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Two distinct domains of Flo8 activator mediates its role in transcriptional activation and the physical interaction with Mss11

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ABSTRACT

Flo8 is a transcriptional activator essential for the inducible expression of a set of target genes such as *STA1*, *FLO11*, and *FLO1* encoding an extracellular glucoamylase and two cell surface proteins, respectively. However, the molecular mechanism of Flo8-mediated transcriptional activation remains largely elusive. By generating serial deletion constructs, we revealed here that a novel transcriptional activation domain on its extreme C-terminal region plays a crucial role in activating transcription. On the other hand, the N-terminal LisH motif of Flo8 appears to be required for its physical interaction with another transcriptional activator, Mss11, for their cooperative transcriptional regulation of the shared targets. Additionally, GST pull-down experiments uncovered that Flo8 and Mss11 can directly form either a heterodimer or a homodimer capable of binding to DNA, and we also showed that this formed complex of two activators interacts functionally and physically with the Swi/Snf complex. Collectively, our findings provide valuable clues for understanding the molecular mechanism of Flo8-mediated transcriptional control of multiple targets.

1. Introduction

The acute responses of yeast against the fast-changing environmental ques such as glucoamylase production, haploid invasion, and pseudohyphal differentiation ensure its survival in harsh conditions and are mediated by many transcriptional activators such as Flo8, Mss11, Ste12, and Tec1 [1–9]. As one of crucial target genes associated with these acute responses, the *FLO11* gene encodes a member of cell-surface flocculin involved in the cell–cell adhesion, pseudohyphal growth, and haploid invasion [1,10]. Notably, the promoter of *FLO11* is almost identical to that of another target gene, *STA1* encoding glucoamylase, except for two inserts, 20 and 64 bp, and indeed the expressions of two genes are co-regulated to a large extent in response to various signals [1]. Further studies proposed that the *FLO11* promoter did not contain the functional Filamentation and invasion response element (FRE), a composite DNA sequence for cooperative binding of Ste12 and Tec1, whereas the *STA1* promoter did contain the FRE in UAS2-1 [4,9]. This finding suggests that there are some differences in the regulation of *FLO11* and *STA1* expression even though upstream regions of them are very similar.

Flo8 was originally identified as a transcriptional activator of the *FLO1* gene, which encodes a cell adhesion molecule [11]. Flo8 also plays a critical role in *STA1* and *FLO11* expression. Interestingly, the *FLO8* gene is mutated in most laboratory strains and this causes a defect in *STA1* expression [3,12]. Another transcriptional activator Mss11 was isolated as a multicopy suppressor of *STA10*, a negative regulator of *STA1* expression [8], and two independent domains of Mss11 for its transcriptional activation have been previously characterized [13]. Mutation of *FLO8* and *MSS11* shows very similar phenotypes to each other, suggesting that their function is closely related. Consistently, they indeed co-immunoprecipitated and bound cooperatively to UAS1-2 of the *STA1* promoter [4,9].

In addition to Mss11, possible involvement of another factor in Flo8-mediated transcriptional activation has been also proposed. The Swi/Snf complex promoting nucleosome remodeling is also implicated in *STA1* activation [14,15], and it has been proposed that this complex activates *STA1* expression by promoting Flo8 and Mss11 binding to the *STA1* promoter [4,9]. Furthermore, the fact that the Swi/Snf complex is also required for *FLO1* transcription suggests that this complex is functionally related to Flo8 [16]. This complex interacts with a specific activation domain of several

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transcriptional activators, such as Gcn4, VP16, and Hap4 [17–19]. However, still large proportion of molecular details on the Flo8-mediated transcriptional activation such as information about the domains of Flo8 required for its proper functioning and its binding to interacting partners remains unclear. Therefore, we characterized the specific domains of Flo8 involved in the transcriptional activation and also in the interaction with Mss11 and suggest a possible model explaining the mode of Flo8 action through its novel activation domain and by interacting with Mss11 activator and Swi/Snf complex.

2. Materials and methods

2.1. Strains and plasmids

Yeast strains and plasmids used in this study are listed in Table 1. Yeast cells were grown at 30C in YPD (1% yeast extract, 2% peptone, and 2% glucose) or a synthetic medium containing 0.67% yeast nitrogen base supplemented with appropriate amino acids and carbon sources.

To construct *FLO11p-lacZ* and *FLO1p-lacZ* plasmids, the upstream regions of *FLO11* (2.5 kb) and *FLO1* (1.0 kb) were amplified and inserted into *Xho*I site of pLG-670z. The different truncations of LexA fused Flo8 protein were constructed by replacement of the *MSS11* gene with the different fragments of *FLO8* gene of plasmid pLexA-*MSS11*.

2.2. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously [4,9]. The precipitated DNAs were amplified by 40 cycles of PCR to detect the upstream region of the *FLO1* promoter by using the following primer pairs: 5′-CTTCCTCTGCTCATTAATTGC-3′ and 5′-TCAACTGT GCCGTTAATGCTG-3′.

2.3. Haploid invasion and flocculation ability test

To determine the ability of yeast cells to grow invasively into the agar, cells were grown on YPD plate for 3 days and washed off the surface of the agar plate. Plates were photographed both before and after the washing process. To test flocculation ability, cells were grown in YPD broth to OD_{600} = 1.0 and flocculation was measured as the decrease in OD_{600} of the culture.

2.4. Glucoamylase assay and β-galactosidase assay

Glucoamylase and β-galactosidase assay was performed as described previously [20].

2.5. GST-pull down assay

Total extracts prepared from the integrated *FLO8-HA*, *MSS11-HA*, and *SNF6-HA* strains were incubated for 3 h on ice with 2 μg of purified glutathione S-transferase (GST)-fused proteins. Glutathione–agarose beads (30 μl) were added and incubated for 1.5 h at 4 °C with constant agitation. The beads were pelleted and washed four times. Proteins in the pellet were eluted by boiling the beads in sample buffer and were analyzed by Western blotting with α-HA antibodies. To test direct interaction between Flo8 and Mss11, total extracts of *Escherichia coli* containing His6-Flo8 or His6-Mss11 were used.

Table 1
Yeast strains and plasmids used in this study.

Strains	Genotype	References
KHS 182	*Mata STA1 leu2 his3 trp1 ura3*	Kim et al. (2003) [3]
KHS 182-1	*Mata STA1 leu2 his3 trp1 ura3 flo8Δ::TRP1*	Kim et al. (2003) [3]
KHS 182-2	*Mata STA1 leu2 his3 trp1 ura3 mss11Δ::TRP1*	Kim et al. (2003) [3]
KHS 182-4	*Mata STA1 leu2 his3 trp1 ura3 ste12Δ::TRP1*	Kim et al. (2003) [3]
KHS 182-5	*Mata STA1 leu2 his3 trp1 ura3 tec1Δ::HIS3*	Kim et al. (2003) [3]
KHS 182-10-1	*Mata STA1 leu2 his3 trp1 ura3 snf6Δ::TRP1*	Kim et al. (2004a) [4]
KHS 182-30	*Mata STA1 leu2 his3 trp1 ura3 FLO8-HA::LEU2*	Kim et al. (2004b) [9]
KHS 182-30-1	*Mata STA1 leu2 his3 trp1 ura3 FLO8-HA::LEU2 mss11Δ::TRP1*	Kim et al. (2004a) [4]
KHS 182-30-2	*Mata STA1 leu2 his3 trp1 ura3 FLO8-HA::LEU2 ste12Δ::TRP1*	Kim et al. (2004a) [4]
KHS 182-31	*Mata STA1 leu2 his3 trp1 ura3 MSS11-HA::LEU2*	Kim et al. (2004b) [9]
KHS 182-31-1	*Mata STA1 leu2 his3 trp1 ura3 MSS11-HA::LEU2 flo8Δ::TRP1*	Kim et al. (2004a) [4]
KHS 182-34	*Mata STA1 leu2 his3 trp1 ura3 SNF6-HA::LEU2*	Kim et al. (2004a) [4]
KHS 182-34-3	*Mata STA1 leu2 his3 trp1 ura3 SNF6-HA::LEU2 ste12Δ::TRP1*	Kim et al. (2004a) [4]
Plasmids	Description	References
pLG-FLO11-lacZ	−2500 to −1 region of the *FLO11* promoter in pLG-670z	This study
pLG-FLO1-lacZ	−933 to −1 region of the *FLO1* promoter in pLG-670z	This study
pLexA-Flo8-1	Replacement of *MIG1* with *FLO8* ORF of pLexA-MIG1	Kim et al. (2004a) [4]
pLexA-Flo8-2	1–400 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Flo8-3	401–799 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Flo8-4	401–600 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Flo8-5	601–799 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Flo8-6	601–700 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Flo8-7	701–799 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Flo8-8	701–750 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Flo8-9	751–799 amino acids of Flo8 in pLexA-MIG1	This study
pLexA-Mss11	Replacement of *MIG1* with *MSS11* ORF of pLexA-MIG1	Kim et al. (2004a) [4]
pSH18-34	2u *URA3* 8*lexA*op-*GAL1*$_{TATA}$-*lacZ*	Estojak et al. (1995) [21]
pGEX-FLO8	*FLO8* ORF in pGEX4T-1	Kim et al. (2004a) [4]
pGEX-F1	1–400 amino acids of Flo8 in pGEX4T-1	This study
pGEX-F2	401–799 amino acids of Flo8 in pGEX4T-1	This study
pGEX-F3	1–105 amino acids of Flo8 in pGEX4T-1	This study
pGEX-F4	106–700 amino acids of Flo8 in pGEX4T-1	This study
pGEX-F5	701–799 amino acids of Flo8 in pGEX4T-1	This study
pET-FLO8	*FLO8* ORF in pET30a	This study
pET-MSS11	*MSS11* ORF in pET30a	This study
pGEX-MSS11	*MSS11* ORF in pGEX4T-1	Kim et al. (2004a) [4]

3. Results

3.1. The extreme C-terminal domain of Flo8 directly activates transcription

Flo8 activates transcription of multiple target genes including *STA1* and *FLO11* and functionally interacts with Mss11 [3,9,11]. Although Mss11 is known to have two independent activation domains, the H2 domain and the extreme C-terminus [13] (Fig. 1A), the regions of Flo8 responsible for its activation function has not yet been reported. To identify the specific activation domain(s) of Flo8 protein, we used LexA fusion proteins and pSH18-34 containing the *lacZ* reporter under the control of eight *lexA* operators and the minimal TATA region [21,22]. We first tested two constructs containing amino acids 1–400 and 401–799 of Flo8 protein. The N-terminal region did not activate *lacZ* expression, whereas the C-terminal region did. Furthermore, activation by the latter was more effective than by full-length Flo8 (Fig. 1B and C). Next, we constructed a series of LexA fusions with smaller regions of Flo8. The data in Fig. 1B and C establish that full activation by Flo8 requires C-terminal amino acids 700–799, though the C-terminal half of this region (amino acids 751–799) retains about one quarter of full activity (Fig. 1C). These results clearly show that the extreme C-terminal region of Flo8 mediates transcriptional activation.

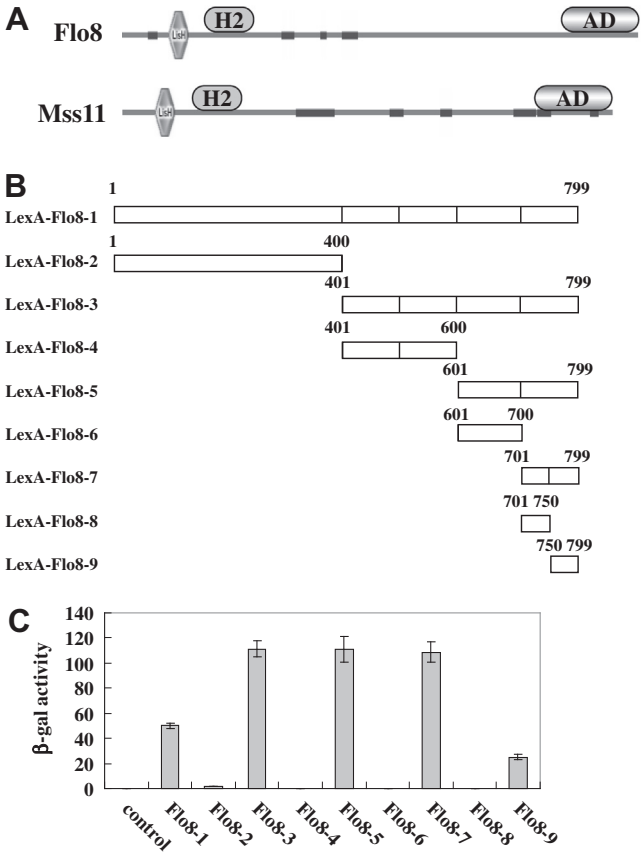

Fig. 1. The extreme C-terminus of Flo8 activates transcription. (A) Schematic representation of the domain architecture of Flo8 and Mss11 as defined by the SMART program. H2 indicates the homologous domains of Flo8 and Mss11, and AD indicates the activation domains of them. (B) Schematic diagram representing the different regions of Flo8 fused to the LexA DNA binding domain. (C) Each construct was transformed together with pSH18-34 into wild type cells, and three independent colonies were grown in 2% glucose medium and tested for β-galactosidase activity.

3.2. Flo8 interacts with Mss11 via the LisH motif

Flo8 also has a distinct domain on its N-terminal region referred to as a Lissencephaly type-1-like (LisH) motif (amino acids 72–105) (Fig. 1A). Although the role of LisH motif is not well understood yet, it was suggested that the LisH motif might be involved in a protein–protein interaction [23,24]. Interestingly, Mss11 also has this motif on its N-terminus (amino acids 50–83) (Fig. 1A). Evidently these motifs of Flo8 and Mss11 are not involved in their activation function.

Next, given the reported physical interaction between Flo8 and Mss11, we performed GST pull-down experiments to test whether the LisH motif of these two proteins mediates the interaction between them. Full-length Flo8 or sub-regions were fused to the 3′ end of the glutathione S-transferase gene and were expressed in *E. coli* (Fig. 2A). Purified GST fusion proteins were mixed with the total proteins extracted from the integrated *MSS11*-HA tagged yeast strain. As shown in Fig. 2B, Mss11-HA precipitated with GST-Flo8 and GST-F1 containing the LisH motif but not GST alone or GST-F2, which does not contain the LisH motif. Furthermore, the full-length Flo8 and the extreme N-terminus carrying the LisH motif (amino acids 1–105, F3) only interact with Mss11-HA (Fig. 2C). These results indicate that the LisH motif of Flo8 mediates the interaction with Mss11 consistent with a previous result showing that Mss11 lacking the LisH motif failed to interact with Flo8 in *Candida albicans* [25].

3.3. Flo8 and Mss11 can form a heterodimer or homodimers

Flo8 and Mss11 bind cooperatively to UAS1-2 of the *STA1* promoter and mutation of either gene prevents the other from binding [9]. On the other hand, multicopy plasmids bearing *FLO8* or *MSS11* suppress *mss11Δ* and *flo8Δ* mutations, respectively [4], suggesting a possibility that both proteins can form a homodimer which is capable of binding to DNA in the absence of its partner. Thus, we wondered whether Flo8 and Mss11 directly interact and each protein forms a homodimer. To address this, we carried out GST pull-down assays with proteins expressed in *E. coli*, and as shown in Fig. 2D, found that His6-Flo8 interacted with both GST-Flo8 and GST-Mss11 but not with GST alone. Furthermore, His6-Mss11 was also bound by GST-Flo8 and GST-Mss11. These results indicate that the interactions are direct and they can form both heterodimer and homodimer.

3.4. Flo8 and Mss11, but not Ste12 and Tec1 activate FLO11 and FLO1 expression

Although multiple transcriptional activators, Flo8, Mss11, Ste12, and Tec1 are known to be associated with the activation of *STA1* and *FLO11*, our previous study suggested that some differences exist in the patterns of their functioning [1–7,11,13]. Moreover, their exact functions in the regulation of another target *FLO1* [11] also remain unknown. Thus, to dissect the exact functions of these activators on their shared targets, we measured *lacZ* reporter expression from the *FLO11* and *FLO1* promoter in cells lacking these activators. Wild type cells showed normal derepression of *FLO11* and *FLO1* promoters in a medium containing 0.05% glucose. However, *flo8Δ* and *mss11Δ* caused a complete repression of these genes in the same condition. Although *lacZ* expression from the *FLO11* promoter was slightly reduced in *tec1Δ* mutant, both wild type and *ste12Δ* mutant showed similar level of *lacZ* expression. Furthermore, the activity of *FLO1* promoter was not affected by either *ste12Δ* or *tec1Δ* (Fig. 3A).

According to our previous study, Flo8 and Mss11 cooperatively bind to UAS1-2 of the *STA1* promoter and the corresponding region of the *FLO11* promoter [9]. Sequence analysis of the *FLO1* promoter

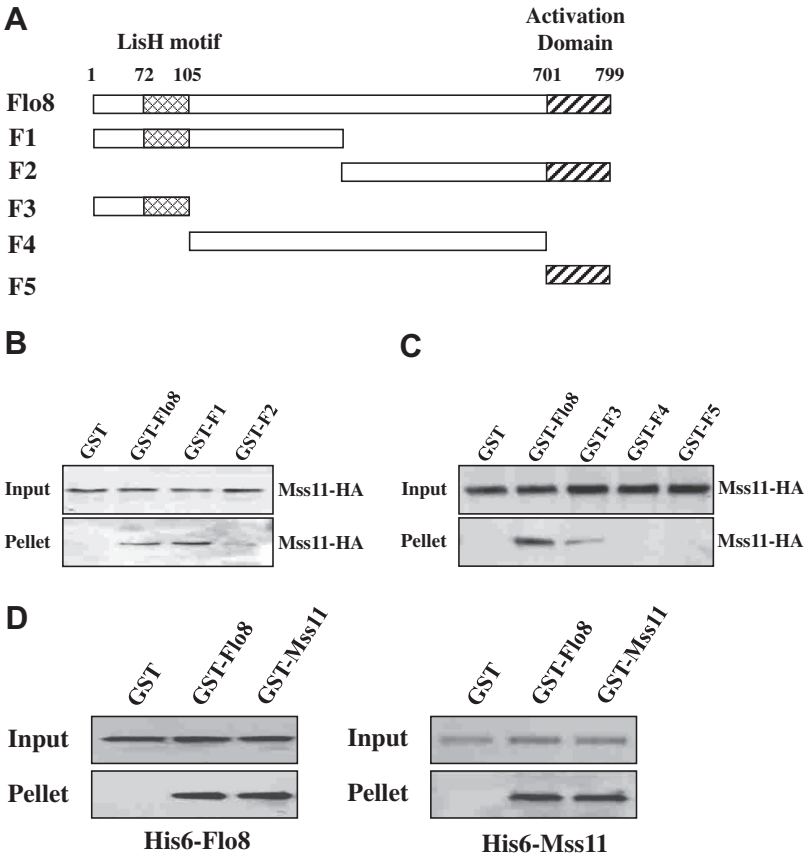

Fig. 2. Flo8 interacts with Mss11 via the LisH motif. (A) Schematic diagram of the segments of Flo8 fused to glutathione S-transferase gene. (B and C) 2 μg of the purified GST fusion proteins were mixed with 1 mg of total extract of a yeast strain harboring integrated *MSS11*-HA, and the GST fusions and their interacting proteins were collected with glutathione–agarose beads and pelleted. Total (1/10) and pellet (1/2) fractions were analyzed by Western blotting with specific antibodies. (D) Flo8 and Mss11 form homodimers and heterodimers. His6-Flo8 and His6-Mss11 were expressed in *E. coli* and mixed with purified GST, GST-Flo8, and GST-Mss11. The GST fusions and their interacting proteins were precipitated with glutathione–agarose beads. Total (1/30) and pellet (1/3) fractions were analyzed by Western blotting with anti-His6 antibody.

shows that the inverted repeat sequence, TTTGC-n-GCAAA, a putative binding site of Flo8 and Mss11 in the *STA1* promoter is replaced with TTTGG-n-GCAAA in the *FLO1* promoter. To see if Flo8 directly binds to the *FLO1* promoter, we performed chromatin immunoprecipitation assay. As previously reported [9], Flo8 strongly bound to the *STA1* promoter in wild type cells, but not in both *mss11Δ* and *ste12Δ* mutants (Fig. 3B). Even though Flo8 binds to the *FLO1* promoter to a lesser extent, this binding was still observed in *ste12Δ* mutants but not in *mss11Δ* cells (Fig. 3B).

Consistent with *lacZ* reporter assay, wild type, *ste12Δ*, and *tec1Δ* cells invaded the agar surface (Fig. 3C) and they also exhibited flocculation phenotypes (Fig. 3D); *ste12Δ* and *tec1Δ* cells show more increased flocculation phenotypes. However, both invasive growth and flocculation were completely eliminated in both *flo8Δ* and *mss11Δ* mutants (Fig. 3C and D). All these results indicate that Flo8-Mss11 heterodimer, but not Ste12 and Tec1, directly activate *FLO11* and *FLO1* transcription in our strain background.

3.5. Flo8-Mss11 heterodimer interacts physically and functionally with the Swi/Snf complex

We showed previously that the Swi/Snf complex was required for binding of each individual protein of Flo8 and Mss11 to UAS1-2 of the *STA1* promoter [4,9]. Given our current finding of heterodimer formation between Flo8 and Mss11 (Fig. 2D), we then investigated the interrelationship between Flo8-Mss11 heterodimer and the Swi/Snf complex. According to the previous work, the *STA1* gene was activated by introduction of multicopy plasmids bearing Flo8 and Mss11 into *mss11Δ* and *flo8Δ* mutant, respectively [9]. However, this was not observed in *snf6Δ* mutant, even though this mutation did not affect the levels of Flo8 and Mss11 (Fig. 4A). Next, GST pull-down assays were carried out to examine whether Flo8 and Mss11 interact physically with the Swi/Snf complex. As shown in Fig. 4B, Snf6-HA, a component of the Swi/Snf complex, precipitates with GST-Flo8 and GST-Mss11 but not with GST alone. We then examined the recruitment of the Swi/Snf complex to the *FLO1* promoter and found that Snf6 directly bound to both *STA1* and *FLO1* promoter (Fig. 4C). Interestingly, although the binding to the *STA1* promoter is known to require Ste12 [9], *ste12Δ* mutation did not affect the interaction between Snf6 and the *FLO1* promoter (Fig. 4C). This finding indicates that physical interaction between Flo8-Mss11 heterodimer and the Swi/Snf complex is critical for activation of *STA1*, *FLO11*, and *FLO1* expression.

4. Discussion

We show here that Flo8 has two separated domains required for transcriptional activation and interaction with Mss11. Mss11 has two independent activation domains, the H2 and the extreme C-terminus, and the H2 domain is also conserved in Flo8 [13]. Therefore it was previously suggested that activation by DNA-bound Flo8 might require this domain. However, we uncovered in the current study that only the extreme C-terminal region of Flo8 activated transcription. Interestingly, despite of strong transcriptional activation activity of amino acids 700–799 of Flo8, we

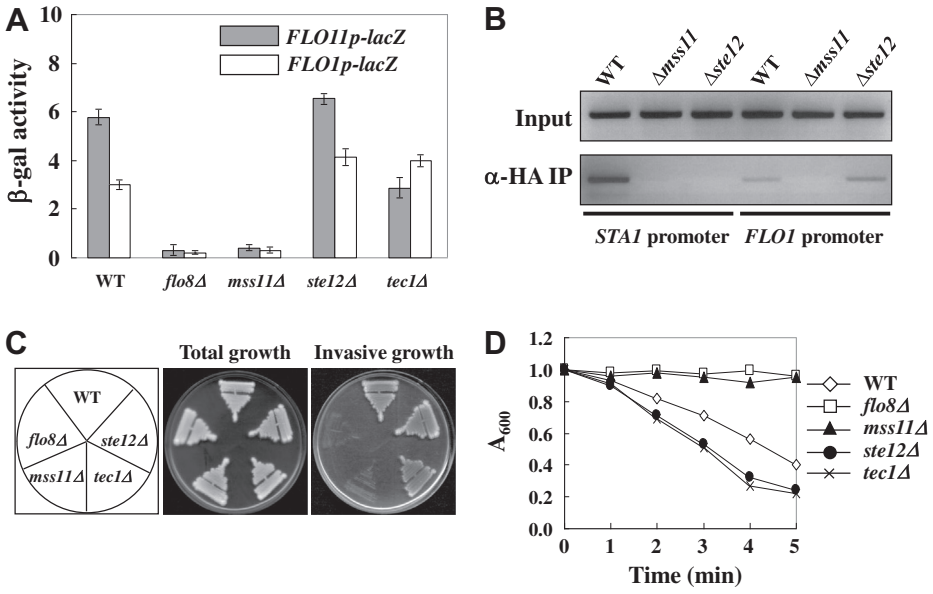

Fig. 3. Flo8 and Mss11 activate *FLO11* and *FLO1* expression. (A) *FLO11p-lacZ* and *FLO1p-lacZ* plasmids were transformed into wild type and each mutant, and the resulting transformants were grown in 2% glucose and shifted to 0.05% glucose for 4 h. Three independent transformants of each type were examined for β-galactosidase activity. (B) *FLO8*-HA wild-type, *FLO8*-HA *mss11Δ*, *FLO8*-HA *ste12Δ*, and untagged strains were grown in 2% glucose medium and shifted to 0.05% glucose for 4 h and treated with formaldehyde to cross-link DNA and proteins. Anti-HA chromatin IP was performed using 1 mg of total extracts and the upstream regions of *STA1* and *FLO1* genes containing the inverted repeat sequence were amplified using the purified DNA to detect binding of Flo8 to this region. (C) Wild type and each mutant were patched on YPD plates, incubated for 2 days and photographed before (total growth) and after (invasive growth) noninvasive cells were washed off from the agar surface. (D) Cells were grown in YPD broth to OD_{600} = 1.0, and flocculation rate was measured as the reduction in optical density.

did not find any homology with activation domains in other transcriptional activators, suggesting that the C-terminal region of Flo8 may be a novel transcription-activating domain. In addition, we also demonstrated that the LisH motif of Flo8 is important for interaction with Mss11 (Fig. 2). Notably, it has been reported that Lis1 and Nudf, both of which contain LisH motifs, function as a homodimer and that interaction between monomeric Lis1 and Nudf occurs via their N-terminal coiled-coil domains similar to our findings [23,24,26]. RanBPM and Twa1 (two hybrid-associated with RanBPM protein No. 1) also contain LisH motifs and are reported to form a protein complex [27]. Our finding that the LisH of Flo8 is required for interaction with Mss11 suggests a new possibility that the LisH motifs of RanBPM and Twa1 might promote heterodimerization [27].

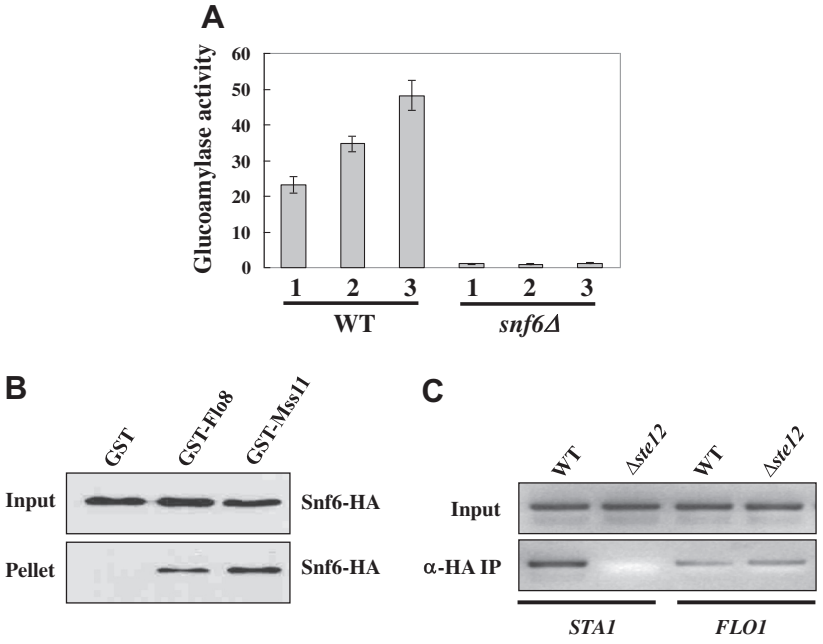

Fig. 4. Flo8-Mss11 heterodimer interacts functionally and physically with the Swi/Snf complex. (A) pRS326 (1), pRS326-*FLO8* (2), and pRS326-*MSS11* (3) were transformed into wild type and *snf6Δ* mutant. Three independent colonies were grown in 2% glycerol/ethanol media and tested for glucoamylase activity. (B) Yeast total extracts were prepared from integrated *SNF6*-HA tagged strain. Purified GST fusion proteins were mixed with 500 μg of yeast extract and the GST fusions and their interacting proteins were collected with glutathione–agarose beads. Fractions of total (1/10) and pellet (1/2) were analyzed by Western blot analysis. (C) *SNF6*-HA wild-type, *SNF6*-HA *ste12Δ*, and untagged strains were grown in 2% glucose medium and shifted to 0.05% glucose for 4 h and treated with formaldehyde to cross-link DNA and proteins. Anti-HA chromatin IP was performed using 1 mg of total extracts and the upstream regions of *STA1* and *FLO1* genes were amplified using the purified DNA.

The physical interaction between Flo8 and Mss11 appears to be critical for the function of these activators, but it is still unknown how this interaction is regulated. We have previously shown that Flo8 expression is induced in the absence of glucose [9], and this could be one of mechanism that controls the formation of Flo8-Mss11 heterodimer. Alternatively, it is also possible that posttranslational modifications of these proteins directly affect interaction between Flo8 and Mss11. Flo8 is known to be phosphorylated by Tpk2, a catalytic subunit of cyclic AMP-protein kinase A pathway and this phosphorylation promotes its binding to DNA [28]. Since Flo8 interaction with Mss11 is important for its DNA binding activity, it might be interesting to see if phosphorylation of Flo8 by Tpk2 affects its interaction with Mss11. Interestingly, Mss11 also contains at least seven phosphorylated sites (http://ptmfunc.com/) but the roles of these modifications on its transcriptional activation and interaction with Flo8 have not been yet uncovered. Unveiling how the interaction between Flo8 and Mss11 is regulated will help us fully understand the complicated feature of the transcriptional activation by Flo8 and Mss11 heterodimer.

Although we mapped two important domains of Flo8 required for activation of target genes, its DNA binding domain still needs to be characterized. Flo8 seems to have two potential DNA binding motif, basic region leucine zipper (BRLZ) (amino acids 36–79) and high mobility group 17 (HMG17) (amino acids 434–510). It will be interesting to test whether these two domains of Flo8 directly bind to DNA. Identifying DNA binding domain of Flo8 will provide a more detailed molecular mechanism of Flo8-dependent activation of target genes.

Acknowledgments

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