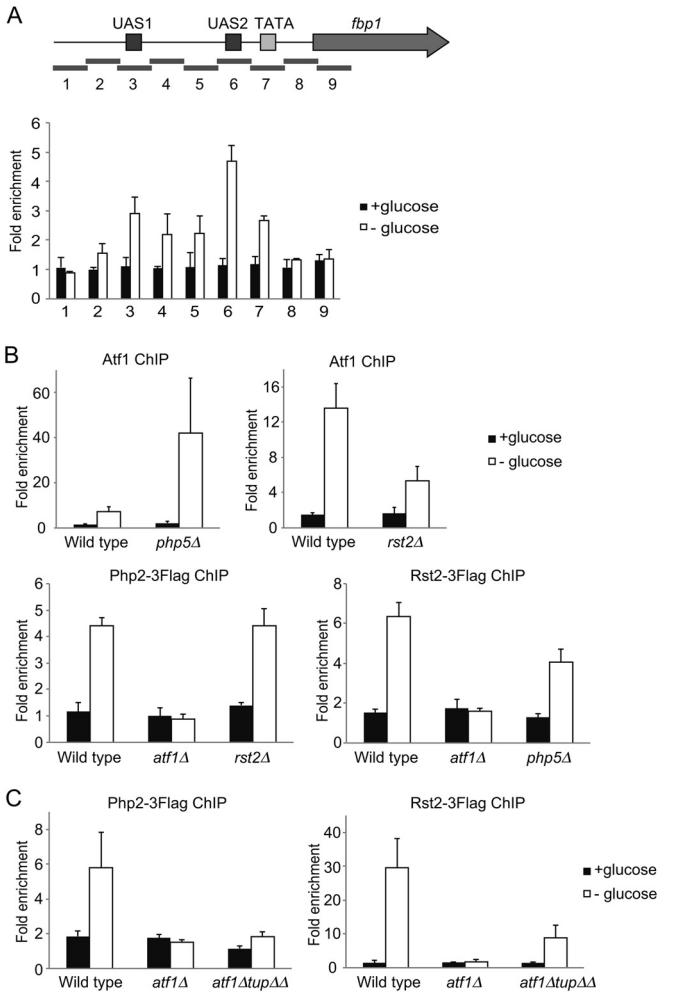

FIG 5 Atf1 is required for Php2 and Rst2 binding to *cis*-acting elements in the *fbp1*$^+$ promoter. (A) Schematic drawing of the segments quantifying DNA precipitated with the indicated activators. Primer sets covering each segment were used for quantitative PCR. Segments 3, 6, and 7 contain UAS1, UAS2, and the TATA box, respectively. Cells expressing Php2-3Flag were cultured in YER medium to 1.0×10^7/ml cells and then transferred to YED medium. Cells were cross-linked at 120 min after glucose starvation. Coprecipitated DNA was quantified using primers corresponding to segments 1 to 9. (B, C) ChIP analysis of Atf1, Php2, and Rst2. Cells were cultured and cross-linked as described in the legend to panel A. ChIP samples were quantified using primer sets for UAS1 (in the ChIP for Atf1), UAS2 (in the ChIP for Php2 and Rst2), and *prp3* (as a control site). The ChIP efficiency for each transcription activator was normalized to that for the control *prp3* amplification. The error bars show the standard deviations from at least two independent experiments.

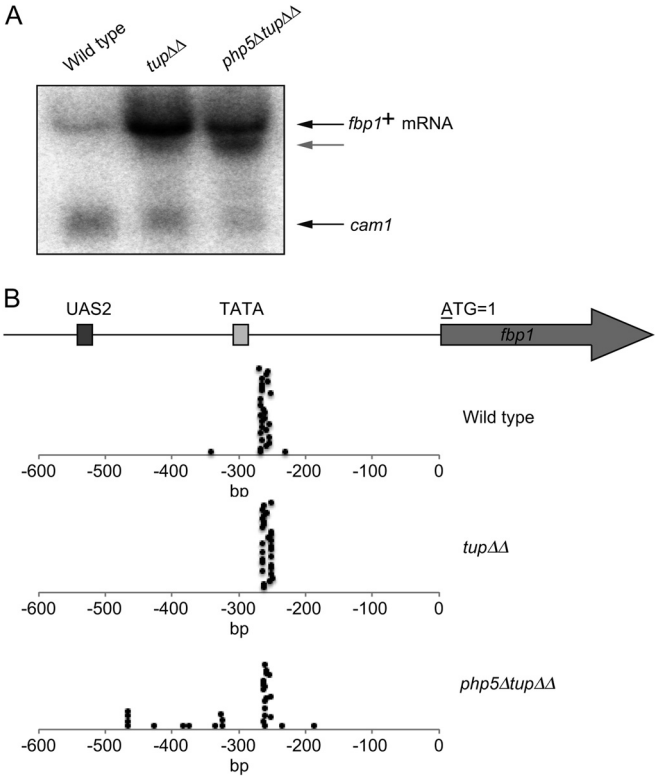

FIG 6 Php5 determines the precise TSS of *fbp1*$^+$ transcription. (A) Northern analysis to detect *fbp1*$^+$ transcripts in wild-type, *tup*ΔΔ, and *php5*Δ *tup*ΔΔ cells. The cells were cultured as described in the legend to Fig. 1 and harvested at 120 min after glucose starvation. (B) The TSS of the *fbp1*$^+$ transcript in wild-type, *tup*ΔΔ, and *php5*Δ *tup*ΔΔ cells was determined using 5′ RACE. The cells were cultured as described in the legend to Fig. 1 and harvested at 120 min after glucose starvation. The 28, 28, and 30 cloned 5′ RACE products from wild-type, *tup*ΔΔ, and *php5*Δ *tup*ΔΔ mRNAs, respectively, were sequenced. The black dots indicate the TSS. The A residue of the first ATG is represented as 1.

cerevisiae (43). Upon glucose starvation, Atf1 binds UAS1 and suppresses Tup-mediated chromatin repression at UAS1 and UAS2 (Fig. 7ii). The CBF complex could then bind at UAS2 and induce further chromatin remodeling around the TATA box by suppressing the repressive function of the Tup corepressors (Fig. 7iii). Finally, after the chromatin around the TATA box takes on an open configuration, Rst2 might suppress the Tup-mediated repression of the basic transcription machinery, including RNA polymerase II (RNAPII), as suggested previously (35) (Fig. 7iv). Such a stepwise activation mechanism results in a massive induction of *fbp1*$^+$ transcription and preserves the stress response specificity. Since the CBF complex and Rst2 appear to bind to UAS2 independently, the order in which these activators bind to UAS2 cannot be determined. However, our data clearly indicate that these two types of transcriptional activators participate in *fbp1*$^+$ transcription in distinct steps: formation of an open chromatin configuration at the TATA box, which is a prerequisite to mRNA start site determination and stabilization of the transcriptional machinery.

We have previously shown that Tup11/12 bind persistently at UAS1-UAS2 (24). Thus, Tup proteins might be required for the precise regulation of transcription through chromatin modulation under either repressive or nonrepressive conditions, rather than by simply inducing transcriptional repression. This idea seems reasonable, since *S. cerevisiae* Tup1 also resides at promoters and can activate chromatin alteration by recruiting a histone acetyltransferase complex (44). More importantly, we previously demonstrated that *tup*ΔΔ cells lose their stress-selective activation of several stress response genes (20). Thus, it seems likely that antagonistic regulation by Tup11/12 and the transcriptional activators might be needed to ensure a stress-selective response. Indeed, the concurrent loss of Atf1 and Tup11/12 makes cells critically sensitive to stress (R. Asada and K. Hirota, unpublished results).

We demonstrate herein the role of the CBF complex in the determination of the TSS. In *php5*Δ cells, the *fbp1*$^+$ mRNA does

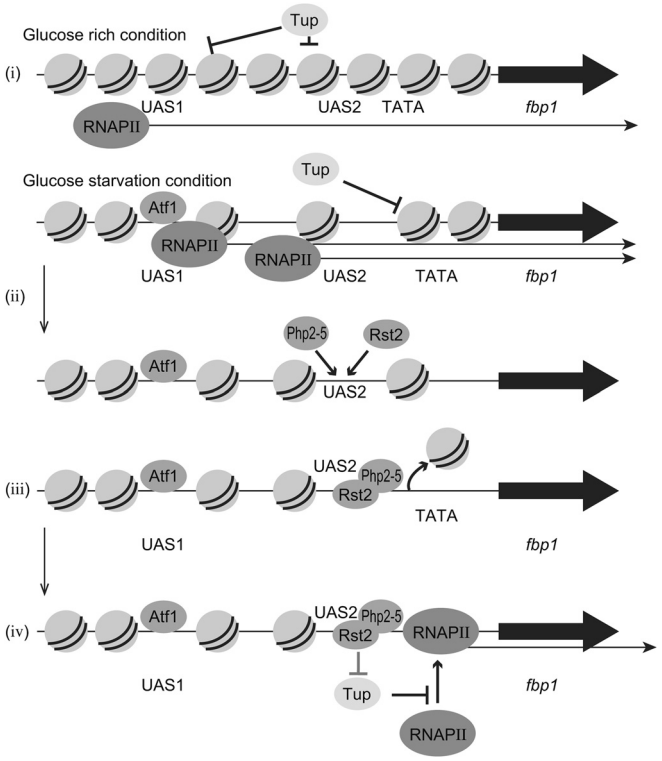

FIG 7 Model for the regulation of *fbp1*$^+$ transcription. (i) Tup11/12 repress chromatin remodeling at UAS1-UAS2 under glucose-rich conditions. (ii) Under conditions of glucose starvation, Atf1 suppresses the repressive role played by the Tup corepressor and induces chromatin remodeling at UAS1-UAS2, after which Php5 and Rst2 bind to UAS2. (iii) Php5 then suppresses Tup-mediated chromatin repression at the TATA box and induces the formation of open chromatin at the TATA box. (iv) Rst2 further suppresses the repressive effect of the Tup corepressors on the transcription machinery, including RNA polymerase II (RNAPII), and induces *fbp1*$^+$ transcription.

not appear, but transcript c (one of the mlonRNAs) is strongly induced. In *php5*Δ *tup*ΔΔ cells, induction of *fbp1*$^+$ mRNA is restored, but the TSSs of the *fbp1*$^+$ mRNA in this triple mutant are distributed more widely than they are in wild-type cells (Fig. 6). In *php5*Δ cells, the formation of the open chromatin at the TATA box is critically impaired and the loss of Tup11/12 partially restores this defect (Fig. 4). Thus, a possible rationale for the mechanism determining the genuine TSS for mRNA is that the Tup corepressor and CBF regulate nucleosome positioning around the TATA box and thereby control transcription initiation from a particular point. This is consistent with the fact that the architecture of the CBF-DNA assembly is very similar to that of the histone H2A/H2B-DNA assembly in the nucleosome (45), suggesting a possible regulatory role as an H2A/H2B-like variant (46). Moreover, CBF interacts with histone acetyltransferases, suggesting that CBF may play an important role in local chromatin modulation by means of histone modification (47–51).

This study demonstrates the previously unappreciated role of the Tup corepressors and CBF/NF-Y in the determination of the TSS through chromatin modulation at the TATA box. Since *fbp1*$^+$ transcription is associated with mlonRNA-mediated chromatin remodeling (35), understanding how these factors and the mlonRNA-associated mechanism interact to regulate chromatin structure is an important question to be addressed in the future.

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