Deciphering the Transcriptional-Regulatory Network of Flocculation in \textit{Schizosaccharomyces pombe}

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Abstract

In the fission yeast \textit{Schizosaccharomyces pombe}, the transcriptional-regulatory network that governs flocculation remains poorly understood. Here, we systematically screened an array of transcription factor deletion and overexpression strains for flocculation and performed microarray expression profiling and ChIP–chip analysis to identify the flocculin target genes. We identified five transcription factors that displayed novel roles in the activation or inhibition of flocculation (Rfl1, Adn2, Adn3, Sre2, and Yox1), in addition to the previously-known Mbx2, Cbf11, and Cbf12 regulators. Overexpression of \textit{mbx2}\(^{+}\) and deletion of \textit{rfl1}\(^{-}\) resulted in strong flocculation and transcriptional upregulation of \textit{gsf2}\(^{+}\)/\textit{pfl1}\(^{-}\) and several other putative flocculin genes (\textit{pfl2}\(^{-}\)--\textit{pfl9}\(^{-}\)). Overexpression of the \textit{pfl}\(^{+}\) genes singly was sufficient to trigger flocculation, and enhanced flocculation was observed in several combinations of double \textit{pfl}\(^{+}\) overexpression. Among the \textit{pfl}\(^{+}\) genes, only loss of \textit{gsf2}\(^{+}\) abrogated the flocculent phenotype of all the transcription factor mutants and prevented flocculation when cells were grown in inducing medium containing glycerol and ethanol as the carbon source, thereby indicating that Gsf2 is the dominant flocculin. In contrast, the mild flocculation of \textit{adn2}\(^{+}\) or \textit{adn3}\(^{+}\) overexpression was likely mediated by the transcriptional activation of cell wall–remodeling genes including \textit{gas2}\(^{+}\), \textit{psu1}\(^{+}\), and \textit{SPAC4H3.03c}. We also discovered that Mbx2 and Cbf12 displayed transcriptional autoregulation, and Rfl1 repressed \textit{gsf2}\(^{+}\) expression in an inhibitory feed-forward loop involving \textit{mbx2}\(^{+}\). These results reveal that flocculation in \textit{S. pombe} is regulated by a complex network of multiple transcription factors and target genes encoding flocculins and cell wall–remodeling enzymes. Moreover, comparisons between the flocculation transcriptional-regulatory networks of \textit{Saccharomyces cerevisiae} and \textit{S. pombe} indicate substantial rewiring of transcription factors and cis-regulatory sequences.

Introduction

Flocculation is an inherent characteristic of yeasts involving asexual aggregation of cells into flocs that separate rapidly from the medium (reviewed recently in [1,2]). Individual yeast cells transition into this morphological state as an adaptation to various environmental stresses by shielding the inner cells of the flocs [3]. The flocculent trait has also proven highly beneficial in industrial yeast applications by allowing efficient and cost-effective removal of cells [4]. The ability of yeast strains to flocculate is dependent on the expression of specific cell surface glycoproteins known as flocculins. Cell-to-cell adhesion occurs via binding between the flocculin and surface carbohydrates in a calcium-dependent manner [5]. The bound carbohydrates consist of various sugars including mannose, glucose, and galactose that are specific to the type of flocculin and yeast species [6–8]. There has been considerable interest in elucidating the genetic control of flocculation to better understand this phenomenon and generate biotechnological advances in yeast-based industries.

In \textit{Saccharomyces cerevisiae}, a transcriptional-regulatory network composed of interactions between transcription factors and their flocculin gene targets is central in controlling flocculation. The primary flocculins that function in flocculation are encoded by the \textit{FLO1}, \textit{FLO5}, \textit{FLO9}, and \textit{FLO10} genes [9–11]. Overexpression of the individual \textit{FLO} genes is sufficient to trigger flocculation [8,12]. However, the degree of flocculation by \textit{FLO} overexpression varies from \textit{FLO1} to \textit{FLO10} exhibiting the strongest to weakest flocculation, respectively. The flocculin \textit{FLO11} also exhibits weak flocculation when overexpressed [8], but its function is mainly in cell-to-surface adhesion [13], diploid pseudohyphal growth [14], and haploid invasive growth [15]. The transcription factors required for flocculation include Flo8p and Mss11p, which primarily activate \textit{FLO1} transcription [16]. The \textit{Sac. cerevisiae} laboratory strain \textit{S288C} containing a nonfunctional \textit{FLO8} gene is not able to flocculate, but flocculation is restored in this strain by the overexpression of \textit{FLO8} or \textit{MSS11} [16,17]. In addition, Sha1p has been shown to inhibit transcription of \textit{FLO1} in the \textit{W303-1A} strain and not in \textit{S288C}, likely through interactions with the Snf6p-Tup1p global repressor and components of Mediator [18,19].

The control of flocculation is much less known in \textit{Schizosaccharomyces pombe}. The ability of the heterothallic wild-type strains...
972 h− and 975 h+ to flocculate has not been observed presumably because the inducing environmental conditions have not been identified. Phenotypic analysis of constitutive flocculent mutant strains show that flocculation is dependent on the presence of calcium, but unlike Sac. cerevisiae, the flocculin-carbohydrate interactions involve galactose rather than mannose and glucose residues [7]. Moreover, the transcriptional-regulatory network governing flocculation in S. pombe remains poorly characterized. Only a single interaction between the Mbx2 MADS box transcription factor and the gsf2+ flocculin gene is currently known [20,21]. The gsf2+ gene was initially identified as highly upregulated in response to heterologous expression of FLO8 [20]. Overexpression of gsf2+ is sufficient to trigger flocculation while its deletion abrogates the flocculent phenotype of tup1/2A, lkb1/2, and gsf1 mutants. In addition, gsf2+ displays additional roles in cell-to-surface adhesion and invasive growth [20]. The induction of gsf2+ during flocculation and invasive growth is mediated by Mbx2 [21]. Two other transcription factors implicated in flocculation have been reported. The CSL transcription factors Cbf11 and Cbf12 play opposing roles in flocculation where mutant strains lacking cbf11+ or overexpressing cbf12+ flocculate [22]. The direct targets of these transcription factors functioning in flocculation have not been identified, but could be several putative flocculin genes that show protein sequence homology to other yeast-related proteins [23]. Indeed, these putative flocculin genes, as well as gsf2+ are transcriptionally upregulated in certain Mediator mutants that flocculate indicating that these genes are likely repressed by Mediator [24]. Similar to Sac. cerevisiae, the global transcriptional regulators Tup11 and Tup12 function in flocculation but their influence on the expression of these flocculin genes has not been addressed [25]. Importantly, it has not been directly demonstrated that these putative flocculin genes in S. pombe actually play a role in flocculation and the identity of the transcription factors that regulate them remains unknown.

In this study, we have initiated an extensive characterization of the transcriptional-regulatory network of S. pombe flocculation by identifying the relevant transcription factors and their flocculin gene targets. Importantly, we have also determined that heterothallic wild-type S. pombe is able to flocculate when grown in rich medium containing ethanol and glycerol as a carbon source. A screen of transcription factor deletion and overexpression strains for flocculent phenotypes revealed five novel transcriptional regulators of flocculation (Rfl1, Adn2, Adn3, Sre2, Yox1) in addition to our independent finding of Mbx2, Cbf11, and Cbf12. The strongest flocculation was observed upon overexpression of mbx2+ and deletion of gsf2+ (SPBC15D4.02) which encodes an uncharacterized fungal Zn2+–Cys6+ transcription factor. Microarray expression profiling of the mbx2OE and rfl1A strains revealed good overlap in the upregulation of several flocculin genes, while ChIP-chip analysis of HA-tagged Mbx2 and Rfl1 under control of the nmt41 promoter indicated that these transcription factors bound to some of the flocculin gene promoters. Nine flocculin gene targets (gsh2−/gsh3−) including gsf2+ were identified. The single overexpression of these genes triggered flocculation to varying degrees and cumulative effects on flocculation were observed in double overexpression experiments. Only loss of gsf2+ could abrogate the flocculent phenotype of all the transcription factor mutants indicating that gsf2+ encodes the dominant flocculin in S. pombe. Interestingly, we discovered that certain cell wall-remodeling enzymes can also function in flocculation, and some of these genes are likely regulated by the LisH transcription factors Adn2 and Adn3. In addition to the identification of target genes within the transcriptional-regulatory network, autoregulatory and inhibitory feed-forward loops involving several transcription factors were also detected. These results provide a significant insight into the transcriptional control of flocculation in S. pombe.

**Results**

**Screening for novel transcription factors functioning in fission yeast flocculation**

Our understanding of the transcriptional-regulatory network that governs flocculation in S. pombe remains limited. To further decipher this network, we sought to systematically identify transcription factors that play a role in flocculation. A list of 101 genes encoding sequence-specific transcription factors containing a bona-fide DNA-binding domain was assembled from [26] and GeneDB [27]. From this gene list, we constructed 101 nmt41-driven overexpression strains and 92 nonessential deletions in which the entire ORF was replaced with the KanMX6/NatMX6 cassette. A detailed description of the construction and phenotypic characterization of this transcription factor mutant collection will be described elsewhere (unpublished data). The transcription factor array of overexpression and deletion strains were screened for flocculation in EMM lacking thiamine and YES media, respectively. We recovered a total of eight transcription factors in which four overexpression strains (mbx2OE, adn2OE, adn3OE and cbf12OE) and four deletions (gsh1Δ, sre2Δ, yox1Δ and cbf11Δ) exhibited flocculation. These transcription factors represent positive and negative regulators of flocculation, respectively. Among these transcription factors, only the overexpression of cbf12+ and mbx2+ and deletion of cbf11+ have been reported to cause flocculation [20,22].

The strongest flocculation was observed in the mbx2OE and rfl1A strains. The flocs of the rfl1Δ strain in YES medium were larger and sedimented faster than the flocs produced in the mbx2OE strain after 48 hour induction (Figure 1A). The mbx2+ gene encodes a MADS-box transcription factor which was originally isolated in a screen for genes functioning in the biosynthesis of cell surface pyruvated galactose residues [28]. Recently, Mbx2 has been shown to function in flocculation and invasive growth by regulating the flocculin gene gsf2+ [20,21]. The
rfl1 is the repressor of flocculation gene that encodes an uncharacterized fungal Zn(2)-Cys(6) transcription factor.

The flocculation exhibited by these overexpression and deletion transcription factor mutants recovered from our screens could be abolished with the addition of galactose, but not mannose or glucose (data not shown). The amount of galactose required to completely deflocculate cells depended on the degree of flocculation. For example, the mbx2OE strain could be deflocculated with 2% galactose while the rfl1Δ strain required 5–10 times more galactose to completely deflocculate. Reflocculation of these strains was achieved in CaCl2 or in YES medium (data not shown).

The growth conditions that trigger flocculation in heterothallic wild-type S. pombe are not well known. To identify the inducing conditions, 972 h− and 975 h+ cells were tested on different carbon sources at different cell densities for flocculation. We determined that heterothallic wild-type cells were able to flocculate when cultured for five days at an initial concentration of 1 × 10^6 cells/ml in medium containing 1% yeast extract, 3% glycerol and 4% ethanol (referred to as flocculation-inducing medium, Figure 1B). The degree of flocculation was slightly enhanced in strains auxotrophic for leucine, uracil, and/or adenine indicating that nutrient status may also play a role in triggering flocculation (data not shown). However, these wild-type strains flocculated significantly less in flocculation-inducing medium than the mbx2OE and rfl1Δ mutants in EMM and YES media, respectively. The weaker flocculation in these strains was more easily observed in

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**Figure 1. Flocculation induction by rfl1 deletion, mbx2 overexpression, or wild type grown in flocculation-inducing medium.** (A) The flocculation of rfl1Δ mutant was visualized after culturing in YES medium for 24 hours at 30°C. The flocs produced in the nmt1-driven mbx2OE strain after 48 hour induction. Due to fast settling of flocs, the culture tubes were shaken vigorously immediately prior to image capture. (B) Heterothallic wild-type cells (972 h−) flocculate when cultured in flocculation-inducing medium (1% yeast extract, 3% glycerol and 4% ethanol). However, deletion of mbx2 or gsf2 abolishes flocculation. Cells were inoculated in inducing medium at 10^6 cells/ml and cultured for 5 days at 30°C, followed by petri dish assay (see materials and methods). (C) The rfl1Δ mutant exhibits enhanced adhesion to agar and invasive growth. Wild type (972 h−) and the rfl1Δ mutant were grown on LNB medium overlaid on YE+ALU medium without glucose for 10 days as per procedure outlined by Dodgson et al. [29]. Adhesion and invasive growth were determined by the amount of cells resistant to removal from the agar by gentle washing and more rigorous washing by rubbing cells off the agar with a finger under a stream of water, respectively.

doi:10.1371/journal.pgen.1003104.g001
petri-dishes incubated on an orbital rotator than in test tubes. In contrast to wild type, deletion of mbx2OE did not produce any visible flocs in the flocculation-inducing medium (Figure 1B).

Fungal genes that function in flocculation are usually associated with filamentous invasive growth [17,20]. We hypothesized that the rfl1Δ strain would exhibit hyperfilamentous invasive growth because of its strong flocculent phenotype. Indeed, the amount of cells resistant to removal from the agar by washing in the invasive assay on LNB medium with an underlayer of YE+ALU was much greater in the rfl1Δ strain than in wild type (Figure 1C). Under the microscope, the filamentous growth like those detected by Dodson et al. [29] was observed below the agar surface for both wild type and rfl1Δ strain with the latter showing much larger and more frequent formation of filamentous growth (data not shown). Similarly, adn2Δ and adn3Δ which were previously observed to have defects in invasive growth when deleted were recovered in our screens as flocculent when overexpressed [29].

**Mbx2 and Rfl1 are opposing transcription factors that regulate putative flocculin genes**

The strongest flocculation observed in the mbx2OE and rfl1Δ strains indicated that these two genes encode the major regulators of flocculation. Therefore, we initially focused on the characterization of these two transcription factors and proceeded to identify their target genes involved in flocculation. The nmt41-driven mbx2-HA strain was subjected to microarray expression profiling with a custom-designed *S. pombe* 8×15 K Agilent expression microarray (Table S2). The intermediate strength nmt41 promoter was sufficient for mbx2OE flocculation and was utilized in the microarray experiments in order to reduce possible secondary transcriptional effects compared to the strong nmt1 promoter. To better distinguish the direct target genes, ChIP-chip was also carried out concurrently on the same strain using the *S. pombe* 4×44 K Agilent Genome ChiP-on-chip microarray (Table S3). For the rfl1Δ expression profiling and ChiP-chip experiments, the flocculent deletion mutant and nmt41-driven rfl1-HA strain were used, respectively (Tables S4 and S5). The highly-induced putative target genes identified by microarray expression profiling of these transcription factor mutant strains were validated by qPCR (Table S13).

The list of genes that were induced at least two fold in the mbx2OE or rfl1Δ strain was subjected to gene ontology analysis using the Princeton GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder). These induced genes were highly enriched in cell wall components with p-values of 9.0e-9 and 6.3e-6 for the mbx2OE and rfl1Δ strains, respectively. Strikingly, the most-induced genes in the mbx2OE strain encoded cell surface glycoproteins. The cell surface glycoprotein genes up-regulated above two-fold were SPAC186.01, gfs2Δ, SPAC977.07c/SPBC1348.08c, SPCC138.09c/fas2Δ, SPBC947.04, SPBC359.04c, SPBC1289.15, SPAPB2C8.01, SPAPB18F9.04c, SPCC553.10, and SPBPJ1466.02, which all but gfs2Δ and the last 4 genes were predicted to be *pombe* adhesins based on BLAST sequence analysis (Figure 2A; [23]). SPAC977.07c and SPBC1348.08c are gene duplications with 100% sequence identity. To our knowledge, these genes with the exception of gfs2Δ have not been characterized further. The induction of these genes in the mbx2OE strain ranged from 2 to 112-fold relative to the empty vector control (Figure 2A, Table S13). In addition, several genes (gfs2Δ, pso1Δ, SPAGH43.03c and gas2Δ) encoding cell wall-remodeling enzymes such as glucan glucosidases and a beta glucanase transferase were induced up to 91-fold compared to the empty vector control when mbx2OE was overexpressed (Figure 2A). In the rfl1Δ expression data, a similar set of cell surface glycoprotein genes were upregulated at a comparable level as the mbx2OE expression data except for SPAC11F0.02, SPBC359.04c, SPAPB15E9.04c and SPBPJ1466.02 (Figure 2A, Table S13). In contrast to the mbx2OE strain, the same genes encoding the cell wall-remodeling enzymes were not highly upregulated in the rfl1Δ strain (Figure 2A).

Of the thirteen highly-induced cell surface glycoprotein genes in the mbx2OE expression data, nine of them were detected with ChIP-chip indicating that these genes are very likely the direct transcriptional targets of Mbx2 (Figure 2A). Four of the nine highly-induced cell surface glycoprotein genes in the rfl1Δ strain were detected with ChIP-chip confirming that these genes are probably direct transcriptional targets of Rfl1 (Figure 2A). For both Mb2x and Rfl1, gfs2Δ, fas2Δ and SPAPB2C8.01 were detected in the expression microarray and ChiP-chip experiments (Figure 2A).

Next, we sought further evidence that these cell surface glycoprotein genes were targets of Mbx2 and Rfl1 by epistasis studies. We decided to study a subset of these genes, which included the majority of the gene sequences analyzed by Linder and Gustafsson [23,24]. The mbx2Δ gene was overexpressed in single deletions of these putative target genes and their degree of flocculation was determined visually in petri-dishes, as well as quantitatively (Table S14). The putative glycoprotein gene SPAPB15E9.01c was included in these studies, because even though the transcript was downregulated in both mbx2OE and rfl1Δ strains, ChiP-chip analysis detected Mbx2 and Rfl1 association with its promoter (Figure 2A). Deletion of gfs2Δ decreased mbx2OE flocculation to the greatest extent while the reduction of flocculation was less extensive in the other single deletion mutants (Figure 2B, Table S14). The degree of reduction in mbx2OE flocculation roughly corresponded to the *pfl* numbers, which were assigned based on the degree of flocculation when overexpressed (see below). Moreover, mbx2OE flocculation was completely abrogated in the gfs2Δ pfl9A double mutant indicating that the reduction of mbx2OE flocculation in these mutants were additive in some cases (Figure 2B). Similar experiments were performed for fas2Δ in which flocculation was assayed in the same putative target deletions in the rfl1Δ background. The flocculation exhibited in the rfl1Δ strain was completely abolished by the deletion of gfs2Δ, but not by the deletion of pfl9A (Figure 2C).

To further analyze the expression microarray datasets of Mbx2 and Rfl1, the promoter regions of the differentially-expressed genes were subjected to the motif-finding algorithms RankMotif** and MEME to identify their binding specificities [30,31]. Mbx2 is a member of the MEF2-MADS box transcription factor family which has been shown to bind to the consensus sequence 5'- (C/T)TA(T/A)4TA(G/A)-3' [28,32,33]. The Mbx2 binding specificity obtained by RankMotif** closely resembled this known consensus sequence (Figure 2D). Similarly, RankMotif** generated an Rfl1 binding specificity that resembled known consensus sequences of several members of the fungal Zn(2)-Cys(6) transcription factor family (Figure 2E). The binding specificity of Zn(2)-Cys(6) DNA-binding domains is composed of conserved GC-rich trinucleotides spaced by a variable sequence region differing in length among members of the transcription factor family [34]. Analyses of the Mbx2 and Rfl1 expression microarray and ChiP-chip datasets by MEME did not generate any candidate DNA motifs.

Altogether, these results demonstrate that Mbx2 and Rfl1 are transcription factors responsible for regulation of flocculation in fission yeast by activating or repressing the transcription of candidate *S. pombe* flocculin genes, respectively.
Figure 2. Transcriptional regulation of putative flocculin genes by Mbx2 and Rfl1. (A) The heat map shows the induction of several cell surface glycoprotein genes in mbx2OE and rfi1Δ strains and detection of their promoter occupancy by Mbx2 and Rfl1 (middle panel). Several genes encoding cell wall-remodeling enzymes were also induced in the mbx2OE strain, but not in the rfi1Δ strain (lower panel). Microarray expression profiling was performed with dye reversal on an nmt41-driven mbx2-HA strain and rfi1Δ mutant, while ChIP-chip analysis was carried out on nmt41-driven mbx2-HA and rfi1-HA strains. The color bars reflect relative expression and ChIP enrichment ratios between experimental and control strains. Light grey in the ChIP-chip clustergram indicates detection is below the threshold. (B) The deletion of putative flocculin target genes of Mbx2 and Rfl1 reduces the degree of flocculation as a result of mbx2Δ+ overexpression. An nmt1-driven mbx2Δ+ was overexpressed in single and double deletion strains of various putative flocculin genes in EMM minus thiamine medium for 24 hours and the degree of flocculation examined. (C) Deletion of gsf2Δ+ abrogates the flocculation of rfi1Δ cells. The mutant strains were grown to log phase in YES medium for 24 hours to assay flocculation. (D & E)
The putative flocculin gene targets of Mbx2 and Rfl1 are sufficient to induce flocculation when overexpressed

Besides gsf2, the other putative target genes of Mbx2 and Rfl1 that encode for cell surface glycoproteins share some amino acid sequence homology with domains found in other fungal adhesins [23]. However, the role of these glycoprotein genes in flocculation has not been demonstrated. Overexpression studies were employed to the aforementioned set of putative flocculin target genes of Mbx2 and Rfl1 to determine whether they function directly in flocculation. Each single overexpression of these flocculin genes was able to induce flocculation to varying degrees with the strongest flocculation observed in the gsf2OE strain which produced visible flocs within one day (Figure 3A; Table S14). Weaker flocculation was observed from the overexpression of the other flocculin genes after total incubation of 2–7 days in EMM minus thiamine medium with sub-culturing into fresh medium in Day 3. The flocculation images of these overexpression strains shown in Figure 3A were captured after total of 7 days of induction. As a result of these observations, we named these genes pfl for Pombe Flocculins and numbered them according to their degree of flocculation when overexpressed: pfl1+/gsf2 (referred as gsf2+ hereafter), pfl2+/SPAPB15E9.01c, pfl3+/SPBC947.04, pfl4+/SPCC188.09c, pfl5+/SPBC1289.15, pfl6+/SPBC977.05c, pfl7+/SPBC359.04c, pfl8+/fu5 (referred as fu5+ hereafter) and pfl9+/SPAC186.01. Furthermore, we overexpressed some double combinations of the weaker flocculin genes to determine whether flocculation could be additive. Indeed, the pfl4+pfl9+, pfl6+pfl9+, and fu5+pfl9+ double overexpression strains flocculated earlier and formed larger flocs than their corresponding single overexpressors, thus, demonstrating the additive effect of these flocculins (Figure 3B, Table S14). We next tested the single deletions of the pfl+ genes for their ability to flocculate in flocculation-inducing medium. No visible flocculation was observed in the gsf2Δ strain while wild type was flocculent (Figure 1B).

Figure 3. Overexpression of putative flocculin target genes of Mbx2 and Rfl1 induces flocculation. (A) Single overexpression of the Mbx2 and Rfl1 flocculin target genes (gsf2+/pfl1+ and pfl2–pfl9+) with the nmt1 promoter induces flocculation to varying degrees. The number assigned to the pfl+ genes corresponds roughly to the relative strength of flocculation upon overexpression (i.e. gsf2+/pfl1+ to pfl9+ showing strongest to mildest flocculation, respectively). The overexpression strains were cultured for total of 7 days (sub-cultured into fresh medium on third day) in EMM minus thiamine medium at 30°C. (B) The flocculin genes exhibit additive effects on flocculation. Double overexpression of various flocculin genes resulted in greater flocculation than the overexpression of the single corresponding genes.
In contrast, flocculation still occurred in the pfl2Δ-pfl9Δ strains in the inducing medium indicating that gsf2+ encodes the dominant flocculin and the other flocculin genes are dispensable for flocculation (data not shown).

These observations revealed that the contribution in flocculation by these pfl+ genes varied and certain combinations of pfl+ were additive. The strength of flocculation by the single overexpression of pfl+ genes was directly correlated with the reduction of mbx2OE flocculation in the corresponding deletion strains (Figure 2B and Figure 3A, Table S14). For example, the mbx2OE strain which produced larger flocs than the pfl3OE, pfl9OE strains exhibited a greater inhibition of mbx2OE flocculation when deleted. Similarly, the flocculation of the rfl1Δ strain was completely abrogated by the deletion of gsf2+, but not at all by the deletion of pfl9+ (Figure 2C). Consistent with the above results, the deletion of both gsf2+ and pfl9+ led to a greater abrogation of mbx2OE flocculation compared to each deletion alone (Figure 2B).

In summary, we have demonstrated that these pfl+ genes encode for S. pombe flocculins and Gsf2 is the dominant flocculin.

Positive and negative autoregulation of mbx2+ and rfl1+, respectively

Interestingly, ChIP-chip analysis also detected binding of Mbx2 and Rfl1 to their own promoters, as well as Rfl1 binding to the mbx2+ promoter (Figure 2A), indicating autoregulation and mbx2+ regulation by Rfl1 within the transcriptional-regulatory network of S. pombe flocculation. Mbx2 also appeared to be associated with the rfl1+ promoter, but this interaction was marginal as it was found just above the detection threshold for ChIP-chip (Figure 2A). To investigate the autoregulation of mbx2+, the gene was C-terminal tagged with GFP at its native locus (mbx2-GFP). However, the GFP-tagged strain resulted in a hypermorphic allele that displayed constitutive flocculation and nuclear localization of Mbx2-GFP (see below). We speculated that the removal of the 3′-untranslating region of mbx2+ during the C-terminal tagging may be the cause of the hypermorphic allele. To bypass this potential problem, we created an N-terminal GFP-tagged allele (GFP-mbx2) with an intact 5′-untranslated region and approximately 1 kb of native promoter sequence. In contrast to the C-terminal tagged hypermorphic allele, the N-terminal tagged GFP-Mbx2 expression was comparable to background levels and the strain did not exhibit constitutive flocculation (Figure 4A). Moreover, the GFP-mbx2 strain flocculated when grown in glycerol-inducing medium indicating that the tagged protein is functional (Table S14). When mnt1-driven mbx2+ expression was induced for 9 hours in the GFP-mbx2 strain, nuclear GFP-Mbx2 expression was detected, indicating that Mbx2 can activate its own expression (Figure 4A). As expected, this strain was now flocculent. Longer induction of mnt1-driven mbx2+ expression resulted in greater GFP-Mbx2 expression with multi-nucleated GFP foci (data not shown). The positive autoregulation of mbx2+ is likely to be direct as several putative MEF2-binding sequences (e.g. 5′-TTAAAAATAG-3′) are located within 1000 bp upstream from the mbx2+ start codon (data not shown).

To determine whether negative autoregulation occurs with rfl1+, a C-terminal GFP-tagged strain under native control was generated (rfl1-GFP). The localization of Rfl1-GFP was nuclear in the rfl1-GFP strain (Figure 4B). The induction of mnt1-driven rfl1+ expression for 18 hours in the rfl1-GFP strain led to a reduced nuclear Rfl1-GFP signal and a slightly increased cytoplasmic Rfl1-GFP signal (Figure 4B). However, overall Rfl1-GFP expression in the cell was reduced when Rfl1 was overexpressed compared to the empty vector control (Figure 4B; two-tailed t-test; p value<0.01). In contrast to our observations with the Rfl1-GFP protein expression, we found that there was no decrease of the Rfl1-GFP transcript when rfl1+ was overexpressed (Table S13). These results indicate that although Rfl1 can bind to its own promoter, negative autoregulation appears marginal or may not be occurring.

Rfl1 represses mbx2+ expression

The observation that Rfl1 is associated with the mbx2+ promoter by ChIP-chip suggests that Rfl1 may oppose Mbx2 function in flocculation by repressing its expression. To test this hypothesis, we first examined the genetic interactions between mbx2+ and rfl1+. The mbx2Δ rfl1Δ double mutant did not display flocculation indicating that mbx2+ is epistatic to rfl1+ (Figure 3A). In addition, the flocculation associated with mbx2OE was abrogated by overexpression of rfl1+ (Figure 5A). These results are consistent with mbx2+ being downstream of rfl1+ and that rfl1+ opposes mbx2+ function in flocculation.

We next utilized the C-terminal and N-terminal GFP-tagged mbx2+ strains to further determine if Rfl1 represses mbx2+ expression. First, Rfl1 was overexpressed in the hypermorphic C-terminal tagged mbx2-GFP allele which shows constitutive nuclear Mbx2-GFP expression and flocculation. This resulted in the near-abolishment of both the GFP signal (Figure 5B) and flocculation (data not shown) in the hypermorphic mbx2 allele. Second, when the N-terminal tagged GFP-mbx2 strain was crossed into the rfl1Δ background, the resulting strain displayed dramatic increase in nuclear GFP-Mbx2 expression (Figure 5C) and flocculation strength equivalent to the rfl1Δ strain (data not shown). These results support the hypothesis that mbx2+ expression is repressed by Rfl1 in non-flocculent cells.

Overexpression of cbf12+ causes flocculation due to up-regulation of gsf2+

Cbfl2, a member of the CSL transcription factor family has previously been reported to trigger flocculation when overexpressed [22]. However, the target genes of Cbf12 that function in flocculation have not been identified. To further elucidate the role of cbf12+ in flocculation, we took a similar approach to identify its direct target genes by concurrent expression microarray profiling and ChIP-chip analysis of the mnt41-driven cbf12-HA strain (Tables S6 and S7, respectively).

When cbf12+ was deleted and cultured in flocculation-inducing medium, flocculation was abolished (Figure 6A). In contrast, overexpression of cbf12+ by the mnt1 promoter triggered flocculation (Figure 6C) and produced a bowling pin–shaped phenotype after 24 hours in medium lacking thiamine (data not shown). Further induction of the mnt1-driven cbf12+ caused the strain to become sick and granulated, eventually leading to growth arrest (data not shown). To reduce the toxic effects of cbf12+ overexpression, an mnt41-driven cbf12-HA strain was used for concurrent expression profiling and ChIP-chip analysis.

Gene ontology analysis was carried out separately on the top 50 most highly-induced genes and all 160 promoter-occupied genes by Cbf12 with the Princeton GO Term Finder. Functional enrichment of genes in cell surface (p = 1.3e-7) and plasma membrane (p = 5.7e-4) were detected for the highly-induced and promoter-occupied genes, respectively. These genes included several flocculin genes, (Figure 6B). Both gsf2+ and pfl9+ were among the five highest induced genes (18.1 and 27.6-fold, respectively) in the cbf12+OE strain and were also detected by ChIP-chip (Figure 6B) suggesting that Cbf12 directly activates the transcription of gsf2+ and pfl9+ for flocculation. The flocculation triggered by cbf12+ overexpression was completely abrogated in the gsf2A background, whereas deletion of pfl9+ had little effect.
Transcription Control of Flocculation in *S. pombe*

**A**

*N-ter. GFP-mbx2 Empty vec.*

*N-ter. GFP-mbx2 mbx2OE*

**B**

*rf1-GFP Empty vec.*

*rf1-GFP rf1OE*

GFP Intensity per area

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PLOS Genetics | www.plosgenetics.org 8 December 2012 | Volume 8 | Issue 12 | e1003104
**Figure 4. mbx2**<sup>+</sup> **and rfl1**<sup>+</sup> undergo positive and negative autoregulation, respectively. (A) Positive autoregulation of mbx2<sup>+</sup>. A strain containing N-terminal GFP-tagged mbx2<sup>+</sup> under the control of its native promoter displayed increased GFP expression when mbx2<sup>+</sup> was ectopically expressed with the nmt1 promoter. Nuclear GFP-Mbx2 signal and flocs in liquid culture were detected at the 9-hour induction of nmt1-driven mbx2<sup>+</sup> in EMM minus thiamine medium. Cells were deflocculated in 2% galactose prior to fluorescence microscopy to facilitate image acquisition. The presence of galactose does not affect the GFP signal (data not shown). The bar graph compares the mean and standard deviation of cellular GFP-Mbx2 signal resulting from nmt1-driven mbx2<sup>+</sup> and empty vector control with a significant difference of p < 0.001 (Welch's two tailed t-test; n = 50, df = 52). (B) Negative autoregulation of rfl1<sup>+</sup>. A strain containing C-terminal GFP-tagged rfl1<sup>+</sup> under native control exhibited nuclear expression (empty vector). Ectopic expression of nmt1-driven rfl1<sup>+</sup> for 18 hours in EMM minus thiamine medium reduced the nuclear GFP signal with a slight increase in cytoplasmic GFP signal. The bar graph compares the mean and standard deviation of overall cellular rfl1<sup>+</sup>-GFP signal resulting from nmt1-driven rfl1<sup>+</sup> and empty vector control with a significant difference of p < 0.001 (Welch's two tailed t-test; n = 27, df = 44). Cells were stained with DAPI to visualize nuclei. Scale bar, 10 µm. doi:10.1371/journal.pgen.1003104.g004

(figure 6C, Table S14). This was consistent with the hypothesis that gsf2<sup>+</sup> encodes the dominant flocculin. In addition, loss of gsf2<sup>+</sup> or mbx2<sup>+</sup> did not alter the bowling-pin cell shape or the reduced fitness phenotypes of the cbf12OE strain indicating that these two phenotypes were not due to the upregulation of the flocculin genes (data not shown). The much weaker flocculation observed in the cbf12OE strain in comparison to the mbx2OE and gsf2OE strains may be attributed to additional defects in cell and nuclear division, which would cause early growth arrest before the full flocculation potential could be reached [22].

Consistent with previous findings, C-terminal GFP-tagged Cbf12 under native control was expressed predominantly in the nucleus in stationary phase cells while expression in logarithmic cells was comparable to background (Figure 6D; [22]). Compared to logarithmic growth in rich medium, Cbf12-GFP nuclear expression increased in cells grown in flocculation-inducing medium, thus supporting its role in flocculation (Figure 6D). Interestingly, Cbf12 was also detected by Chip-chip to bind to its own promoter (Figure 6B). Indeed, positive autoregulation appears to occur as native Cbf12-GFP expression increased greater than three-fold when nmt1-driven cbf12<sup>+</sup> was ectopically expressed in logarithmically growing cells (Figure 6E).

Recently, it was demonstrated that an N-terminal-truncated Cbf12 bound to probes containing a canonical CSL binding motif (5′-GTGGGGA-3′) by gel mobility shift assay [35]. We next searched for a similar DNA binding sequence for Cbf12 from the logarithmically growing cells (Figure 6E).

The microarray expression profile of the cbf11A strain revealed greater than 2-fold increase of gsf2 and pbf3 transcripts and a 60-fold increase of the SPAC1F8.02c transcript suggesting that these two flocculin genes and their putative regulators were induced in the cbf11A and sre2A strains. In the sre2A strain, gsf2, pbf3<sup>+</sup> and fus5<sup>+</sup> transcripts were upregulated 3.7, 2.5 and 3.1-fold, respectively, indicating that the expression of these genes could be contributing to the flocculent phenotype (Figure 7C). In contrast, mbx2<sup>+</sup> and cbf12<sup>+</sup> transcripts were downregulated approximately 2-fold suggesting that the elevated levels of gsf2<sup>+</sup>, pbf3<sup>+</sup> and fus5<sup>+</sup> transcripts in the sre2A strain were not mediated by Mbx2 and Cbf12 (Figure 7C). Similarly in the yox1D strain, we observed that gsf2<sup>+</sup> and pbf3<sup>+</sup> transcripts were upregulated although less than in the sre2A strain, and mbx2<sup>+</sup> and cbf12<sup>+</sup> were also downregulated (Figure 7C). Therefore, this suggests that sre2<sup>+</sup> and yox1<sup>+</sup> may be involved in the repression of flocculation through a pathway independent from mbx2<sup>+</sup> and cbf12<sup>+</sup>.

The role of flocculation by Adn2 and Adn3 is influenced by genes encoding cell wall–modifying enzymes and gsf2<sup>+</sup>

The transcription factor genes adn2<sup>+</sup> and adn3<sup>+</sup> are orthologous to Saccharomyces cerevisiae FLO8 (http://www.pombase.org/) and exhibit defects in invasive growth and cell-to-surface adhesion when deleted during nitrogen starvation [29]. From our screens, we discovered that the overexpression of adn2<sup>+</sup> and adn3<sup>+</sup> triggered
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**Figure A**

Images showing different conditions:
- **rfl1Δ**
- **mbx2Δ**
- **mbx2Δ rfl1Δ**
- **mbx2OE rfl1OE**
- **mbx2OE rfl1OE**

**Figure B**

- **Bright Field**
- **DAPI**
- **GFP**

Comparison of C-terminal mbx2-GFP:
- Empty vec.
- rfl1OE

**Figure C**

- **Bright Field**
- **DAPI**
- **GFP**

Comparison of N-terminal GFP-mbx2:
- Empty vec.
- rfl1Δ

**Graphs**

- GFP intensity per area for C-terminal mbx2-GFP.
- GFP intensity per area for N-terminal GFP-mbx2.

*Note: Specific data and values are not transcribed here.*
minor flocculation while loss of adn2+ and adn3+ prevented flocculation in flocculation-inducing medium (Figure 8A and 8B, respectively). The flocculent phenotype of adn2OE and adn3OE strains was disrupted by the addition of galactose (data not shown). To identify the target genes of Adn2 and Adn3 that are involved in flocculation, expression microarray profiling was performed on nmt1-driven adn2OE and adn3OE strains (Tables S1 and S12). Surprisingly, gsf2+ transcript levels were relatively unchanged and the majority of pff+ genes were downregulated in both overexpression strains (Figure 8C). Consistent with these results were the observations that mbx2+ and cfh12+ transcripts were downregulated greater than 2-fold in both adn2OE and adn3OE strains, whereas rfl1+ transcript levels were not differentially regulated (Figure 8C). Therefore, it appeared that the flocculent phenotype of adn2+ and adn3+ overexpression could not be attributed to the pff+ genes identified in this study. These results led us to consider that perhaps the expression of other genes besides these encoding for floculins could be responsible for triggering flocculation in adn2OE and adn3OE strains.

Interestingly, some of the aforementioned cell wall-remodeling enzymes (gas2+, pwi1+ and SPAC4H3.03c) were also highly upregulated in both adn2OE and adn3OE strains (Figure 8C, Table S13). For example, gas2+ and SPAC4H3.03c were the highest induced genes in the adn2OE strain (17.9 and 36.8-fold, respectively) and also appeared within the top 20 most induced genes in the adn3OE strain. These genes were also induced in the mbx2OE strain except for pwi1+ (Figure 2A). Overexpression analysis was subsequently carried out to determine if these genes possessed some role in flocculation. Although agn2+ was not upregulated in the adn2OE and adn3OE strains, it was included in the overexpression analysis because it was the second most induced gene (91-fold), as well as detected by ChIP-chip in the mbx2OE strain. Indeed, the single overexpression of these four genes resulted in flocculation after 5-days (including 3rd day subculturing into fresh medium) in medium lacking thiamine, implicating the involvement of these cell wall-remodeling enzymes in flocculation (Figure 8D, Table S14). Since deletion of adn2+ and adn3+ results in defects of invasive growth and cell-to-surface adhesion in response to nitrogen starvation, we wanted to determine if the single overexpression of gas2+, agn2+, pwi1+ and SPAC4H3.03c could cause enhancement of these processes. We discovered that the single overexpression of these four cell wall-remodeling genes increased cell-to-surface adhesion, but not invasive growth relative to wild type under the nitrogen-deprivation condition (Figure S1). Because gsf2+ encodes the dominat floculcin, we also investigated whether the flocculation caused by adn2+ and adn3+ overexpression was dependent on gsf2+. Deletion of gsf2+ completely abrogated the flocculation in adn2OE and adn3OE strains (Figure 8A, Table S14).

In addition, the adn2OE and adn3OE strains exhibited cell separation defects such as the formation of multisepta and forhead phenotypes (Figure 8E). The cell separation defect was more severe when adn3+ was overexpressed. We next determined whether the putative target genes involved in the flocculation of adn2OE and adn3OE strains also played a role in the multisepa phenotype. Overexpression of adn2+ and adn3+ in the gsf2+ background did not alter the multisepa phenotype (Figure 8E), while the overexpression of gas2+, SPAC4H3.03c, pwi1+ and agn2+ did not lead to formation of multisepta (data not shown). These results suggest that Adn2 and Adn3 may regulate cell separation and flocculation independently through different sets of target genes. Our microarray expression data suggests that Adn2 and Adn3 may control cell separation through ace2+, which encodes a major transcriptional activator of this process (Alonso-Núñez et al., 2005). Overexpression of adn2+ and adn3+ resulted in the down-regulation of ace2+ and many of its known target genes such as adg1+, adg2+, adg3+, cfh4+, agn1+, eng1+, and mid2+ by 1.5 to 3.4-fold (Figure 8C).

In summary, the regulation of flocculation by adn2+ and adn3+ is likely mediated by the induction of genes encoding the cell wall-remodeling enzymes Gas2, SPAC4H3.03c, and Pwi1. The regulation of these genes is independent from Mbx2 because mbx2+ was downregulated in the adn2OE and adn3OE strains. Although gsf2+ transcript level was not significantly upregulated by adn2+ and adn3+ overexpression, it was sufficient to abrogate the flocculation when deleted. However, it is possible that other cell surface glycoprotein genes not investigated in this study but were upregulated may also play a significant role in the flocculation function of adn2+ and adn3+.

Discussion

In this study, we have deciphered a significant portion of the transcriptional-regulatory network governing flocculation in S. pombe. To date, few transcription factors and their target genes that function in flocculation have been identified. The MADS box transcription factor Mbx2 positively regulates flocculation by induction of the floculcin gene gsf2+, while the CSL transcription factors Cbf11 and Cbf12 repress and activate flocculation, respectively, but their target genes are not known (21,22). We have substantially expanded our limited knowledge of the flocculation transcriptional-regulatory network by the identification of several novel transcriptional activators (Adn2 and Adn3) and repressors (Ril1, Yox1 and Sre1), and their putative target genes that function in flocculation. In addition, novel target genes of Mbx2, Cbf11 and Cbf12 were identified. The putative target genes of the transcription factors implicated in flocculation encode for several cell surface glycoproteins (gsf2+ and pff2+–pfl9+) and cell wall-remodeling enzymes (agn2+, pwi1+ SPAC4H3.03c and gas2+). These target genes were sufficient to trigger flocculation when overexpressed. Moreover, instances of regulation between transcription factors (Ril1 repression of mbx2+), as well as positive
Figure 6. Regulation of flocculation by Cbf12. (A) Loss of cbf12+ prevents flocculation under inducing conditions. Wild type and the cbf12Δ mutant were cultured in flocculation-inducing medium for 5 days at 30 °C. (B) Cbf12 regulates putative flocculin genes. The heat map shows induction of several flocculin genes and their promoter occupancy by Cbf12 from microarray expression profiling and ChiP-chip analysis, respectively, of an nmt1-driven cbf12Δ strain grown at 30 °C. (C) The absence of gsf2+ abolishes the flocculation triggered by cbf12Δ overexpression. Strains containing nmt1-driven cbf12Δ in wild type, gsf2Δ or pfl7A backgrounds were cultured for 24 hours in EMM minus thiamine medium at 30 °C. Approximately 1/16 of the petri-dish was magnified to reveal more details of the flocs. (D) ctf12Δ is expressed in wild-type cells grown in rich medium at stationary phase and in flocculation-inducing medium, but not in rich medium at log phase. A C-terminal GFP-tagged ctf12Δ strain under the control of its native promoter was grown to log or stationary phase in YES or in the inducing medium at 30 °C. The bar graph compares the mean and standard deviation of cellular Cbf12-GFP signals. (E) Positive autoregulation of ctf12+. Ectopic expression of nmt1-driven ctf12+ results in the upregulation of native-controlled Ctf12-GFP expression in log-phase cells. The bar graph compares the mean and standard deviation of cellular Cbf12-GFP signals between induced and uninduced ctf12OE cells with significant difference of p < 0.001 (Welch’s two tailed t-test; n = 19, df = 25). (F) A DNA motif closely matching the binding specificity of CSL transcription factors was retrieved from the ctf12OE microarray expression data. The promoter region (1000 base pairs upstream of the start codon) of 11 highly-induced genes encoding for cell surface proteins as identified by the Princeton GO Term Finder was applied to MEME using default settings. The orange line indicates bases that match with the known binding site of CSL transcription factors. Cells were stained with DAPI to visualize nuclei. Scale bar, 10 µm.

doI:10.1371/journal.pgen.1003104.g006

(mbx2+ and cbf12+) autoregulation were detected within the flocculation network. Mbx2 and Rfl1 appeared to be the major positive and negative regulators of flocculation, respectively, based on the largest flocs observed in the mbx2OE and rfl1Δ strains compared to the other flocculant mutants in this study. Our initial efforts to identify the target genes of Mbx2 and Rfl1 revealed several putative flocculin genes that were strikingly upregulated in the mbx2OE and rfl1Δ flocculant mutants. Previously, Gs2 was the only S. pombe flocculin demonstrated to be directly involved in flocculation, and its transcription was influenced by the activity of Mbx2 [20,21]. Similar to these studies, we also found that overexpression of gsf2+ triggers flocculation while loss of gsf2− abrogates the flocculent phenotype of several mutants including mbx2OE. Here, we identified an additional eight flocculin genes (pfl2+, pfl3+, pfl9+, ada4+, pgd3, adh4, pgk1, fba1, eno1, and tpi1) that positively regulate flocculation. Strains containing single gsf2+ overexpression or null mutations in each of these eight genes exhibited antagonistic roles in flocculation (Table S4). From these data, we speculate that Rfl1 could serve as a negative transcriptional regulator of several enzymes involved in the glycolysis and gluconeogenesis. Because flocculation and invasive growth are associated with nutritional limitation, Rfl1 may coordinate the expression of genes involved in flocculation and carbohydrate metabolism in fission yeast.

Previously, the CSL proteins Cbf11 and Cbf12 were shown to exhibit antagonistic roles in flocculation [39]. Overexpression of ctf12+ or loss of ctf11+ triggers flocculation. However, none of their target genes have been identified. We present supportive evidence that Cbf12 induces flocculation by directly activating the transcription of gsf2+. In addition, gsf2+ expression is up-regulated approximately 2.4-fold in the ctf11Δ strain suggesting that the repressive flocculation function of Cbf11 may also be directly mediated through gsf2+. The activation and repression of gsf2+ transcription by Cbf12 and Cbf11, respectively, may occur by competitive binding to promoter sites since both transcription factors have been shown to interact with a canonical CSL
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A

\[ \begin{align*}
yox1\Delta & \quad yox1\Delta \text{gsf2}\Delta \\
sre2\Delta & \quad sre2\Delta \text{gsf2}\Delta \\
cbf11\Delta & \quad cbf11\Delta \text{gsf2}\Delta
\end{align*} \]

B

\[ \text{Ribosomal and mitochondrial genes} \]

C

\[ \begin{align*}
yox1 & \quad sre2 & \quad cbf11 \\
\Delta & \quad \Delta & \quad \Delta
\end{align*} \]

\[ \text{Log}_2 \text{ ratio change} \]

\[ \begin{align*}
yox1^* & \quad sre2^* & \quad cbf11^* \\
gsf2^* & \quad pfl3^* & \quad pfl4^* & \quad pfl6^* & \quad fta5^* & \quad fta9^* \\
\text{SPAC1F8.02c} & \quad \text{mbx2^*} & \quad \text{cbf12^*} & \quad \text{rf11^*}
\end{align*} \]
consensus sequence in vitro [39]. Several putative sites with six out of seven nucleotide match to the canonical CSL consensus sequence are located within 900 base pairs of the gsf2 + promoter (data not shown). Further experimentation would be required to verify this proposed mechanism of gsf2 + transcriptional regulation by Cbf11 and Cbf12. It is likely that cjb12 + plays a lesser role in activating flocculation compared to mbx2 + since the floc size resulting from cjb12 + overexpression is considerably smaller than the mbx2OE strain. Also, unlike mbx2 +, deletion of cjb12 + is not sufficient to abrogate the flocculation of the yfl1Δ strain (data not shown). These data suggest that the flocculent phenotype of the cjb12Δ yfl1Δ double mutant is probably caused by the presence of mbx2 + activity.

CSL transcription factors are components of the conserved Notch signaling pathways in metazoans which primarily function in cell-to-cell communication during development [40]. Although multiple fungal CSL proteins have been discovered, their exact roles remain unclear in unicellular organisms [39]. Flocculation has been described as a manifestation of social behaviour in yeast [39]. Several putative sites with six out of seven nucleotide match to the canonical CSL consensus sequence [39]. Flocculation has been described as a manifestation of social behaviour in yeast [39]. Several putative sites with six out of seven nucleotide match to the canonical CSL consensus sequence.

Figure 7. Regulation of flocculation by Yox1, Sre2, and Cbf11. (A) The regulation of flocculation by Yox1, Sre2 and Cbf11 is dependent on gsf2 +, yox1Δ, sre2Δ and cbf11Δ cells flocculate in late log and stationary phase when grown in YES medium (left panels). The flocculation of these mutant strains was abrogated in the gsf2Δ background (right panels). (B) Clustergram of microarray expression profiles of flocculent transcription factor mutants. The microarray expression profiles of the yox1Δ and sre2Δ strains are most similar and show upregulation of ribosomal and mitochondrial genes. (C) The yox1Δ, sre2Δ and cbf11Δ flocculent strains show upregulation of several flocculin genes. The color bars reflect relative expression between experimental and control. doi:10.1371/journal.pgen.1003104.g007

Sre2 is an uncharacterized membrane-tethered helix-loop-helix transcription factor predicted to be an ortholog of mammalian SREBP-1a, which is responsible for the transcriptional activation of genes needed for uptake and synthesis of cholesterol, fatty acids, triglycerides, and phospholipids [50]. While sre1 +, a paralog of sre2 + has been shown to function in the transcriptional activation of sterol-biosynthetic and hypoxic-adaptation genes, there has been no direct evidence that sre2 + plays similar biological roles [37]. Loss of sre2 + results in the upregulation of gsf2 +, pfl3 + and fla5 + transcripts (3.76, 2.51 and 3.08-fold, respectively) (Figure 7C, Table S9) which may contribute to its flocculent phenotype. The sre2Δ flocculent phenotype requires gsf2 + activity and is independent of mbx2 + and cjb12 + since these transcripts are downregulated in the deletion mutant.

In addition, the microarray expression profiles of yox1Δ and sre2Δ strains displayed similar differential gene expression despite the supposedly different functions of these transcription factors (Figure 7B). Mitochondrial genes were found to be highly upregulated in both deletion mutants (Tables S8 and S9). This occurrence may not be unexpected for Sre2 if it has a similar role in hypoxia as Sre1 where mitochondrial function is probably impaired [37]. It is currently not clear whether the mitochondrial genes are direct targets of Yox1 and Sre2 or induced in response to an altered physiological state in the deletion mutants. Interestingly, mitochondrial activity has been reported to be important for flocculation and invasive growth in Sacc. cerevisiae [48,51]. Disruption of mitochondrial activity has been shown to alter the synthesis and structure of the cell wall, possibly by interfering with the interactions of flocculins and their substrates [52]. Based on these observations, the flocculent phenotype of yox1Δ and sre2Δ strains could be partially the result of enhanced mitochondrial activity from the upregulation of mitochondrial genes.

A genome-wide systematic deletion screen previously uncovered a cell-to-surface adhesion function that is sensitive to the presence of galactose for the Adn2 and Adn3 transcription factors [29]. Here, we discovered that adn2Δ and adn3Δ have additional functions in flocculation. Overexpression of adn2 + and adn3 + induced minor flocculation while loss of these genes prevented flocculation in inducing glycerol medium (Figure 8A and 8B). However, the flocculent phenotype of the adn2OE and adn3OE strains appeared to be primarily caused by the differential regulation of genes encoding cell wall-remodeling enzymes rather than flocculins. Several genes encoding cell wall-remodeling enzymes (gas2 +, agn2 +, psu1 + and SPAC4H3.03c) were highly induced when mbx2 +, adn2 + or adn3 + was overexpressed. In the adn2OE strain, gas2 + and SPAC4H3.03c were the most highly induced genes (17.9 fold and 36.8-fold, respectively) (Figure 8C, Table S11) while in the adn3OE strain, these two genes and psu1 + appeared within the top 20 up-regulated genes (Table S12). Similarly, in the mbx2OE strain, gas2 +, agn2 +, and SPAC4H3.03c appeared within the top 100 up-regulated genes (greater than 3.7-fold increase, Figure 2A). We found that the single overexpression of these four genes could trigger flocculation (Figure 8D). Cell wall remodeling is an essential process for proper growth and adaptation to environmental stresses in yeast cells. Part of the cell wall-remodeling process involves the dissolution of sugar
Figure 8. Regulation of flocculation by Adn2 and Adn3 is dependent on gsf2+ and cell wall–remodeling genes. (A) Overexpression of adn2+ or adn3+ triggers weak flocculation, which is abrogated in the gsf2Δ background. The mutant strains were cultured for 3 days in EMM minus thiamine medium. Approximately 1/8 of the petri-dish was magnified and shown for each strain. (B) Deletion of adn2+ or adn3+ prevents flocculation in flocculation-inducing medium. (C) Several cell wall-remodeling genes were upregulated while the majority of pf10 genes and known target genes of Ace2 (bottom panel) were downregulated upon adn2+ or adn3+ overexpression. Microarray expression profiling was performed on nmt1-driven adn2OE and adn3OE strains and induced in EMM minus thiamine medium. The color bar reflects relative expression ratios between experimental and control strains. (D) Overexpression of the cell wall-remodeling genes agn2+, psu1+, gas2+ and SPAC4H3.03c triggers flocculation. These overexpression strains were cultured for total of 5 days (subcultured on third day into fresh medium) in EMM minus thiamine medium. (E) The multiseptation phenotype resulting from adn2+ or adn3+ overexpression is not dependent on gsf2+. The strains were induced for 34 hours in EMM minus thiamine medium and stained with calcofluor white. Scale bar, 10 μm.

doi:10.1371/journal.pgen.1003104.g008

Figure 9. A model of the transcriptional-regulatory network of flocculation in S. pombe

A model of the transcriptional-regulatory network of flocculation in S. pombe. Interactions of transcriptional activation and repression are indicated by arrows and bars, respectively. The major regulators of flocculation, Mbx2 and Rfl1 are in bold. Dash lines denote transcription factor-target gene interactions that may not be direct. See main text for a detailed description of the transcriptional-regulatory network.

doi:10.1371/journal.pgen.1003104.g009
Altogether, these results suggest that Gsf2 is likely expressed in the cell wall as an inactive flocculin, and the cell wall remodeling resulting from adn2+ and adn3+ overexpression alters the arrangement of Gsf2 and possibly other flocculins that now becomes favorable for flocculation.

The single overexpression of the cell wall-remodeling genes triggered flocculation to a greater extent than the adn2OE and adn3OE strains. A possible explanation for the different degrees of flocculation between the transcription factor and its target genes could be that overexpression of adn2+ and adn3+ causes reduced fitness due to toxicity effects associated with a greater misregulation of genes compared to the aberrant production of a single enzyme. Consistent with this theory is that adn2OE and adn3OE strains exhibited additional phenotypes including septation defects (Figure 8E) which were not observed when gas2+, gsu2+, psu1+ and SPAC4H3.03c were overexpressed (data not shown). Furthermore, a systematic overexpression analysis of 5280 genes in *S. cerevisiae* revealed that genes encoding for transcription factors, signalling molecules and cell cycle regulators were more likely to cause reduced fitness [60].

In *S. pombe*, cell separation involves the transcriptional activation of adg1+, adg2+, adg3+, agg1+, eng1+, cfh4+ and mid2+ by the Ace2 transcription factor, which is in turn regulated by the Sep1 forhead transcription factor [61–64]. We discovered that the adn2OE or adn3OE strains displayed multisepta and forhead phenotypes similar to loss-of-function mutations of these cell separation genes. The cell separation defect in adn2OE and adn3OE strains is likely due to the downregulation of ace2+ transcription since ace2+ and its target genes were substantially downregulated in these strains (Figure 8C). However, sept1+ transcript levels remained unchanged in the adn2OE and adn3OE strains indicating that their involvement in cell separation phenotype could be either downstream of sept1+ or parallel to the sept1+ pathway. In addition to its flocculation role, Adn2 and Adn3 appear to have a separate function in cell separation perhaps by directly or indirectly repressing ace2+ transcription. Experiments are planned in the future to address these possibilities.

Interestingly, we also found some evidence that supports a role of Mbx2 and Cbf12 in cell separation perhaps through repression of ace2+ activity. Overexpression of mbx2+ and cbf12+ results in significant down-regulation of all seven Ace2 target genes approximately 1.5 to 3.4-fold relative to the empty vector control. (Tables S2 and S6). The mbx2OE strain indeed showed septation defects but were slightly different in nature than the adn2OE and adn3OE strains with less multi-septation and more mislocalization of septum material (data not shown). Moreover, overexpression of cbf12+ has been reported to produce multisepta phenotypes albeit at a low frequency [39]. These observations indicate the possible existence of crosstalk between flocculation and cell separation pathways mediated by the Mbx2, Cbf12, Adn2 and Adn3 transcription factors (Figure 9).

A comparison between the flocculation network of budding and fission yeast revealed both conserved and divergent features within the transcriptional circuitry. In *Sacc. cerevisiae*, the positive and negative transcriptional controls of the dominant flocculin gene FLO1 by Flo8p or Miss1p, and Sfl1p, respectively, draw parallel to FLO11 transcription through physical interactions with Sn6p and Srb proteins (Srb6p, Srb9p and Srb11p) [19,63,66]. Moreover, Sfl1p has been reported to repress FLO10 in *S. pombe* [24]. In addition, the flocculent phenotype of tup11+//tup12+ mutants [25] and the abrogation of *fkh1A* flocculation in the absence of mbx2+ [21] supports the role of Tup11/12 corepressor in Mbx2-Rfl1-mediated flocculation. Taken together, we speculate that Srb10 and Tup11/12 activity and binding may be required for Rfl1-mediated repression of *gsf2* and *mbx2* expression, future experiments focusing on the interactions between Rfl1, Tup11/12 and Srb8-10 in relation to flocculation would provide clarification to our speculation.

Our analyses of the transcription factors implicated in flocculation of *S. pombe* revealed the possible existence of several network motifs including positive autoregulation of *mbx2* and *cbf12* and regulation of *gsf2* by an inhibitory feed-forward loop (coherent type 2). The latter involves the Rfl1 transcriptional repression of *gsf2* directly and indirectly by inhibition of *mbx2* expression. Autoregulatory motifs have not been detected so far for FLO8, MISS11 and SFL1. The discovery of these network motifs in *S. pombe* suggests that the transcriptional inhibition of *gsf2* could occur more rapidly than its transcriptional activation. Experimental and modeling studies have proposed that positive and negative autoregulation of transcription factors generate slow and fast response times, respectively, within a transcriptional-regulatory network [67]. Under positive autoregulation, the synthesis rate of the transcription factor is initially slow at low concentrations, but increases as the concentration of the transcription factor reaches the activation threshold of the promoter, while negative autoregulation accelerates the attainment of steady state levels of the
transcription factor [67]. Moreover, the inhibitory feed-forward motif of Rfl1 seems to indicate that repression of gfl2 expression likely happens in a shorter period compared to its activation. Altogether, these data suggest that the onset of flocculation may occur gradually while repression of the flocculation pathway is a much faster process. Consistent with this speculation is the observation that it requires several days for wild-type S. pombe cells to undergo flocculation when grown in inducing medium.

In summary, we have provided an initial and substantial view of the transcriptional-regulatory network governing flocculation in S. pombe. Found within this network are the master regulators Mbx2, Cbf12, Adn2 and Adn3, which are able to trigger flocculation when overexpressed by the activation of their target genes encoding for flocculins and cell wall-remodeling enzymes. In addition, several repressors including Rfl1 were uncovered that play a major role in the regulation of these target genes. However, significant gaps of knowledge surrounding the transcriptional-regulatory network still remain. The environmental cues that impinge upon the activity of the positive and negative regulators, as well as the dynamics of transcription factor binding and regulation of target genes during the onset of flocculation remain to be elucidated. Also, although gfl2 encodes the dominant flocculin, it is currently unclear whether the other flocculins have nonessential or more specialized roles during flocculation. Detailed analyses of the temporal and spatial expression of the gfl genes would be required to address these questions. Moreover, the exact mechanism of how other biological processes such as cell wall restructuring and mitochondrial function influence flocculation is unknown. Further studies to expand our knowledge of this transcriptional-regulatory network would provide a more comprehensive understanding of flocculation control and contribute to a valuable resource for the improvement of industrial yeast applications.

Materials and Methods

Yeast strains, media, and general methods

All strains used in this study are listed in Table S1 and were maintained on YES or EMM medium. Geneticin, nourseothricin, and thiamine hydrochloride were added to media at a concentration of 150 μg/L, 100 μg/L, and 15 μM, respectively. EMM medium was supplemented with amino acids when necessary at 225 mg/L each for adenine, leucine, and uracil. Matings were performed on SPAS medium. Wild type and deletion strains were assayed for flocculation in YEGlycerol (flocculation-inducing) medium containing 1% (w/v) yeast extract, 3% (v/v) glycerol, and 4% (v/v) ethanol. Overexpression strains containing ORFs under control of the mnt1 or mnt41 promoter were grown in EMM minus thiamine medium. Standard genetics and molecular biology techniques were performed as described in [68].

Construction of deletion and GFP-tagged strains

A PCR-based stitching method was utilized to construct the deletion and epitope-tagged strains. For construction of deletion strains, ~500 bp fragments upstream and downstream of the ORF and the KanMX6 or NatMX6 cassette were PCR-amplified and gel-purified. The 3’ end of the upstream fragment and 5’ end of the downstream fragment contained ~25 bp homology to the selectable marker cassette sequence. Approximately equimolar amounts (~40 ng) of each PCR fragment were combined and stitched together in a 20 μl PCR reaction (0.2 mM dNTPs and 0.4 units of Phusion HF DNA polymerase (New England Biolabs), and subjected to one cycle of 98°C (30 sec), 5 cycles of 98°C (15 sec), 60°C (1 min), and 72°C (1–2 min) and a final extension at 72°C (5 min). The stitched product was then amplified in a 50 μl PCR reaction by combining the entire stitched reaction with 6 nmol dNTPs, 0.6 units of Phusion HF DNA polymerase and 20 pmol each of the outer pair of primers and then subjected to one cycle of 98°C (30 sec), 30 cycles of 98°C (10 sec), 60°C (30 sec) and 72°C (2 min), and a final extension at 72°C (5 min). The amplified product was gel-purified and transformed into the appropriate strain by lithium acetate transformation. A similar strategy was used to construct GFP-tagged transcription factors under the control of the native promoter. To tag the transcription factor with GFP at the C-terminus, ~500 bp upstream and downstream fragments flanking the stop codon and the GFP-KanMX6 cassette (amplified from pYM27 plasmid, [69]) were PCR-amplified for the stitching reaction as described above. To conserve the native promoter in the N-terminal GFP fusion of Mbx2, 1 kb upstream of the mnt2 promoter was amplified along with four other fragments for PCR stitching: (1) ~500 bp upstream of the aforementioned 1 kb fragment; (2) ~500 bp downstream of the mnt2 promoter; (3) KanMX6 cassette and; (4) the GFP ORF with its stop codon removed and a GDAGL linker added (adapted from [70]). All five fragments contained ~25 bp overlapping homology to their respective flanking fragments and were PCR-stitched as described above. Proper gene deletion and GFP tagging were confirmed by colony PCR screen and the resulting amplicons sequenced.

Construction of overexpression strains

Genes were overexpressed with the nmt1 promoter by cloning the entire ORFs of interest into the pREP1 or pREP2 vector. For ChIP-chip experiments, C-terminal triple HA-tagged Mbx2, Rfl1, and Cbf12 were expressed with the nmt11 promoter by cloning the corresponding ORFs into pSIP272 [71]. All the clones were PCR-confirmed, sequenced, and transformed into appropriate strains by the lithium acetate method. Expression of the HA-tagged proteins was verified by western blotting with anti-HA F-7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Microarray expression profiling

Strains overexpressing the triple HA-tagged Mbx2, Rfl1, and Cbf12 were grown in 200 ml of EMM medium containing appropriate supplements without thiamine for 18-20 hr to induce the nmt41 promoter. The empty vector control strain was cultured concurrently to a matching cell density of ~8×10^6 cells/ml prior to harvesting. The experimental culture was divided into two, each for ChIP-chip and microarray expression profiling while the control culture was only utilized in the latter. The expression profiling cultures were harvested by centrifugation (1800× g, 3 min, 20°C), followed by immediate freezing of the cell pellets in liquid nitrogen. Culturing of adn2OE and adn3OE strains were performed similarly except that these genes were driven by the nmt11 promoter and were not epitope-tagged. For transcription factor deletion strains (gfl1Δ, rfl1Δ, se2Δ, and ydi1Δ), the mutant and an isogenic wild-type strain were concurrently grown in YES medium and harvested at a similar cell density as described above. Total RNA extraction, mRNA isolation, reverse transcription with aminomethyl-dUTP (Sigma-Aldrich, Oakville, ON), and Cy5SM/Cy3SM (GE Healthcare, Buckinghamshire, UK) dye coupling of cDNA were performed with dye reversal as previously described [72]. Purified Cy5SM- and Cy3SM-labelled cDNA (1 μg in total) was hybridized onto custom-designed 8×15 K Agilent expression microarrays containing 60mer probes to all S. pombe ORFs in 2–3 times coverage per gene. The hybridization procedure was carried out according to the manufacturer’s instructions (Agilent Technology, Santa Clara, CA) with the exception for the use of Human
Cot-1 DNA. The microarrays were washed in 6× SSPE/0.005% sodium N-lauroylsarcosine at room temperature for 5 min followed by a second wash in pre-heated 42 °C 0.6× SSPE for 2 min.

The microarrays were scanned with a GenePix4200A scanner (Molecular Devices, Sunnyvale, CA). The raw microarray data was lowest normalized [73] and the average log2 ratios with the corresponding t-test p values [74] from the dye-swap experiments were obtained using the R Bioconductor Limm package. Heat map images of the microarray expression and ChIP-chip data were constructed with Cluster 3.0 [75] and Java Treeview 1.1.6.2 [76]. The microarray expression data has been submitted to the NCBI Gene Expression Omnibus Database (GSE41730).

ChIP–chip experiments and data analysis

Culturing of the HA-tagged transcription factor strains are described above. The culture was fixed by the addition of a final concentration of 1% formaldehyde and agitation for 30 min at room temperature. The formaldehyde was quenched by the addition of 2.5 M glycine to a final concentration of 125 mM and agitation for 5 min at room temperature. The cells were then centrifuged (800 × g, 5 min, 4 °C), washed twice in 25 ml 1× ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 pH 7.4), washed once with 2 ml 1× iced cold lysis buffer (50 mM NaCl, 50 mM HEPES-KOH pH 7.5, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate and 1 tablet/50 ml Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN)). The cell pellet was resuspended in 1.6 ml lysis buffer and stored at −80 °C.

The cell suspension was transferred to two 2 ml bead beating vials containing 800 μl of 0.5 mm Zirconia/Silica beads (BioSpec Products, Bartlesville, OK) and subjected to 3 cycles of alternating 2 min beating and 2 min incubation on ice with a Mini Beadbeater 16 (BioSpec Products, Bartlesville, OK). The lysed cells were collected by puncturing the bottom of the bead-beating vial with a flame-heated inoculating needle and placing the vial on a sonication tube in 10 ml disposable culture tubes prior to centrifugation (800 × g, 3 min, 4 °C). The cell pellet was resuspended, transferred to chilled microcentrifuge tubes, centrifuged (16,000 × g, 15 min, 4 °C) to remove unbound soluble proteins, and the resulting pellet resuspended in 800 μl of fresh lysis buffer in a sonication tube. Total cell lysate volume was adjusted to 2.2 ml with lysis buffer and subjected to 4 cycles of sonication and 1 min on ice incubation at 30% amplitude, 30 sec setting using a Sonic Dismembrator with a 1/8 tapered microtip probe (Thermo Scientific, Waltham, MA). The sonicated cell lysate, and shaken gently for 2 hr at 4 °C on a Labquake Tube Shaker (Thermo Scientific, Waltham, MA). The beads were washed twice in 1 ml cold deoxycholate buffer (100 mM Tris-HCl pH 8.1, 1 mM EDTA, 0.5% (w/v) sodium deoxycholate, 0.5% (v/v) NP-40, 250 mM LiCl) and twice in 1 ml cold lysis buffer. The beads were resuspended in 200 μl 1× PBS-BSA, combined with 400 μl of sonicated cell lysate, and shaken gently for 2 hr at 4 °C. Four washes of 5 min each were next carried out: (1) 1.4 ml cold lysis buffer at 4 °C; (2) 1.4 ml cold lysis buffer with 400 mM NaCl at 4 °C; (3) 1.4 ml deoxycholate buffer at room temperature and; (4) 1.4 ml TE (pH 8) at room temperature. The transcription factor and bound DNA were eluted twice from the Dynabeads by incubating with 250 μl TES each (TE pH 8, 1% (w/v) SDS) at 65 °C for 6 min. Dynabead washing and the supernatant collection were performed using DynaMag®3−2 (Invitrogen, Carlsbad, CA). For the input DNA, 200 μl of the cell lysate was added to 300 μl TES. Both the immunoprecipitated and input cell lysates were incubated at 65 °C overnight to reverse the DNA-protein cross-linking. Western blotting with anti-HA antibody was performed to confirm proper pull-down of the transcription factor.

For protein removal, both immunoprecipitated and input samples were incubated with 200 μg Proteinase K (Promega, Madison, WI) and 20 μg glycogen (Roche Applied Science, Indianapolis, IN) at 56 °C for 2 hr. The DNA was then extracted by phenol-chloroform extraction, ethanol-precipitated overnight, washed once with 70% EtOH, resuspended in 42 μl TE containing 0.1 μg DNAse-free RNaseA (Roche Applied Science, Indianapolis, IN), and incubated for 30 min at 37 °C.

Blunt ends were generated in the entire immunoprecipitate and input DNA samples with 1 unit of T4 DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA), 1× NEB Buffer #2 (New England Biolabs, Ipswich, MA), 5 μg NEB BSA, and 10 nmol dNTPs in a 110 μl reaction by incubation at 12 °C for 20 min, followed by phenol-chloroform extraction and ethanol precipitation with 10 μg glycogen and 1/10 volume 3 M NaOAc. The DNA pellets were washed in 70% EtOH and resuspended in 25 μl water. Approximately 1/5 of precipitated input DNA was used in the subsequent ligation reaction as input DNA concentration was >100 times greater than that of immunoprecipitated DNA. For ligation of linkers to blunt ends, the resuspended DNA was incubated with 1000 units of concentrated T4 DNA Ligase (New England Biolabs, Ipswich, MA), 1× T4 DNA Ligase Buffer (Invitrogen Life Technologies, Carlsbad, CA), and 200 pmol annealed linker (15 μM Oligo #1 5′-GCGGTGACCCGGGAGATCTGGAATTC-3′ and 15 μM Oligo #2 5′-GAATTCGATCG-3′ in 250 mM Tris) at 16 °C overnight. The annealed linker and the ligation mix were kept on ice at all times prior to overnight incubation. The DNA was ethanol-precipitated, washed, and resuspended in 25 μl water as described above.

The ligated DNA was PCR-amplified by adding 15 μl of labeling mix (2 μl aa-dUTP dNTP mix containing 5 mM each ddATP, ddCTP and ddGTP, 3 mM dTTP, and 2 mM aminoallyl-dUTP (Sigma-Aldrich, St. Louis, MO)), 1.25 μl 40 μM Oligo #1 (5′-GCGGTGACCCGGGAGATCTGGAATTC-3′), 4 μl 10× ThermoPol Buffer (New England Biolabs, Ipswich, MA) and 7.75 μl water in a PCR cycler paused at 55 °C. A 10 μl enzyme mix containing 5 units of GoTaq DNA polymerase (Promega, Madison, WI), 0.001 units of Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA), and 1× ThermoPol Buffer (New England Biolabs, Ipswich, MA) was added and the PCR proceeded with one cycle of 55 °C (4 min); 72 °C (5 min); 95 °C (2 min) and 30 cycles of 95 °C (30 sec); 55 °C (30 sec); 72 °C (1 min), followed by a final extension at 72 °C (4 min). The PCR products were purified (QIAGEN, Valencia, CA) with a few modifications: (1) buffer PE was replaced with phosphate wash buffer (5 mM KPO4 pH 8.5, 80% ethanol) and (2) buffer EB was replaced with phosphate elution buffer (4 mM KPO4 pH 8.5). A sample of the purified PCR product was run on an agarose gel to check for fragment sizes ranging between 100 bp and 1 kb. The purified PCR products were quantified, and equal amounts of
immunoprecipitated samples and corresponding input samples were coupled to Cy5 and Cy3 dyes as described above.

The labelled samples (total amount of 3–5 μg) were hybridized onto an Agilent 4 × 44 K S. pombe Genome ChIP-on-chip microarray according to the manufacturer’s instructions (Agilent Technology, Santa Clara, CA) except for the use of Human Cot-I DNA. The washing and scanning of the microarrays were performed as described above. The ChIP-chip data was normalized by scaling in Limma [73] and analyzed by ChIPOTe Peak Finder Excel Macro [77] with the default setting of log2 ratio cutoff of 1. Peaks located within 3 kb upstream of a start codon and 2 kb downstream of a start codon within a coding region or 3' UTR, in the case of short ORFs, were assigned to the gene. ChIP-chip data sets are found in Tables S3, S5, and S7. Genes with multiple peaks are noted in the data set with the peak values. The ChIP-chip data has been submitted to the NCBI Gene Expression Omnibus Database (GSE41730).

Motif-finding analysis

The transcription factor binding specificities were determined by RankMotif++ [31] and MEME [30]. S. pombe promoter sequences 1000 bp upstream of the translational start site were used for these motif-finding algorithms. For MEME, promoter sequences of genes with various log ratio thresholds from expression microarray and ChIP-chip experiments were input into the MEME online server. RankMotif++ was applied to the entire expression microarray data since its motif-searching algorithm is threshold independent. The consensus sequences of the transcription factor binding sites were displayed by submitting the position weight matrices obtained from RankMotif++ analysis into the enoLOGOS online server [78].

Flocculation assays

Strains were grown in flasks at 30°C for the appropriate time, and 10 ml of culture was transferred to culture tubes for strains with larger floc sizes. Images were acquired immediately after vigorous shaking in glass culture tubes with a Canon G10 digital camera. For strains with mild flocculation, flocs were harder to visualize in culture tubes, and therefore, were observed in 90 mm plastic petri dishes. 10–15 ml of culture was transferred to petri dishes, followed by gentle shaking [8] on an orbital low-speed shaker (Labnet International, Woodridge, NJ) at maximum speed for one hour in room temperature. Floc images in petri dishes were captured using a SPImager (S&P Robotics Inc., Toronto, ON). Deflocculation of flocculent strains was performed by the addition of 2–20% D(-)-galactose or 10 mM EDTA. The reflocculation of the deflocculated cells was performed by washing with water, resuspending the cells in YES or EMM medium or 100 mM CaCl2 and allowing the culture to sit for 30 min at room temperature.

For the overexpression of pft genes, the strains were inoculated at a concentration of 107 cells in 100 ml of EMM without thiamine and cultured for 3 days at 30°C. For the weaker flocculent strains (pft2– pmf9), 5 ml of the 3-day culture was then inoculated into 100 ml of fresh EMM without thiamine and incubated for another 3–4 days at 30°C followed by the petri dish flocculation assay as described above. Fresh EMM medium was added on the third day to prevent cells from remaining in stationary phase. Flocculation assays for the more flocculent overexpression strains were similarly carried out except the induction times were less than three days and did not require refeeding with fresh EMM medium. It should be noted that the empty vector control cells also eventually flocculate after refeeding with fresh EMM medium, but the onset of flocculation and flocc were delayed for several days and less pronounced, respectively, compared to the weakest flocculent overexpression strains. Wild-type strain and deletion mutants mbx2Δ, gsFΔ, shf1Δ, adn2Δ and adn3A were induced to flocculate by inoculating cells at a concentration of 109 cells in 100 ml of YEglycEtOH medium and culturing for 5 days at 30°C followed by the petri dish flocculation assay as described above.

Agar adhesion and invasive growth assay

A patch of cells approximately 1/6 of a 90 mm petri dish was grown on YES medium for two days at 30°C and transferred as described in [29] onto a LNB plate (0.067 g/L yeast nitrogen base without amino acids (Bacto), 20 g/L glucose, 20 g/L agar, salts and vitamins as for EMM) with an underlying layer of YE + ALU (0.5% YE, 225 mg/L adenine, leucine, and uracil each) [79]. The plates were incubated at 30°C for 2 weeks before testing for cell-to-surface adhesion by washing cells off under a gentle stream of water and for invasive growth by rubbing the remaining cells off the agar with a finger under a stream of water. For strains showing resistance to rigorous washing by finger, a small section of the agar was cut out and observed under a Zeiss AxioScope A1 tetrad microscope (Zeiss, Thornwood, NY). Invasive growth was observed by the presence of elongated and branched cells remaining underneath the agar [29,79].

Fluorescence microscopy

Images of GFP-tagged cells were acquired with a Zeiss AxioScope 2 microscope (Zeiss, Thornwood, NY) and Scion CFW Monochrome CCD Firewire Camera (Scion Corporation, Frederick, MD). Fluorescence intensity was quantitated using the open source software ImageJ (version 1.44) [National Institutes of Health]. First, the background signal for each image was subtracted using the “Subtract Background” function (50 pixel rolling ball radius). Individual cells were then selected as regions of interest using the freehand or polygon selection tools. Using the “Set Measurements” function both the area and integrated density were determined for each selected cell (in ranged between 27 and 50). Corrected GFP intensity was determined for each cell and was defined as the quotient of integrated density/area in background subtracted images. The averaged integrated density/area measurements for a given number cells is presented as the mean corrected GFP intensity with standard deviation. Significant differences between means were calculated by the Student t-test. To view nuclei and cell wall material, cells were methanol-fixed and stained with DAPI (1 μg/ml) and calcofluor white (50 μg/ml), respectively.

Supporting Information

Figure S1 Overexpression of cell wall-remodeling genes enhances cell-to-surface adhesion, but not invasive growth. The assays for adhesion and invasive growth were carried out as described in the Materials and Methods. (TIF)

Table S1 Schizosaccharomyces pombe strains used in this study. (DOC)

Table S2 Expression Microarray Profiling of mbx2-HA OE strain versus empty vector strain. (XLSX)

Table S3 mbx2-HA ChIP-chip data. (XLSX)
with SYBR 
tative PCR was performed on a StepOne Real-Time PCR System 
expression of putative targets and overexpressed genes. Quanti-
and the following program: 95

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Table S4  Expression Microarray Profiling of fbl1 deletion strain versus isogenic wild type. (XLSX)
Table S5  fbl1-HA ChIP-chip data. (XLSX)
Table S6  Expression Microarray Profiling of cbf12-HA OE strain versus empty vector strain. (XLSX)
Table S7  cbf12-HA ChIP-chip data. (XLSX)
Table S8  Expression Microarray Profiling of yox1 deletion strain versus isogenic wild type. (XLSX)
Table S9  Expression Microarray Profiling of spe2 deletion strain versus isogenic wild type. (XLSX)
Table S10 Expression Microarray Profiling of cbf11 deletion strain versus isogenic wild type. (XLSX)
Table S11 Expression Microarray Profiling of adn2OE strain versus empty vector strain. (XLSX)
Table S12 Expression Microarray Profiling of adn3OE strain versus empty vector strain. (XLSX)
Table S13 Validation of putative targets and overexpressed genes by qPCR. The log2 ratios determined from expression 
   microarrays are shown for comparison. Culturing, RNA extraction and reverse transcription for each strain were performed 
   independently from the microarray experiments. Primer sets were checked for specificity by the presence of a single amplicon of the 
   correct size using their melting curves and gel electrophoresis. The act1+ gene was used as a reference for determining the relative 
   expression of putative targets and overexpressed genes. Quantitative PCR was performed on a StepOne Real-Time PCR System 
   with SYBR® green master mix (Life Technologies, Carlsbad, CA) and the following program: 95°C for 10 min, 40 cycles of 95°C for 
   15 sec and 58°C for 1 min, followed by a melting curve program of 38°C to 95°C with a heating rate of 0.3°C per second. Three 
   replicates were carried out for each combination of query gene and strain. The relative expression of each query gene was 
   compared between the mutant and the corresponding wild type or empty vector strain. Fold changes were determined by ΔΔCt 
   method according to manufacturer’s recommendation (Life Technologies). (DOC)
Table S14 Degree of flocculation observed in flocculent strains. Strains were grown in EMM minus thiamine medium unless 
   indicated. Cultures were refed with fresh medium on the third day to prevent entry into stationary phase (see Materials and Methods). 
   To semi-quantify the amount of flocculation, 5–10 ml of culture was centrifuged (800× g, 2 min, 25°C), and deflocculated by 
   washing once with 10 ml of 10 mM EDTA. The culture was subsequently washed three times with 15 ml of water and 
   resuspended in water at a final concentration of ~107 cells/ml. Reflocculation was carried out by the addition of CaCl2 at a final 
   concentration of 20 mM to 2.7 ml of resuspended culture in a 60 mm petri dish shaken on an orbital low-speed shaker (Labnet 
   International, Woodridge, NJ) at maximum speed for 30 min in room temperature. The entire culture was pipetted carefully into a 
   3.0 ml cuvette and an OD600 reading close to the top of the cuvette was obtained with a SpectraMax Plus microplate reader 
   (Molecular Devices, Sunnyvale, CA). A control culture was carried out similarly except no CaCl2 was added. The degree of 
   flocculation was determined by subtracting the difference of the optical density of the reflocculation culture and the control culture 
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   flocculation were derived from at least two technical replicates. (DOC)

Author Contributions

Conceived and designed the experiments: E-JGK GC. Performed the experiments: E-JGK AL LV JK. Analyzed the data: E-JGK KC-R. Contributed reagents/materials/analysis tools: E-JGK AL KC-R LV JK. Wrote the paper: E-JGK GC.

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