The Zinc Finger Transcription Factor Fts2 Represses the Yeast-to-Filament Transition in the Dimorphic Yeast *Yarrowia lipolytica*

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ABSTRACT  The yeast-to-filament transition is an important cellular response to environmental stimulations in dimorphic fungi. In addition to activators, there are repressors in the cells to prevent filament formation, which is important to keep the cells in the yeast form when filamentation is not necessary. However, very few repressors of filamentation are known so far. Here, we identify a novel repressor of filamentation in the dimorphic yeast *Yarrowia lipolytica*, Fts2, which is a C_{2}H_{2}-type zinc finger transcription factor. We show that fts2Δ cells exhibited increased filamentation under mild filament-inducing conditions and formed filaments under non-filament-inducing conditions. We also show that Fts2 interacts with YlSsn6, component of the Tup1-Ssn6 transcriptional corepressor, and Fts2-LexA represses a lexAop-FwCCF-LeuZ reporter in a Tup1-Ssn6-dependent manner, suggesting that Fts2 has transcriptional repressor activity and represses gene expression via Tup1-Ssn6. In addition, we show that Fts2 represses a large number of cell wall protein genes and transcription factor genes, some of which are implicated in the filamentation response. Interestingly, about two-thirds of Fts2-repressed genes are also repressed by Tup1-Ssn6, suggesting that Fts2 may repress the bulk of its target genes via Tup1-Ssn6. Lastly, we show that Fts2 expression is downregulated in response to alkaline pH and the relief of negative control by Fts2 facilitates the induction of filamentation by alkaline pH.

IMPORTANCE  The repressors of filamentation are important negative regulators of the yeast-to-filament transition. However, except in *Candida albicans*, very few repressors of filamentation are known in dimorphic fungi. More importantly, how they repress filamentation is often not clear. In this paper, we report a novel repressor of filamentation in *Y. lipolytica*. Fts2 is not closely related in amino acid sequence to CaNrg1 and Rfg1, two major repressors of filamentation in *C. albicans*, yet it represses gene expression via the transcriptional corepressor Tup1-Ssn6, similar to CaNrg1 and Rfg1. Using transcriptome sequencing, we determined the whole set of genes regulated by Fts2 and identified the major targets of Fts2 repression, which provide clues to the mechanism by which Fts2 represses filamentation. Our results have important implications for understanding the negative control of the yeast-to-filament transition in dimorphic fungi.

KEYWORDS  dimorphic transition, dimorphism, filamentation, hyphal growth, C_{2}H_{2} zinc finger proteins

Some fungal species can switch the morphology from the oval-shaped yeast form to filamentous forms in response to environmental stimulations (1–3). This yeast-to-filament transition (also called dimorphic transition or filamentation) is a stress response that helps the cells to expand into new environment. In some pathogenic fungi, including the human pathogen *Candida albicans*, the ability to switch between the yeast and filamentous forms is essential for virulence (1, 2). Therefore, the regulation of dimorphic transition has attracted a lot of attention. Many of these studies have been conducted in.
2 yeast species, *Saccharomyces cerevisiae* and *C. albicans*, and extensive knowledge has been gained from these studies.

*Yarrowia lipolytica* is an industrial yeast utilized in the production of valuable metabolites including organic acids, sugar alcohols, and lipids (4, 5). Under the induction of poor carbon source, nitrogen starvation, and alkaline pH, *Y. lipolytica* cells can switch from oval-shaped yeast form to filamentous forms including rod-like elongated cells, pseudohyphae, and hyphae (6, 7). Previous studies have shown that filamentation in *Y. lipolytica* is positively regulated by evolutionarily conserved pathways such as the Ras/MAPK pathway and the Rim101 pH response pathway (8, 9), which also function similarly in *S. cerevisiae* and *C. albicans* (10–13). Filamentation is also positively regulated by the transcription factors Mhy1 and Hoy1 (14, 15). Mhy1 is a key regulator of filamentation in *Y. lipolytica*. Its deletion abolished filamentation whereas its overexpression caused strong filamentation (15, 16). Furthermore, proteins that regulate cytoskeletal organization including YlBem1, YlCla4, and YlRac1 are also required for filamentation (17–19).

In addition to positive regulators that promote filamentation under filament-inducing conditions, studies in *C. albicans* revealed the existence of negative regulators that repress filamentation under non-filament-inducing conditions. The transcription factors CaNrg1 and Rfg1, and the general transcriptional corepressor Tup1-Ssn6 play a major role in the repression of filamentation in *C. albicans* (20–24). Tup1-Ssn6 is an evolutionarily conserved corepressor complex responsible for the repression of hundreds of genes implicated in a variety of cellular functions in *S. cerevisiae* and *C. albicans* (25–28). Tup1 and Ssn6 do not bind DNA. They are brought to specific promoters by DNA-binding transcription factors to execute the repression of target genes. CaNrg1 and Rfg1 are thought to repress gene expression via Tup1-Ssn6 (21, 22, 28).

Previous studies have identified several negative regulators of filamentation in *Y. lipolytica*, including the Ras/PKA pathway, the high-osmolarity glycerol response (HOG) pathway, the TORC1-Sch9 pathway, and the transcription factors YITec1 and Znc1 (29–33). Among these, the HOG pathway and the TORC1-Sch9 pathway function similarly in *C. albicans*. However, the Ras/PKA pathway and the transcription factor CaTec1 promote, but do not repress, filamentation in *C. albicans* (34, 35). Hence, there are similarities as well as differences between *Y. lipolytica* and *C. albicans* regarding the mechanisms for the control of filamentation.

In this study, we report the identification of a novel repressor of filamentation, Fts2, in the dimorphic yeast *Y. lipolytica* and address how Fts2 represses filamentation. We examined the transcriptional repressor activity of Fts2 and its relationship with the Tup1-Ssn6 corepressor. We also characterized the major target genes controlled by Fts2 using transcriptome sequencing. Lastly, we investigated whether Fts2 expression may change during the yeast-to-filament transition and, if so, its involvement in the induction of filamentation.

**RESULTS**

**Identification of Fts2, a novel repressor of filamentation in *Y. lipolytica*.** Yali0E23518 is a putative transcription factor whose cellular function is not known in *Y. lipolytica*. In a mutant screen, we isolated 2 insertional loss-of-function mutants of YALI0E23518, both of which displayed increased filamentation (data not shown). Subsequent deletion of this gene in the wild-type strain generated an identical phenotype (see below). This finding suggests that Yali0E23518 represses filamentation. Based on the mutant phenotype, Yali0E23518 was named Fts2 (Filamentous 2).

We deleted the *FTS2* gene in the wild-type strain and examined the phenotypes of *fts2Δ* cells. *fts2Δ* cells grew normally as wild-type cells did. They did not exhibit any detectable defect in growth. When grown under mild filament-inducing conditions, such as on YNBG agar (a glycerol-containing synthetic medium), wild-type colonies were slightly wrinkled but not fluffy. In contrast, *fts2Δ* colonies were fluffy, covered with aerial filaments (Fig. 1A, left column). Microscopic examination of the microcolonies grown for a shorter time showed that *fts2Δ* colonies exhibited longer radial filaments
than wild-type colonies (Fig. 1A, second column). Similarly, when grown in liquid YNBD medium, a glucose-containing medium that weakly stimulates filamentation, wild-type cells displayed an elongated, rod-like morphology but very few cells (~1%, n > 400) were longer than 30 μm. No long filaments were observed. In contrast, 64% of fts2Δ cells (n > 400) were longer than 30 μm, and long filaments were readily observed in fts2Δ cells (Fig. 1A, third column). These results indicate that fts2Δ cells exhibit increased filamentation compared with the wild-type cells under mild filament-inducing conditions, suggesting that Fts2 represses filamentation.

Interestingly, when grown under non-filament-inducing conditions, such as in liquid YNBG or YPD media, a significant fraction of fts2Δ cells were still elongated and formed filaments. In liquid YNBG medium, none of wild-type cells were longer than 20 μm. In contrast, 47% and 40% of fts2Δ cells (n > 400) were longer than 20 μm and 30 μm, respectively, and long filaments were readily observed (Fig. 1A, fourth column). In nutrient-rich YPD medium, none of wild-type cells were longer than 20 μm. In contrast, 19% and 10% of fts2Δ cells (n > 400) were longer than 20 μm and 30 μm, respectively, and long filaments were still present (Fig. 1A, right column). These results suggest that Fts2 also represses filamentation under non-filament-inducing conditions. Reintroduction of the FTS2 gene into fts2Δ cells reduced filamentation to the level comparable to that of wild-type cells both on solid media and in liquid media, indicating that the loss of Fts2 function is responsible for increased filamentation in fts2Δ cells.

In addition to increased filamentation, fts2Δ cells also exhibited stronger invasive growth than wild-type cells (Fig. 1B). Moreover, fts2Δ cells exhibited increased resistance to peroxide (Fig. 1C), suggesting that Fts2 may also regulate the oxidative stress response.

Taken together, our results suggest that Fts2 is a novel repressor of filamentation. It also represses invasive growth and oxidative stress response.

**Fts2 is a C2H2 zinc finger transcription factor related to RfeC.** Fts2 is a protein of 301 amino acids featuring at the N-terminus two C2H2 zinc finger domains (a.a. 21 to 69), which are DNA-binding domains found in many transcription factors. Fts2 has no other recognizable domains except for a short QH-rich sequence (a.a. 148 to 192) in the central region.

Fts2 does not share extensive amino acid sequence similarities to proteins in *S. cerevisiae*, *C. albicans*, and other yeasts. The closest homologs of Fts2 in *S. cerevisiae* are

![Image](https://example.com/image.png)
Mig2 and Mig1, whereas in *C. albicans* are Try5, Bcr1, and CaMig1. Fts2 shares 37% to 42% sequence identity to these proteins but the homologous region is limited to the 2 zinc finger domains. Fts2 shares a higher sequence identity (46% to 54%) to RfeC, the C2H2 zinc finger transcription factor widely present in filamentous fungi, and to the RfeC-like proteins in 2 plant species, *Lupinus albus* and *Quercus suber* (Fig. S1). However, the homologous region is still limited to the 2 zinc finger domains plus the flanking several amino acids (Fig. 2A). Outside this region, Fts2 does not share sequence similarity to these proteins. The cellular function of RfeC is not known except that *Aspergillus terreus* RfeC can promote *FLO11* expression when expressed in *S. cerevisiae* (36). Consistent with a predicted role as a transcription factor, GFP-tagged Fts2 localized to the nucleus (Fig. 2B). Thus, Fts2 is a novel RfeC-like C2H2 zinc finger transcription factor that controls filamentation.

**Fts2 interacts with YlSsn6, component of the Tup1-Ssn6 general transcriptional corepressor, and Fts2-LexA represses a reporter gene in a Tup1-Ssn6-dependent manner.** Tup1-Ssn6 is a general transcriptional corepressor in yeast. It is involved in the repression of hundreds of genes directed by specific transcription factors (25, 27, 28). In *C. albicans*, Tup1-Ssn6 represses filamentation (20, 27, 37). Similar to *C. albicans* Catup1Δ cells, Yltup1Δ and Ylssn6Δ cells also displayed increased filamentation (Fig. 3A), indicating that Tup1-Ssn6 also represses filamentation in *Y. lipolytica*. Interestingly, Fts2 interacted with YlSsn6 in co-immunoprecipitation assay in *Y. lipolytica* cells (Fig. 3B), suggesting that Fts2 may interact with the Tup1-Ssn6 corepressor.

To examine whether Fts2 has transcriptional repressor activity, we performed one-hybrid assay in *Y. lipolytica* cells, which tested the ability of a Fts2-LexA fusion (containing the DNA-binding domain of LexA, a.a. 1 to 87) to influence the expression of a *lexAop*·*PYlACT1-lacZ* reporter. Comparison of the expression levels of the reporter in cells expressing Fts2-LexA and LexA alone indicates that Fts2 caused 4.2-fold repression in wild-type cells (Fig. 3C). YlSsn6 caused 3.4-fold repression, comparable to that of Fts2. The repression by Fts2 was significantly reduced in Yltup1Δ and Ylssn6Δ cells (Fig. 3C), indicating that both YlTup1 and YlSsn6 are required for the repression by Fts2. This feature is very similar to that of CaNrg1 (25, 27, 28), a transcriptional repressor in *C. albicans*. Together, our results suggest that Fts2 represses gene expression via Tup1-Ssn6.

**Identification of Fts2-regulated genes by transcriptome sequencing.** To understand how Fts2 represses filamentation, we wanted to identify the whole set of genes regulated by Fts2. To this end, we conducted RNA-Seq-based transcriptome sequencing.
in wild-type and \textit{fts2\Delta} cells grown in liquid YNBD medium. To determine whether the Fts2-regulated genes may also be controlled by Tup1-Ssn6, \textit{Yl\textit{tup1}\Delta} and \textit{Yl\textit{ssn6}\Delta} cells were also included in the analysis. In the RNA-Seq datasets, 6397 protein-coding genes (99.2\%) in the genome (6448 protein-coding genes) were detected to be expressed in both wild-type and \textit{fts2}\Delta cells (Table S1, full data). Among these genes, a total of 888 genes displayed significant differential expression (\(\geq 2\)-fold, \(P < 0.05\)) in \textit{fts2}\Delta cells. Of these, 743 genes (83.7\%) were upregulated whereas just 145 genes (16.3\%) were downregulated (Fig. 4A and Table S1, upregulated and downregulated genes). A total of 202 genes displayed highly differential expression (\(\geq 5\)-fold, \(P < 0.05\)) in \textit{fts2}\Delta cells. Of these, 177 genes (87.6\%) were highly upregulated, whereas only 25 genes (12.4\%) were highly downregulated. In both cases, the number of upregulated genes greatly exceeds that of downregulated genes, suggesting that Fts2 functions mainly as a repressor in the control of gene expression.

The upregulated genes in \textit{fts2}\Delta cells mainly encode nutrient transporters, cell wall proteins and enzymes involved in cell wall organization and biogenesis, metabolic enzymes, transcription factors, as well as proteins involved in cell cycle, signal transduction, cell polarity, and stress response (Fig. 4B and Table S2). In contrast, the downregulated genes mainly encode metabolic enzymes and nutrient transporters. A significant portion of the upregulated genes (41.5\%) and the downregulated genes (32.5\%) do not have a known cellular function. Some have homologs in \textit{S. cerevisiae}, \textit{C. albicans}, or other fungi but without known cellular functions. Many more do not have homologs in these species.

\textbf{Fts2 represses a large number of cell wall protein genes, including YIPHR1 and a set of adhesin-like genes.} During the yeast-to-filament transition, the cell wall undergoes dramatic reorganization to support filamentous growth and modulate adherence
properties (38). This process involves the upregulation and downregulation of some cell wall proteins. In the RNA-Seq data sets, we observed that a large number of cell wall protein genes were upregulated in \textit{fts2}\textsuperscript{D} cells. A total of 68 genes were significantly upregulated (\( \geq 2\)-fold, \( P < 0.05 \)), whereas just 8 genes were significantly downregulated. Of these, 23 genes were highly upregulated (\( \geq 5\)-fold, \( P < 0.05 \)), whereas only 1 gene was highly downregulated (Table 1). To evaluate the accuracy of the RNA-Seq data, 9 highly upregulated genes were examined for their mRNA levels by qRT-PCR. Consistent with the RNA-Seq data (Fig. 5A), all 9 genes were markedly upregulated at the transcription level in \textit{fts2}\textsuperscript{D} cells (Fig. 5B). We tagged the 3 highly upregulated adhesin-like genes \textit{YALI0C11165} (U1), \textit{YALI0B18194} (U30), and \textit{YALI0C23452} (U107) at the 3\textsuperscript{9}-terminus of ORF with GFP to allow the detection of these proteins in live cells (the short C-terminus containing the GPI modification site was deleted from each protein to avoid the removal of tagged GFP during GPI modification). \textit{fts2}\textsuperscript{D} cells carrying the 3 GFP-tagged genes all exhibited brighter GFP fluorescence than wild-type cells carrying the same construct (Fig. 5C), indicating that all 3 adhesin-like genes were upregulated at the protein level as well in \textit{fts2}\textsuperscript{D} cells.

Among the 68 upregulated cell wall protein genes, 9 genes encoding proteins similar to cell surface adhesins (\textit{YALI0C11165}, \textit{YALI0B18194}, and \textit{YALI0C23452}), \textit{S. cerevisiae} cell wall structural proteins Tir1/Tir3 (\textit{YALI0E22088} and \textit{YALI0F07535}) and Cwp1 (\textit{YALI0E11517}, \textit{YALI0E22286}, and \textit{YALI0E31108}), and cell surface glycosidase (\textit{YILPHR1}) were not only highly upregulated (\( \geq 5\)-fold), but also exhibited very high RNA-Seq read counts (>9500) in \textit{fts2}\textsuperscript{D} cells (Table 1 and Table S1). Hence, these genes appear to be the major targets of Fts2 repression. Of these, \textit{YALI0C11165} was upregulated by 338-fold, the highest among all genes. \textit{YALI0E22088} and \textit{YALI0F07535} were upregulated by 88.7-fold and 29.4-fold, respectively. \textit{YILPHR1} was upregulated by 12.8-fold. Among these genes, only \textit{YILPHR1} is known to be required for filamentation (9). The roles of other major target genes in filamentation are not clear. Some of them might play roles in filamentation (see Discussion).

Cell surface adhesins represented by the \textit{S. cerevisiae} flocculin Flo11 and the \textit{C. albicans} adhesins Hyr1 and Hwp1 are GPI-anchored cell surface glycoproteins that direct cell-cell adhesion or cell-surface adhesion (39, 40). \textit{FLO11}, \textit{HYR1}, and \textit{HWP1} are filament-specific genes that are upregulated concomitant with the yeast-to-filament transition (41–43). We observed that a total of 18 adhesin-like genes were upregulated in \textit{fts2}\textsuperscript{D} cells (Table 1, FIG 4 Fts2-regulated genes identified by transcriptome sequencing. (A) The heat map of the differentially expressed genes in \textit{fts2}\textsuperscript{D} cells compared with wild-type cells. (B) The numbers of upregulated and downregulated genes in functional categories. “Unknown cellular function” refers to genes that encode proteins similar to function-unknown proteins. *, in comparison with proteins in \textit{S. cerevisiae}, \textit{C. albicans}, or other fungi.
In contrast, just 1 transcription factor gene was downregulated among these genes. Table S1, but also exhibited very high RNA-Seq read count in genes. Hence, highest among transcription factor genes and very high among all Fts2-repressed genes.

### Table 1: The upregulated and downregulated cell wall protein genes in fts2Δ cells identified by RNA-Seq

| Direction of regulation | Tup1-regulated only | Ssn6-regulated only | Tup1-Ssn6-coregulated | Non-Tup1-Ssn6-regulated |
|-------------------------|---------------------|---------------------|------------------------|-------------------------|
| Up                      | E22286 (U61)        | C11165 (U11)        | C15004 (U2) b, E22088 (U6) b, C18194 (U30) b, YWP1 (E11517, U41) b, PHR1 (D04851, U55) b, C24342 (U107) c, E31108 (U108) c, A08800 (U150) b, E20229 (U159), E22440 (U61) b, A15378 (U3) b, D07535 (U22) c, D09185 (U69) c | F15653 (U26), C08423 (U33), B05654 (U110), A09196 (U111), D26257 (U162) |
| Low (≥2-fold, <5-fold)  | C17875 (U218), D17248 (U545), DFGS (F18722, U704) c, C13970 (U210) c, D18381 (U439) c, E08008 (U467) c, E22440 (U493) c, E25784 (U632) c | F10549 (U306), C10923 (U349), E33363 (U418), D06996 (U483), CHS2 (B16324, U251), CHS1 (D03179, U767), B00132 (U620), F21428 (U712) | | |
| Down                    | E18766 (D12)        | E03938 (D58), C20779 (D135), A20438 (D33), D01331 (D31) | D22957 (D73) b, E28336 (D40) |

For simplicity, "YAL10" in the systematic name of each gene was omitted as “E22286” stands for “YAL10E22286”. Numbers in parentheses indicate each gene’s ranking in the full list of RNA-Seq data set. Genes encoding putative cell surface adhesins that share similarities to the known adhesins such as S. cerevisiae Fio1 and C. albicans Hye1 or Hwp1 are underlined. Genes that displayed significant differential expression (≥2-fold, P < 0.05) in YlTup1Δ or YlSsn6Δ cells compared with wild-type cells are defined as YlTup1-regulated or YlSsn6-regulated. Genes that displayed highly differential expression (≥5-fold, P < 0.05) in YlTup1Δ or YlSsn6Δ cells are in bold.

“Genes that are highly differentially expressed in YlSsn6Δ cells.”

GFS2 represses a large number of transcription factor genes, some of which promote filamentation. transcription factors play important roles in the control of gene expression. Our RNA-Seq analysis revealed that 41 transcription factor genes were significantly upregulated (≥2-fold, P < 0.05) in fts2Δ cells compared with wild-type cells. Six of these transcription factor genes were highly upregulated (≥5-fold, P < 0.05) (Table 2). In contrast, just 1 transcription factor gene was downregulated in fts2Δ cells.

Among the 41 Fts2-repressed transcription factor genes, most of them are not known to promote filamentation except. MHY1 and HOY1. MHY1 is distinctive among these genes. MHY1 is not only highly repressed by Fts2 (Fig. 6A and B, and Table S1), but also exhibited very high RNA-Seq read count in fts2Δ cells, which is the highest among transcription factor genes and very high among all Fts2-repressed genes. Hence, MHY1 is a major target of Fts2 repression. We observed that Fts2 was present at the promoter of MHY1 by chromatin immunoprecipitation (Fig. 6C), suggesting that Fts2 functions at the promoter of MHY1 to repress its transcription. MHY1 overexpression caused strong filamentation in wild-type cells (Fig. 6D), whereas fts2Δ mhylΔ cells did not form filaments (Fig. 6E). Hence, MHY1 is a major target of Fts2 in the repression of filamentation.
To examine whether the remaining transcription factor genes in the top 8 list may also regulate filamentation, we overexpressed 5 of them, except YALI0C09482 and YAL0E34925, which exhibited the lowest RNA-Seq read counts (~80). Interestingly, overexpression of the Rme1-like transcription factor gene YALI0E14971, the 2 Zn(II)$_2$Cys$_6$ zinc cluster transcription factor genes YALI0D10681 and YALI0D14872, and the bHLH
transcription factor gene YALI0B13354 all increased filamentation in wild-type cells (Fig. 6D), whereas overexpression of the GATA zinc finger transcription factor gene Y1Brg1 (YALI0E131757) did not affect filamentation (data not shown). Remarkably, the deletion of YALI0B13354 caused a drastic reduction of filamentation in wild-type cells, as well as in fts2Δ cells (Fig. 6E). This finding indicates that the 4 transcription factor genes YALI0E14971, YALI0D10681, YALI0D14872, and YALI0B13354 promote filamentation. The repression of these genes by Fts2 may contribute to the repression of filamentation.

**Fts2 may repress the bulk of its target genes via Tup1-Ssn6.** To determine whether the Fts2-regulated genes may also be controlled by Tup1-Ssn6, we examined the transcript levels of Fts2-regulated genes in the RNA-Seq data sets of Yltup1Δ and Ylsn6Δ cells (Table S3 and Table S4). The results showed that Fts2 shares many target genes with Tup1-Ssn6 (Fig. 7). There were 1122 and 804 genes upregulated in Yltup1Δ and Ylsn6Δ cells, respectively. Among the 743 genes that were upregulated in fts2Δ cells, 380 genes (51.1%) and 447 genes (60.2%) were also upregulated in Yltup1Δ and Ylsn6Δ cells, respectively, whereas 314 genes (42.3%) were upregulated in both Yltup1Δ and Ylsn6Δ cells. Collectively, 513 genes were upregulated in either Yltup1Δ or Ylsn6Δ cells, indicating that 69.0% of Fts2-repressed genes were also repressed by Tup1-Ssn6. Moreover, among the 68 cell wall protein genes and 41 transcription factor genes repressed by Fts2, 66.2% (45 genes) and 90.2% (37 genes) of them were also repressed by Tup1-Ssn6, respectively (Table 1 and Table 2). In total, about two-thirds of Fts2-repressed genes are also repressed by Tup1-Ssn6. Hence, Fts2 may repress the bulk of its target genes via Tup1-Ssn6.

There were 145 genes downregulated in fts2Δ cells. Of these, 100 genes (69.0%) were also downregulated in either Yltup1Δ or Ylsn6Δ cells (Fig. 7), indicating that the bulk of Fts2-activated genes are also activated by Yltup1 and/or Ylsn6.

**Fts2 expression is downregulated at alkaline pH and the relief of negative control by Fts2 facilitates the induction of filamentation by alkaline pH.** As a repressor that prevents the yeast-to-filament transition, the transcriptional repression imposed by Fts2 is expected to be relieved during the yeast-to-filament transition. Thus, it is

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**TABLE 2** The upregulated and downregulated transcription factor genes in fts2Δ cells identified by RNA-Seq

| Direction of regulation | Tup1-regulated only | Ssn6-regulated only | Tup1-Ssn6-coregulated | Non-Tup1-Ssn6-regulated |
|-------------------------|---------------------|---------------------|------------------------|-------------------------|
| Up                      |                     |                     |                        |                         |
| High (≥5-fold)          | 6 genes             |                     |                        |                         |
| Low (≥2-fold, <5-fold)  | 35 genes            |                     |                        |                         |
|                        | C10010 (U289),      | C10010 (U289),      | E14971 (U37)           |                         |
|                        | C03564 (U366),      | C03564 (U366),      | (E31757, U127)         |                         |
|                        | YAP1 (F03388, U637),| YAP1 (F03388, U637),| (E34925, U515)         |                         |
|                        | PPR1 (B09713, U677),| PPR1 (B09713, U677),| (D14872, U190)         |                         |
|                        | C13178 (U706),      | C13178 (U706),      | (A16841, U203)         |                         |
|                        | B11902 (U530)       | B11902 (U530)       | (A17200, U537) YALI0E22286 |                         |
| Down                   |                     |                     |                        |                         |
| High (≥5-fold)          | 0 gene              |                     |                        |                         |
| Low (≥2-fold, <5-fold)  | 1 gene              |                     |                        |                         |
|                        |                     | E17215 (D99)        |                        |                         |

*For simplicity, “YALI0” in the systematic name of each gene was omitted as “E22286” stands for “YALI0E22286”. Numbers in parentheses indicate each gene’s ranking in the full list of RNA-Seq data set. Genes that displayed significant differential expression (≥2-fold, P < 0.05) in Yltup1Δ or Ylsn6Δ cells compared with wild-type cells are defined as Yltup1-regulated or Ylsn6-regulated. Genes that displayed highly differential expression (≥5-fold, P < 0.05) in Yltup1Δ or Ylsn6Δ cells are in bold.

a Genes that are highly differentially expressed in Yltup1Δ cells.

b Genes that are highly differentially expressed in Ylsn6Δ cells.

c The regulation on this gene by Yltup1 is opposite to the regulation by Fts2.

d The regulation on this gene by Ylsn6 is opposite to the regulation by Fts2.
Fts2 represses a large number of transcription factor genes, some of which promote filamentation. (A) The RNA-Seq read counts of the top eight Fts2-repressed transcription factor genes in wild-type (WT), fts2Δ, Yltup1Δ, and Ylssn6Δ strains. (B) Validation of the RNA-Seq results by qRT-PCR. The transcript levels of YALI0E14971, YALI0D10681, YALI0D14872, and YALI0B13354 in wild-type (WT) and fts2Δ strains grown in YNB medium were determined by qRT-PCR and normalized to YlACT1. Statistically significant differences are indicated by the asterisks (***, P < 0.001). (C) Fts2 is present at the MHY1 promoter detected by chromatin immunoprecipitation (ChIP) assay. Cells of the wild-type strain (No tag) and strain YLX524 (FTS2-3FLAG) carrying pRNA44S vector were grown in YNBG (pH 4.0) medium at 30°C. Cells were collected at OD600 of ~ 0.6 for ChIP.

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likely that the expression level of Fts2 might be downregulated during the induction of filamentation. To test this possibility, we examined the mRNA level and protein level of Fts2 during alkaline pH-induced filamentation. To facilitate the detection of Fts2 protein, we utilized YLX523, a wild-type strain with FTS2 tagged C-terminally by 3 copies of HA (FTS2-HA) at the chromosomal locus. Cells of this strain grown at acidic pH (pH 4.0) in YNBG medium were in the oval-shaped yeast form. After being transferred to fresh YNBG medium buffered at weakly alkaline pH (pH 7.5), cells became elongated and started to filament after 4.5 h at 30°C (Fig. 8A, top row, and Fig. 8B, top chart). In contrast, cells were still in the yeast form during the same period after being transferred to fresh YNBG (pH 4.0) medium (Fig. 8A, bottom row). We observed that the mRNA level of FTS2 decreased rapidly in the first 2 h after cells were transferred to pH 7.5 and remained at low level after 2 h, as detected by qRT-PCR. The steady level of FTS2 mRNA at pH 7.5 was ~2.5-fold lower than that at pH 4.0 (Fig. 8B, bottom chart), indicating that FTS2 transcription was downregulated in response to alkaline pH.

We also examined the protein level of Fts2-HA in the cells by immunoblotting with an anti-HA antibody (Fig. 8C). Unlike the mRNA level of FTS2, the protein level of Fts2 did not decrease in the first 2 h after cells were transferred to pH 7.5. However, it decreased rapidly after 6.5 h and remained at low level after 8.5 h (Fig. 8B, bottom chart, and Fig. 8C). The steady level of Fts2 protein at pH 7.5 was ~4-fold lower than that at pH 4.0, indicating that the protein level of Fts2 was also downregulated at pH 7.5, although it occurred later than the downregulation of FTS2 mRNA. Together, our results demonstrate that Fts2 expression is downregulated in response to alkaline pH.

To investigate whether the downregulation of Fts2 expression at alkaline pH might be involved in the induction of filamentation by alkaline pH, we asked whether an excess of Fts2 in the cells may block filamentation. To this end, we overexpressed Fts2-FLAG on a plasmid under the control of the constitutive YITDH1 (GAPDH) promoter in strain YLX523. This Fts2-overexpressing strain was used to repeat the alkaline-induced filamentation assay. Strain YLX523 (WT) was used as a control. We observed that alkaline-induced filamentation still occurred in cells of this strain after being transferred to YNBG medium buffered at pH 7.5. However, the onset of filamentation was delayed by about 2 h (Fig. 8D and E). Moreover, the strength of filamentation was significantly decreased, as the percentage of cells longer than 20 μm were much lower than that of the control cells (Fig. 8E). Immunoblotting revealed that the protein level of Fts2 (indicated by Fts2-FLAG) in this strain did not decrease after 10.5 h of growth at pH 7.5.
Fts2 expression is downregulated at alkaline pH and the relief of negative control by Fts2 facilitates the induction of filamentation by alkaline pH. (A) to (C) Cells of strain YLX523 (FTS2-3HA) carrying the pINA445 vector were grown in liquid YNBG (pH 4.0) medium to OD$_{600}$ of ~ 0.6. Cells (Continued on next page)
DISCUSSION

The repressors of filamentation play an important role in keeping the cells in the yeast form when filamentation is not necessary. In this study, we identify Fts2, a novel repressor of filamentation in *Y. lipolytica*. We provide evidence that Fts2 is a transcriptional repressor and represses gene expression mostly via Tup1-Ssn6. We also show that Fts2 represses a large number of cell wall protein genes and transcription factor genes, some of which promote filamentation. Lastly, we show that Fts2 expression is downregulated in response to alkaline pH (a strong inducer of filamentation), and the relief from the negative control by Fts2 facilitates the induction of filamentation by alkaline pH. Our results provide new insights into the understanding of these transcriptional repressors and their regulation on filamentation.

We propose that Fts2 is a transcriptional repressor in the cells based on 2 observations: First, a Fts2-LexA fusion protein exhibited transcriptional repressor activity on a reporter gene. Second, transcriptomic analysis indicates that more genes are upregulated than downregulated (743 genes versus 145 genes) in *fts2Δ* cells. How does Fts2 repress gene expression? We propose that Fts2 represses gene expression mainly via the Tup1-Ssn6 corepressor. Three lines of evidence support this idea. First, Fts2-LexA fusion represses a reporter gene in a Tup1-Ssn6-dependent manner, which is similar to *C. albicans* CaNrg1 (25–28). Second, Fts2 interacts with YlSsn6 in co-IP assay. Third, transcriptomic analysis indicates that about two-thirds of Fts2-repressed genes are also repressed by Tup1-Ssn6. Transcriptomic analysis also suggests that Fts2 may repress about one-third of target genes in a Tup1-Ssn6-independent mechanism. Fts2 is the first transcription factor in *Y. lipolytica* shown to be able to repress gene expression via Tup1-Ssn6.

How does Fts2 repress filamentation? Using transcriptomic analysis, we identified the whole set of genes that are controlled by Fts2. From these, we characterized 9 cell wall protein genes and 1 transcription factor gene, *MHY1*, as the major targets of Fts2 repression. These genes were highly upregulated and exhibited very high transcript levels in *fts2Δ* cells. Of these, 7 genes except the 3 genes encoding proteins similar to *S. cerevisiae* Cwp1 or Tlr1/Tlr3 (YALI0F07535, YALI0E11517, and YALI0E31108) were also upregulated during alkaline-induced filamentation. Six genes including the 3 adhesin-like genes (YALI0C11165, YALI0B18194, and YALI0C23452), YALI0E22286 (CWP1-like gene), YIPHR1, and *MHY1* were highly upregulated (≥5-fold) (9). *MHY1* is a key regulator that promotes filamentation (15, 16). *YIPHR1* is required for filamentation at alkaline pH (9). Although cells deleted for the adhesin-like gene YALI0C23452 did not exhibit any defect in filamentation (data not shown), we observed that YALI0C23452 increased the cell length when overexpressed together with YALI0F19030 (9), another adhesin-like gene repressed by Fts2. The CWP1-like gene YALI0E22286 is upregulated by 564-fold whereas the protein level of Fts2-HA in the control strain did (Fig. 8F). These results indicate that an excess of Fts2 significantly impacts alkaline-induced filamentation, suggesting that the relief of transcriptional repression by Fts2 facilitates the induction of filamentation.

**FIG 8** Legend (Continued)
by alkaline pH, which is very high. YALI0E22286 also increased the cell length when overexpressed together with YALI0D09185 (9), another adhesin-like gene repressed by Fts2. The repression of these major targets by Fts2 might be responsible for the repression of filamentation.

In addition to the 10 major Fts2 target genes, some other Fts2-repressed cell wall protein genes and transcription factor genes might also play roles in filamentation. There are a set of 12 genes encoding proteins similar to S. cerevisiae Yps3, a plasma membrane-anchored aspartic protease involved in cell wall organization (44). YMSB2 (YALI0D00627) encodes a protein highly similar to S. cerevisiae Msb2, a mucin that promotes filamentation (45). The 4 transcription factor genes (YALI0E14971, YALI0D10681, YALI0D14872, and YALI0B13354) promote filamentation (Fig. 6D and E). Cell polarity proteins play important roles in filamentous growth. We observed that genes encoding cell polarity proteins such as the S. cerevisiae Rho3-like GTPases Yal0B13662 and Yal0F17270, the polarisome components YIAip5 (Yal0B21626) and YIMsb3 (Yal0B22792), and the axial budding landmark component YIAx2 (Yal0D05379) were upregulated in fts2Δ cells. The repression of these genes by Fts2 may collectively contribute to the repression of filamentation.

In addition to increased filamentation, fts2Δ cells also displayed increased resistance to peroxide, suggesting that Fts2 may also regulate the oxidative stress response. The reactive oxygen species (ROS) has been shown to promote fungal differentiation including filamentation (46–48). In C. albicans, more ROS were found to be generated in hyphal cells than in yeast-form cells (47). In S. cerevisiae, cells lacking Yno1, a NADPH oxidase-like enzyme involved in the production of ROS, displayed reduced invasive growth (48). In the RNA-Seq data sets, we observed that the genes YALI0C20251 and YALI0B06413 that encode oxidoreductases were highly upregulated whereas YICTT1 (YALI0E34749), which encodes the cytosolic catalase involved in the breakdown of hydrogen peroxide, was downregulated in fts2Δ cells. These genes and other Fts2-regulated genes might be involved in the resistance to peroxide.

Prior to this study, the only known transcription factors that repress filamentation in Y. lipolytica are the TEA/ATTS transcription factor YITec1 and the Zn(II)2Cys6 zinc cluster transcription factor Znc1 (Yal0B080538) (30, 31). The identification of Fts2 suggests that multiple repressors of filamentation exist in Y. lipolytica. Hence, the repression of filamentation might be rather complex. Fts2, Znc1, and YITec1 harbor different types of DNA-binding domains. They are expected to bind to different DNA sequences, and thus may regulate different target genes. We observed that fts2Δ cells exhibited increased resistance to peroxide. Moreover, a large number of genes involved in nutrient transport and metabolism were upregulated and downregulated in fts2Δ cells. It was also reported that 161 genes and 247 genes were upregulated and downregulated, respectively, in znc1Δ cells during exponential growth. Most of these genes are involved in metabolism or encode proteins with catalytic, transporter, or transcription factor activity (30). Therefore, Fts2, Znc1, and YITec1 may also have other cellular functions in addition to the repression of filamentation.

Because fts2Δ, znc1Δ, and Yltec1Δ mutants all exhibited increased filamentation, Fts2, Znc1, and YITec1 may share some common target genes. A total of 161 genes were reported to be upregulated (≥2-fold) in znc1Δ cells (30). Of these genes, we observed that 57 genes were also upregulated in fts2Δ cells, and 22 genes were also upregulated in both fts2Δ and Yltec1Δ cells (our unpublished results). This observation indicates that Fts2, Znc1, and YITec1 indeed share a subset of target genes. Remarkably, among the 13 genes highly upregulated (≥5-fold) in znc1Δ cells are 3 adhesin-like genes, YALI0C11165, YALI0C08473, and YALI0C23452. All 3 genes were also highly upregulated in fts2Δ cells, and YALI0C23452 was also upregulated, whereas YALI0C11165 was highly upregulated in Yltec1Δ cells (our unpublished results). These results indicate that the adhesin-like genes YALI0C11165 and YALI0C23452 are shared targets of Fts2, Znc1, and YITec1.

YALI0C11165 encodes a putative GPI-anchored protein with many SSTGGADA and NGNGSGDSGS repeats. The Ser/Thr and Asn residues in these repeats are putative O-
glycosylation and N-glycosylation sites, respectively. Yali0C11165 and its close homolog Yali0B18194, which is also highly repressed by Fts2 (Table 1 and Fig. 5C), resemble C. albicans Hry1, a cell-surface adhesin. Its encoding gene, HYR1, is a filament-specific gene repressed by CaNrg1 and CaTup1 (24), two major repressors of filamentation. YALI0C23452 encodes another putative GPI-anchored protein with many PESSEA, which is a key filament-specific gene in S. cerevisiae (42, 49). Since YALI0C11165, YALI0B18194, and YALI0C23452 are also highly upregulated during alkaline-induced filamentation (9), and the 3 encoded proteins (fused to GFP) were highly expressed in fts2Δ cells, we propose that YALI0C11165, YALI0B18194, and YALI0C23452 are filament-specific genes in Y. lipolytica.

MATERIALS AND METHODS

Strains and media. Y. lipolytica strains used in this study are listed in Table S5 in the supplemental material. PO1a (MATa leu2-270 ura3-302) was used as the wild-type strain. Culture media include yeast extract-peptone-dextrose (YPD) medium (20 g/L peptone, 10 g/L yeast extract, 2% glucose), yeast nitrogen base-dextrose (YNBD) medium (6.7 g/L yeast nitrogen base without amino acid, 1% glucose), or yeast nitrogen base-glycerol (YNBG) medium (6.7 g/L yeast nitrogen base without amino acid, 1% glycerol) supplemented with 80 mg/L of leucine, 20 mg/L of uracil, or both when required. YNBG medium was buffered to pH 4.0 or 7.5 with Na2HPO4-citric acid buffer after autoclave. The strains used in this study are listed in Table S5 in the supplemental material. PO1a (MATa leu2-270 ura3-302) was used as the wild-type strain. Culture media include yeast extract-peptone-dextrose (YPD) medium (20 g/L peptone, 10 g/L yeast extract, 2% glucose), yeast nitrogen base-dextrose (YNBD) medium (6.7 g/L yeast nitrogen base without amino acid, 1% glucose), or yeast nitrogen base-glycerol (YNBG) medium (6.7 g/L yeast nitrogen base without amino acid, 1% glycerol) supplemented with 80 mg/L of leucine, 20 mg/L of uracil, or both when required. YNBG medium was buffered to pH 4.0 or 7.5 with Na2HPO4-citric acid buffer after autoclave. The strains used in this study are listed in Table S5 in the supplemental material.

Plasmid construction. The plasmids used in this study are listed in Table S6. The oligonucleotides are listed in Table S7. To generate pINA445-FTS2, FTS2 carrying the 3684-bp promoter and 571-bp 3′-UTR was amplified by PCR and inserted into HindIII-digested vector pINA445 (CEN, LEU2) using CloneExpress II One Step Cloning Kit (Vazyme Biotech Co.). To examine the localization of Fts2 in the cells, FTS2 carrying the 3684-bp promoter was amplified by PCR and inserted into BamHI-digested vector pYL14 (pINA445 backbone, YLEU2, GFP-YαM) (31), yielding pYL14-FTS2 that expresses Fts2-GFP. The FTS2-GFP construct in pYL14-FTS2 is functional since it restored the filamentation of fts2Δ cells back to the wild-type level. Similarly, to visualize the adhesin-like proteins in the cells, YALI0C111655′-1′000 carrying 3794-bp promoter, YALI0B181941′000 carrying 2233-bp promoter, and YALI0C23452′-9′00 carrying 2674-bp promoter were amplified by PCR and inserted into BamHI-digested pYL14, yielding pYL14-YALI0C111655′, pYL14-YALI0B181941′, and pYL14-YALI0C23452′, respectively. To overexpress the transcription factor genes including MHY1, the ORF of each gene plus the 500-bp 3′-UTR was amplified by PCR and inserted into pYL13 (CEN, LEU2, PαM) (31), yielding pYL13-Gene.

To detect the interaction between Fts2 and YLS56 in vivo, YISSN6-1′HA carrying 2000-bp YISSN6 promoter was generated by PCR amplification of Y. lipolytica genomic DNA using primer pairs 445-Ssn6-F and 445-Ssn6-HA-R. Then, it was inserted into HindIII-digested pINA445 using CloneExpress II One Step Cloning Kit, yielding pINA445-YISSN6-HA. To generate pYL26-FTS2-3FLAG for the overexpression of Fts2-FLAG, 2 steps were employed. First, the 696-bp YITDH1 glycolaldehyde-3-phosphate dehydrogenase (GAPDH) promoter (nucleotides -696 to -1 relative to the first nucleotide in the start codon) was amplified by PCR and inserted into NcoI/XbaI-digested pYL13 (31), yielding pYL26. Then, the FTS2 ORF was tagged by 3′-FLAG by overlapping PCR and inserted into Sall-digested vector pYL26.

To generate pINA443-PαM-lexA for one-hybrid assay in Y. lipolytica, the 254-bp YITEF1 promoter (nucleotides -257 to -4 relative to the first nucleotide in the start codon) was amplified by PCR from genomic DNA and fused to the DNA-binding domain (aa. 1 to 87) of the E. coli lexA gene by overlapping PCR. PαM-lexA was then inserted into pINA443, yielding pINA443-PαM-lexA. Similarly, PαM-YISSN6-lexA and PαM-FTS2-lexA were generated and inserted into pINA443, yielding pINA443-PαM-YISSN6-lexA and pINA443-PαM-FTS2-lexA, respectively. To generate pINA445-lexAop-PαM-αM containing the lacZ gene under the control of 4 copies of the lexA operator and the YIACT1 promoter, 575-bp YIACT1 promoter (nucleotides -578 to -4 relative to the first nucleotide in the start codon) was amplified by PCR and inserted into Sall/BamH1-digested pINA445-lexAop-YLEU2-lacZ (31). YLEU2 mini promoter was replaced by YIACT1 promoter.

Yeast strain construction. Genes including FTS2, YALI0B13354, and MHY1 were deleted in Y. lipolytica strains by homologous recombination. Briefly, the ~1.0-kb sequence upstream of the gene ORF (Pgene) and the ~1.0-kb sequence downstream of the gene ORF (Tgene) were amplified by PCR from genomic DNA, and then inserted into the flanking sites of lexA-YIURA3-loxP in pYL8 (31). The resulting pαM-yiura3-loxP-YIURA3-loxP-Tgene deletion cassette was used to transform yeast cells. The transformational cassettes were examined by PCR to identify the ones bearing the correct gene deletion. The YIURA3 marker was later removed by Cre-mediated DNA recombination between loxr and loxP sites. To tag the chromosomal copy of FTS2 with 3HA tag, FTS2-3HA was generated by overlapping PCR. Then, FTS2-3HA-loxr-YIURA3-loxP-TαM was constructed and used to tag the chromosomal copy of FTS2 C-terminally with 3 copies of HA tag by homologous recombination, yielding the yeast strain YLY523 (FTS2-3HA-loxr/R), Strain YLY524 (FTS2-3FLAG-loxr/R)
was constructed similarly. The FTS2-HA and FTS2-FLAG fusion constructs are functional since the yeast strains displayed phenotypes similar to that of the wild-type strain in filamentation.

**Identification of FTS2 in a mutant screen.** To look for the mutants that displayed increased filamentation, cells of the wild-type strain were transformed with the zeta-based mutagenesis cassette (MTC), zeta-URA3, as reported previously (50). The transformants were grown on YNB agar. Random insertion of MTC into the chromosome created mutations. The transformants that formed fluffy or wrinkled colonies were examined for their capacity to filament in liquid medium. Those that displayed increased filamentation were analyzed by TAIL-PCR to determine the insertion site of URA3 on the chromosome. Two insertional mutants of yali0E23518 (fts2) were, thus, isolated.

**Detection of Fts2-HA and Fts2-FLAG by immunoblotting.** For the detection of the protein levels of Fts2-HA and Fts2-FLAG in the cells during alkaline-induced filamentation, cell lysates were prepared by the NaOH/TCA method. Briefly, yeast cells were collected and resuspended in 1 mL sterile water. A total of 150 μL 1.85 N NaOH and 7.4% β-mercaptoethanol was added and incubated on ice for 15 min. Then, 64 μL of 100% TCA (trichloroacetic acid) was added and incubated on ice for 15 min. Cell lysates were centrifuged at high speed. The pellets were washed by 1.5 mL acetone and suspended in 2× SDS sample buffer. Standard immunoblotting procedures were used. Primary antibodies used were mouse monoclonal antibodies against HA and against GFP (Covance Research Products), and against FLAG and against GAPDH (Proteintechn, Rosemont), Horseradish peroxidase-conjugated goat anti-mouse IgG (Bioxy Corporation) was used as the secondary antibody.

**Co-immunoprecipitation.** Cells were collected and lysed in immunoprecipitation (IP) buffer (25 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 5% glycerol, 1% NP-40, pH 7.4) containing protease inhibitor cocktail (Bimake Company) for 30 min on ice. The anti-FLAG affinity beads (Smart-Lifesience) were added into the lysates and incubated overnight at 4°C with rotation. After washing three times with IP buffer, the beads were resuspended into 2× SDS sample buffer, boiled, and centrifuged. Samples were analyzed by immunoblotting with the indicated antibodies.

**β-Galactosidase assay.** The β-galactosidase activity in the cells was determined by the crude cell extract assay with o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate, as reported previously (31). Crude cell extracts were prepared by vortexing in the presence of glass beads. Protein concentration in the cell extracts was measured by the Bradford method. The specific β-galactosidase activity was normalized by the amount of total protein in each extract and was calculated according to the following formula: U = (OD420 × 1.7)/0.0045 × protein concentration (mg mL⁻¹) × sample volume (mL) × time (min). The assays were performed in triplicate.

**RNA-Seq analysis.** RNA-Seq was conducted at Berry Genomics Corporation (Beijing, China) as described previously (9). Differential expression analysis between 2 conditions was performed using the DESeq R package (1.20.0). Differentially expressed genes were defined as those whose expression level was changed by >1.5-fold with a false discovery rate (FDR) of ≤0.05 and the fold change ≥ 2.0.

**RNA extraction and quantitative real-time PCR analysis.** Yeast cells were grown to OD₆₀₀ of ~0.6 in 50 mL cultures. Cells were collected by centrifugation at 3000 rpm for 10 min and washed twice with 10 mL sterile water. Cells were resuspended in 0.4 mL RNA isolater (Vazyme Biotech Co.), snap-frozen in liquid nitrogen, and stored at −80°C freezer. Total RNAs were extracted using RNA isolater, and purified via chloroform extraction method. The purity and yield of RNAs were examined by measuring the A₂₆₀/A₂₈₀ ratio using NanoDrop One Spectrometer (Thermo Scientific). RNA integrity was examined by agarose gel electrophoresis. One microgram RNA from each sample was subjected to reverse transcription using the HScr ipt II Q RT SuperMix for qPCR (Vazyme Biotech Co.) according to the manufacturer’s protocol. Quantitative PCR (qPCR) experiments were carried out as the manufacturer’s protocol suggested. The Cq values of all amplification curves were less than 28 and the melt curves of each primer set only contain a single peak. Three technical replicates data were analyzed using Bio-Rad CFX Manager software (version 3.1) with normalized mode (ΔΔCq). The unpaired two-tailed Student’s T-test was used to examine the statistical significance of difference in two samples.

**Chromatin immunoprecipitation.** Yeast cells were cross-linked with 1% formaldehyde for 15 min at room temperature when OD₆₀₀ reached about 0.6 to 0.8, and were then quenched with glycine for 5 min. Cells were collected, washed twice, and lysed in chromatin immunoprecipitation (ChIP) lysis buffer I (50 mM HEPES-KOH, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, pH 7.5) containing protease inhibitor cocktail. After beads beating, chromatin was sonicated for 10 cycles (high output, 10 sec on, 10 sec off) using the Bioruptor (Diagenode) to produce fragments of ~300-bp length. Cell lysates were centrifuged at 16,000 × g for 15 min at 4°C to remove the debris. Immunoprecipitation was performed overnight at 4°C with anti-FLAG affinity beads. Beads were washed once with ChIP lysis buffer I for 10 min, once with ChIP lysis buffer II (50 mM HEPES-KOH, 1 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, pH 7.5) for 20 min, and once with LiCl/NP40 buffer (10 mM Tris-HCl, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, pH 8.0) for 10 min. Elution was performed with 2 consecutive 10 min incubations with elution buffer (100 mM NaHCO₃, 1% SDS) at room temperature. After reverse cross-linking and proteinase K digestion, DNA was column-purified using the Universal DNA purification kit (TIANGEN).

**Data availability.** RNA-Seq data can be found in the supplemental material.
TABLE S2, PDF file, 0.5 MB.

TABLE S3, XLSX file, 1.3 MB.

TABLE S4, XLSX file, 1.2 MB.

TABLE S5, PDF file, 0.7 MB.

TABLE S6, PDF file, 0.5 MB.

TABLE S7, PDF file, 0.6 MB.

ACKNOWLEDGMENTS
We thank Drs. Claude Gaillardin, Jean-Marie Beckerich, Jean-Marc Nicaud, and Richard Rachubinski for kindly providing yeast strains and plasmids. This work was supported by the National Natural Science Foundation of China (grant numbers 31870062 and 32070072 to X. G.).

The authors have no conflicts of interest.

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