RESEARCH ARTICLE

Functional divergence of a global regulatory complex governing fungal filamentation

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Abstract

Morphogenetic transitions are prevalent in the fungal kingdom. For a leading human fungal pathogen, Candida albicans, the capacity to transition between yeast and filaments is key for virulence. For the model yeast Saccharomyces cerevisiae, filamentation enables nutrient acquisition. A recent functional genomic screen in S. cerevisiae identified Mfg1 as a regulator of morphogenesis that acts in complex with Flo8 and Mss11 to mediate transcriptional responses crucial for filamentation. In C. albicans, Mfg1 also interacts physically with Flo8 and Mss11 and is critical for filamentation in response to diverse cues, but the mechanisms through which it regulates morphogenesis remained elusive. Here, we explored the consequences of perturbation of Mfg1, Flo8, and Mss11 on C. albicans morphogenesis, and identified functional divergence of complex members. We observed that C. albicans Mss11 was dispensable for filamentation, and that overexpression of FLO8 caused constitutive filamentation even in the absence of Mfg1. Harnessing transcriptional profiling and chromatin immunoprecipitation coupled to microarray analysis, we identified divergence between transcriptional targets of Flo8 and Mfg1 in C. albicans. We also established that Flo8 and Mfg1 cooperatively bind to promoters of key regulators of filamentation, including TEC1, for which overexpression was sufficient to restore filamentation in the absence of Flo8 or Mfg1. To further explore the circuitry through which Mfg1 regulates morphogenesis, we employed a novel strategy to select for mutations that restore filamentation in the absence of Mfg1. Whole genome sequencing of filamentation-competent mutants revealed chromosome 6 amplification as a conserved adaptive mechanism. A key determinant of the chromosome 6 amplification is FLO8, as deletion of one allele blocked morphogenesis, and chromosome 6 was not amplified in evolved lineages for which FLO8 was re-located to a different chromosome. Thus, this work highlights rewiring of key morphogenetic regulators over evolutionary time and aneuploidy as an adaptive mechanism driving fungal morphogenesis.
Author summary

Fungal infections pose a severe burden to human health worldwide. Candida albicans is a leading cause of systemic fungal infections, with mortality rates approaching 40%. One of the key virulence traits of this fungus is its ability to transition between yeast and filamentous forms in response to diverse host-relevant cues. The model yeast Saccharomyces cerevisiae is also capable of filamentous growth in certain conditions, and previous work has identified a key transcriptional complex required for filamentation in both species. However, here we discover that the circuitry governed by this complex in C. albicans is largely distinct from that in the non-pathogenic S. cerevisiae. We also employ a novel selection strategy to perform experimental evolution, identifying chromosome triplication as a mechanism to restore filamentation in a non-filamentous mutant. This work reveals unique circuitry governing a key virulence trait in a leading fungal pathogen, identifying potential therapeutic targets to combat these life-threatening infections.

Introduction

The fungal kingdom is recognized for its vast morphological plasticity, with many species capable of undergoing morphogenetic transformations in response to diverse environmental cues. For instance, filamentous fungi such as Aspergillus fumigatus undergo spore germination and branching throughout their life cycle, dermatophyte fungal pathogens produce arthroconidia, and thermally dimorphic fungi such as Histoplasma capsulatum exist as filamentous mycelia at ambient temperatures and transition to a yeast form upon exposure to mammalian physiological temperatures [1–3]. The purposes of such transformations are equally diverse, with morphogenesis playing critical roles in sexual reproduction, nutrient acquisition and virulence. For example, the basidiomycete Cryptococcus neoformans forms elongated filaments for the purpose of mating or monokaryotic fruiting [4], and the model yeast Saccharomyces cerevisiae undergoes invasive or pseudohyphal growth for nutrient acquisition under starvation conditions [5]. Finally, the fungal pathogen Candida albicans transitions from a yeast to hyphal state in response to a variety of host-relevant cues [2,6], which aids in tissue invasion, immune cell evasion, and biofilm formation, such that morphogenesis is critical for virulence of this pathogen [2,7,8]. Thus, fungal morphogenesis comprises a diversity of processes required for cellular survival, proliferation, and pathogenesis.

The past several decades have witnessed a surge in the frequency of life-threatening human fungal infections, making mycotic disease a serious public health problem. Candida species comprise one of the leading genera of human fungal pathogens, with C. albicans being the most prevalent [9]. Those most susceptible to these infections are the increasing population of immunocompromised patients, including those undergoing chemotherapy or transplantation, as well as those infected with HIV. C. albicans can cause fatal bloodstream infections, with mortality rates approaching 40% despite therapeutic intervention [9,10]. This is in part due to a limited number of antifungals available to treat systemic infections coupled with the frequent emergence of antifungal drug resistance in the clinic [11]. Further, the capacity of C. albicans to cause life-threatening disease in its human host is enabled by a complex repertoire of virulence factors, including the expression of surface structures that mediate adherence to epithelial cells, the secretion of hydrolytic enzymes that induce host cell damage, the capacity to produce biofilms that are intrinsically resistant to antifungal drugs, and the ability to transition between yeast and filamentous growth states [2,12–14]. Given the dearth of new antifungal
classes uncovered by traditional approaches of targeting essential proteins required for viability, a complementary approach of targeting proteins required for pathogen virulence may prove to be a useful strategy to combat fungal infections [15].

The ability of *C. albicans* to transition between yeast and filamentous forms occurs upon exposure to a variety of different cues, including host febrile temperature, serum, and nutrient depletion [2,6]. Complex genetic circuitry underpins this transition, and distinct mechanisms are involved in initiating and maintaining filamentous growth [8,16–19]. One of the core pathways required for filamentation in *C. albicans* is the cAMP-protein kinase A (PKA) signaling cascade, for which multiple components of the pathway are required for filamentous growth in response to diverse conditions [2,6]. In *C. albicans*, the PKA complex is composed of the regulatory subunit Bcy1 and two catalytic subunits, Tpk1 and Tpk2 [20]. Increased cAMP levels produced by the adenyl cyclase Cyr1 activate the PKA complex, resulting in phosphorylation and activation of the transcription factor Efg1, which governs the expression of many hyphal-specific genes [6,21,22]. Other signaling pathways including the Cek1-mitogen activated protein kinase (MAPK) pathway [23], Rim101 pH sensing pathway [24], and protein kinase C (Pkc1) cascade [25] also play important roles in enabling filamentation in response to diverse stimuli. Despite the continued identification and characterization of genes involved in *C. albicans* morphogenesis, an understanding of the regulatory networks governing morphogenesis remains largely elusive.

Systematic analyses of genes enabling filamentous growth have been performed on a genomic level in both *S. cerevisiae* and *C. albicans* [18,26]. Such analyses have revealed a striking divergence in the sets of genes required for filamentation between these species [18,26]. A recent study with the *S. cerevisiae* Σ1278b strain defined genes required for: a) diploid pseudo-hyphal formation in response to low nitrogen; b) haploid invasive growth in response to glucose depletion; and c) biofilm formation on semi-solid agar [26]. Despite largely distinct gene sets being important for each of these filamentation programs, there was a core gene set required for morphogenesis, including the previously uncharacterized protein Mfg1 [26]. In *S. cerevisiae*, Mfg1 forms a complex with the transcriptional regulators Flo8 and Mss11 to control the expression of hundreds of genes, including *FLO11*, which encodes a cell surface glycoprotein essential for filamentation [26,27]. Members of the *S. cerevisiae* Flo8-Mfg1-Mss11 complex primarily function in concert to promote morphogenesis. Of the 152 promoters bound by *S. cerevisiae* Mfg1, 89% are also bound by Flo8 and 78% are bound by Mss11 [26]. Further, deletion of *S. cerevisiae MFG1* reduces Flo8 binding to 90.8% of its target promoters [26]. Mfg1 is also a key regulator of morphogenesis in *C. albicans*, and the physical interaction with Flo8 and Mss11 is conserved under basal conditions [26]. However, the mechanisms through which this complex regulates filamentous growth in *C. albicans* remains largely enigmatic given that the transcriptional targets of this complex remain unknown and that no *FLO11* homolog has been identified in this fungal pathogen.

In this study, we characterized the role of the Flo8-Mfg1-Mss11 complex in regulating *C. albicans* filamentation. Although Flo8 and Mfg1 were required for filamentous growth in response to numerous conditions, they were not required for morphogenesis induced by compromised function of the molecular chaperone Hsp90, demonstrating that these mutants are capable of filamentation in response to specific cues. Further, overexpression of *FLO8* resulted in constitutive hyphal growth yet overexpression of *MFG1* did not, suggesting distinct functions of these regulators in the yeast-to-hyphal transition. We observed functional divergence of the complex members, as Mss11 was dispensable for filamentous growth and had a reduced physical interaction with Mfg1 and Flo8 in filament-inducing cues. Surprisingly, *MSS11* overexpression was sufficient to induce morphogenesis in the absence of an inducing cue, highlighting the complex functional relationships. Chromatin immunoprecipitation (ChIP) of
TAP-tagged Flo8 or Mfg1 followed by microarray analysis (ChIP-chip) revealed significant divergence between the transcriptional targets of Flo8 and Mfg1 in *C. albicans*, and highlighted dynamic temporal changes in promoter occupancy and gene expression in response to serum as a filament-inducing cue. However, Mfg1 and Flo8 cooperatively bound to a subset of key targets involved in morphogenesis, including TEC1, for which overexpression is sufficient to drive filamentation in the absence of either Mfg1 or Flo8. Finally, to gain further mechanistic insight into the circuitry through which Mfg1 regulates morphogenesis, we employed a novel experimental evolution strategy based on introduction of a drug resistance marker under the control of a filament-specific promoter to select for mfg1Δ/mfg1Δ mutants with a restored ability to filament in response to serum. Whole genome sequencing of mutants capable of filamentation in the absence of Mfg1 uncovered an adaptive mechanism involving amplification of chromosome 6, on which FLO8 is located. Thus, we highlight Mfg1 as a critical regulator of *C. albicans* morphogenesis, uncover distinct roles of Flo8, Mfg1, and Mss11 in promoting filamentation, and provide a striking example of aneuploidy formation as a mechanism of adaptive evolution to enable filamentous growth, offering broad insights into the biology, pathogenicity, and evolutionary strategies of a leading fungal pathogen.

Results

Divergence in function among *C. albicans* Flo8-Mfg1-Mss11 complex members

Flo8 and Mfg1 are critical regulators of filamentation in *C. albicans*, and they physically interact with each other and with the transcriptional regulator Mss11 under basal conditions [26]. However, the mechanisms by which this complex regulates morphogenesis in *C. albicans* remain largely enigmatic. To characterize the role of the Flo8-Mfg1-Mss11 complex in the yeast-to-hyphal transition, we examined the morphology of *C. albicans* flo8Δ/flo8Δ, mfg1Δ/mfg1Δ, and mss11Δ/mss11Δ mutants in response to a variety of filament-inducing cues. Similar to previous reports [28], we observed that flo8Δ/flo8Δ mutants were completely blocked in filamentous growth in response to 10% serum, RPMI, Spider medium, or high temperature (Fig 1A). However, this strain was capable of forming robust filaments in response to Hsp90 inhibition with geldanamycin (Fig 1A). Similar to the flo8Δ/flo8Δ mutant, the mfg1Δ/mfg1Δ mutant was largely blocked in filamentation in the majority of cues examined, but was capable of filamenting in response to Hsp90 inhibition (Fig 1A). Notably, restoration of one allele of MFG1 [26] or FLO8 (S1 Fig) restored the capacity of the respective nulls to filament. In contrast to previous reports [29], no observable defect in filamentous growth was observed in two independently generated MSS11 mutants (Fig 1A and S2 Fig). As Flo8 and Mfg1 play central roles in filamentation induced by diverse cues, we also assessed their impact on filamentation induced by deletion of the repressors of filamentation, NRG1 [30] and LRG1 [25]. Strikingly, FLO8 was required for filamentation induced by loss of either NRG1 or LRG1, while MFG1 was largely dispensable (Fig 1B). Collectively, these results highlight that although Flo8 and Mfg1 are important for filamentation, mutants lacking these regulators are capable of polarized growth under specific environmental conditions and complex members have distinct roles in *C. albicans* filamentation.

Given that complex members are important to enable *C. albicans* filamentation in response to many cues, we next assessed if overexpression of any single component was sufficient to drive the filamentous growth program. Notably, overexpression of *S. cerevisiae* FLO8 or *MSS11* has previously been shown to result in hyperfilamentous growth [31,32]. We overexpressed each of the three transcriptional regulators individually in *C. albicans* by replacing the native promoter of one allele with a tetracycline-repressible promoter, tetO, which drives
### A) Divergence of a regulatory complex governing fungal filamentation

|                  | Wild type | flo8Δ/flo8Δ | mfg1Δ/mfg1Δ | mss11Δ/mss11Δ |
|------------------|-----------|-------------|--------------|---------------|
| **YPD, 30°C**    | ![Image]  | ![Image]    | ![Image]     | ![Image]      |
| **10% serum, 37°C** | ![Image] | ![Image]    | ![Image]     | ![Image]      |
| **RPMI, 37°C**   | ![Image]  | ![Image]    | ![Image]     | ![Image]      |
| **Spider, 37°C** | ![Image]  | ![Image]    | ![Image]     | ![Image]      |
| **YPD, 39°C**    | ![Image]  | ![Image]    | ![Image]     | ![Image]      |
| **10 μM, GdA, 30°C** | ![Image] | ![Image]    | ![Image]     | ![Image]      |

### B) Additional data

|                  | flo8Δ/flo8Δ | mfg1Δ/mfg1Δ |
|------------------|-------------|--------------|
| **ng1Δ/ng1Δ**    | ![Image]    | ![Image]     |
| **lng1Δ/lng1Δ**  | ![Image]    | ![Image]     |
strong and constitutive expression of the target gene in the absence of tetracycline (Fig 2A and 2B). Overexpression of FLO8 was sufficient to induce robust filamentous growth in the absence of an inducing cue, and resulted in a ~1,200-fold induction of the filament-specific transcript HWP1 relative to the wild-type strain (Fig 2A and 2C). This is reminiscent of other reports in which overexpression of C. albicans FLO8 resulted in wrinkly colony formation in 5% CO2 [33]. Consistent with previous reports [29], overexpression of C. albicans MSS11 was sufficient to induce some filamentation in the absence of an inducing cue and resulted in a 350-fold increase in expression of HWP1 relative to the wild-type strain (Fig 2A and 2C), highlighting that although this regulator is not necessary for filamentation, it plays a role in regulating this morphogenetic trait. Notably, the filaments induced by overexpression of FLO8 appeared distinct from those resulting from MSS11 overexpression, where the former resembled true hyphae, and the latter included many yeast-form cells, suggesting distinct mechanisms. Intriguingly, overexpression of C. albicans MFG1 did not result in filamentous growth in the absence of a filament-inducing cue, nor did it result in an induction of HWP1 (Fig 2A and 2C). This further supports our model that Flo8, Mfg1, and Mss11 have distinct roles in governing the yeast-to-hyphal transition.

Next, we examined if filamentation induced by overexpression of FLO8 or MSS11 was contingent upon Mfg1, given that MFG1 is required for filamentation in response to several inducing cues (Fig 1A). We replaced the native promoter of one allele of FLO8 or MSS11 with the tetO promoter, as above, in an mfg1Δ/mfg1Δ background (S3 Fig). Overexpression of FLO8 resulted in robust filamentous growth even in the absence of MFG1, and HWP1 transcript levels were induced by ~450-fold compared to the wild type (Fig 2C and 2D); this is less than half of the increase in HWP1 that was observed in the presence of MFG1, suggesting that Mfg1 promotes filamentation induced by Flo8. In contrast, overexpression of MSS11 was unable to drive filamentation in the absence of Mfg1, and did not result in induction of HWP1 (Fig 2C and 2D). Taken together, although Flo8, Mfg1, and Mss11 can exist as a complex in C. albicans under basal conditions, they also have distinct functions that influence morphogenesis.

C. albicans Mfg1 partially complements the activity of its S. cerevisiae ortholog

Given our findings that C. albicans Flo8 and Mfg1 are key for filamentation in response to most cues (Fig 1A), we assessed if they displayed a conserved function with those complex members in S. cerevisiae. C. albicans FLO8 has been previously demonstrated to complement the filamentous growth defect of S. cerevisiae haploid flo8Δ and diploid flo8Δ/flo8Δ mutants [28]. Here, we cloned C. albicans MFG1 or FLO8 as a control, into an expression vector and expressed the constructs in the filamentation-competent S. cerevisiae background Σ1278b. Diploid cells were plated on nitrogen-limiting SLAD medium to monitor pseudohyphal growth. When cells expressed the empty vector, the wild-type diploid formed pseudohyphae and the flo8Δ/flo8Δ and mfg1Δ/mfg1Δ mutants were blocked in pseudohyphal growth (Fig 3A). As previously described, pseudohyphal growth was restored in the flo8Δ/flo8Δ mutant when C. albicans FLO8 was expressed (Fig 3A), demonstrating that it functionally
complements the *S. cerevisiae* ortholog. We also observed that *C. albicans MFG1* was able to functionally complement a *S. cerevisiae* strain lacking MFG1, resulting in pseudohyphal growth (Fig 3A). Similarly, expression of *C. albicans FLO8* in the corresponding *S. cerevisiae* haploid deletion mutant was sufficient to restore haploid invasive growth, while expression of

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Fig 2. Overexpression of *FLO8* or *MSS11* results in filamentous growth in the absence of an inducing cue, but overexpression of *MFG1* does not. A) *FLO8*, *MSS11* or *MFG1* were overexpressed by replacing the native promoter of one allele with a tetracycline-repressible promoter, *tetO*. Cells were grown in YPD at 30˚C for 6 hours. Scale bar is 20 μm. B) Individual overexpression of genes encoding each complex member was achieved by replacing the native promoter of one allele with a tetracycline-repressible promoter, *tetO*, which in the absence of tetracycline drives constitutive expression of the target gene. Cells were grown in YPD at 30˚C for 3 hours. Transcript levels were normalized to ACT1 and TEF1 and error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.0001 (***') relative to the wild type (two tailed unpaired t-test). C) Filamentation was quantified by monitoring expression of the filament-specific transcript *HWP1* using qRT-PCR, and normalizing to ACT1 and TEF1. Cells were grown for 4 hours in YPD at 30˚C. Error bars represent standard error of technical triplicates as is representative of two biological replicates. Asterisks indicate P < 0.0001 (***') relative to parental strain (one-way ANOVA, Bonferroni Multiple Comparison Test). D) Filamentation induced by overexpression of *FLO8* is largely independent of Mfg1, whereas filamentation induced by overexpression of *MSS11* is completely dependent on Mfg1. Cells were grown in YPD at 30˚C for 6 hours. Scale bar is 20 μm.

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Fig 3. *C. albicans Mfg1* partially complements the activity of its *S. cerevisiae* ortholog. A) *C. albicans FLO8* or *MFG1* were expressed in diploid cells of the filamentation-competent *S. cerevisiae* Δ1278b strain (Sc Wild type) and in the *S. cerevisiae* strains lacking the relevant orthologs to assess the ability of these complex members to complement the block in *S. cerevisiae* diploid pseudohyphal growth. Cells were grown on SLAD agar plates and incubated at 30˚C for 11 days. Scale bar is 1.0 mm. B) *C. albicans FLO8* or *MFG1* were expressed in *S. cerevisiae* Δ1278b haploid cells (Sc Wild type) to assess their abilities to complement lack of *FLO8* or *MFG1* in *S. cerevisiae* haploid invasive growth. Cells were spotted onto YPD agar plates and incubated at 30˚C for 4 days at which point they were washed with water and images of the plate were taken with a Canon Power Shot A610. Scale bar is 1.0 cm.
C. albicans MFG1 was able to partially restore invasive growth in the mfg1 haploid mutant (Fig 3B). Thus, C. albicans Mfg1 is able to at least partially functionally complement its S. cerevisiae ortholog.

Mfg1 functions downstream of PKA to drive filamentation

In both S. cerevisiae and C. albicans, Flo8 functions downstream of PKA to regulate morphogenesis [28,34]. To determine if Mfg1 also acts downstream of the PKA complex in C. albicans, we overexpressed one of the PKA catalytic subunits, TPK2, by replacing the native promoter of one allele of TPK2 with the tetracycline-repressible promoter, tetO, to drive strong constitutive expression in the absence of tetracycline in the wild-type, flo8Δ/flo8Δ and mfg1Δ/mfg1Δ strains (Fig 4A). Cells were grown in rich medium at 30˚C or 34˚C, conditions that did not induce filamentous growth in wild-type cells, but caused substantial filamentation when TPK2 was overexpressed (Fig 4B). As expected, cells remained in the yeast form in the absence of FLO8 despite overexpression of TPK2 at 30˚C or 34˚C (Fig 4A and 4B), confirming that Flo8 acts downstream of PKA. Similarly, overexpression of TPK2 was insufficient to restore filamentation in the absence of MFG1 at either temperature (Fig 4A and 4B), suggesting that Mfg1 also acts downstream of PKA to enable C. albicans morphogenesis. Interestingly, TPK2 overexpression in the mfg1Δ/mfg1Δ background resulted in an enlarged cellular morphology. This demonstrates another phenotypic difference between flo8Δ/flo8Δ and mfg1Δ/mfg1Δ null mutants.

Flo8 and Mfg1 possess both shared and distinct transcriptional targets

Given that perturbation of C. albicans Flo8 and Mfg1 had distinct consequences on filamentous growth (Fig 1 and Fig 2), we next pursued a global analysis of genes bound and regulated by complex members to further probe their contributions to morphogenesis. Notably, both Flo8 and Mfg1 are localized to the nucleus in basal conditions and upon treatment with serum (S4A Fig). We performed chromatin immunoprecipitation coupled with microarray analysis (ChIP-chip) with strains harboring Flo8 or Mfg1 tagged with a tandem affinity purification (TAP) epitope. Functionality of the TAP-tagged proteins was verified (S4B Fig). DNA binding of Flo8 or Mfg1 was measured in either untreated conditions, or upon exposure to a filament-inducing cue, 10% serum, for one hour or three hours, to identify promoter regions that were bound by these transcriptional regulators (S1 Table). This demonstrated a substantial increase in promoter occupancy of both Flo8 and Mfg1 upon exposure to serum, consistent with their roles as transcriptional regulators of morphogenesis (Fig 5A, S5A Fig and S5B Fig). To identify the Flo8 and Mfg1 targets that are also transcriptionally modulated by these regulators, we performed microarray analysis comparing the gene expression profiles of flo8Δ/flo8Δ, mfg1Δ/mfg1Δ, and flo8Δ/flo8Δ mfg1Δ/mfg1Δ mutants with a wild-type strain under basal and filament-inducing conditions (S2 Table). We noted stark differences in transcriptional regulation by Flo8 and Mfg1 in response to serum, where large sets of genes were specifically altered in expression in either the flo8Δ/flo8Δ or mfg1Δ/mfg1Δ mutant, or in the double mutant (S5C Fig and S5D Fig). There was a considerable temporal component to the effects on gene expression, where we observed significant differences between the one-hour and three-hour serum-exposed conditions (S5C Fig and S5D Fig).

In order to identify key downstream targets of Flo8 and Mfg1 through which they regulate C. albicans filamentation, we compared those genes both bound and transcriptionally regulated by Flo8 and Mfg1 (Fig 5A, S3 Table). In basal conditions, we identified only 17 and 11 genes whose promoters were bound by either Flo8 or Mfg1 respectively, and whose expression was altered (Fig 5A). These sets were expanded in the presence of serum, where we identified
77 genes whose promoters were bound by Flo8 and 101 bound by Mfg1 in filament-inducing conditions, and for which expression was altered in the respective null mutants at the respective time point (Fig 5A). Surprisingly, unlike *S. cerevisiae* Flo8 and Mfg1 which bind to overlapping targets [26], *C. albicans* Flo8 and Mfg1 bind and regulate largely distinct sets of targets (Fig 5A, S5A Fig and S5B Fig, S1 Table), which is striking since Mfg1 does not have a characterized DNA-binding motif. This highlights the unique functions of Flo8 and Mfg1 in
regulating gene expression and identifies dynamic transcriptional changes that occur in response to serum.

To further explore the divergence in Flo8 and Mfg1 targets and to identify any potential additional Mfg1-binding partners, we performed affinity purification coupled to mass spectrometry (AP-MS) with C-terminally GFP-tagged Mfg1. Functionality of the tagged protein was verified (S4C Fig). Our previous AP-MS experiments demonstrated that C. albicans Mfg1 physically interacts with both Flo8 and Mss11 under standard conditions [26]. Here, we expanded the analysis to identify Mfg1 interaction partners in filament-inducing conditions (1 hour or 3 hours of growth at 37˚C), and included sonication and benzonase-treatment to aid in the identification of chromatin-bound proteins. In untreated conditions, Mfg1 interacts strongly with Flo8 and Mss11, as expected (Fig 5B, S4 Table). Under filament-inducing conditions, Flo8 continues to interact with Mfg1, suggesting a key role for their interaction in filamentation.
interaction. However, interaction with Mss11 is decreased in filament-inducing conditions, consistent with it being dispensable for filamentation (Fig 1A, Fig 5B, S4 Table). Interestingly, while the AP-MS identified other proteins as Mfg1 interactors, there were no additional DNA-binding proteins that could mediate Mfg1 binding to DNA, even in response to serum (Fig 5B, S4 Table).

Flo8 and Mfg1 cooperatively regulate TEC1 and BRG1 expression to promote filamentation, and overexpression of FLO8 is sufficient to drive an increase in their expression

Many of the genes bound and transcriptionally regulated by Flo8 or Mfg1 upon exposure to serum have been implicated in morphogenesis (Fig 5A), suggesting that they could be key downstream targets through which Flo8 and Mfg1 regulate C. albicans filamentation. We leveraged this data to probe circuitry downstream of the relatively uncharacterized regulator, Mfg1. We initially focused on a subset of genes that were direct targets of Mfg1 and for which expression was altered in the corresponding condition in the mfg1Δ/mfg1Δ mutant, including the positive regulators of filamentation TEC1, ROB1, HSP21, IHD1 and SUR7, as well as the negative regulator CUP9. We determined if the effectors bound and regulated by Mfg1 were sufficient to promote filamentous growth in the absence of MFG1 by overexpressing each positive regulator in an mfg1Δ/mfg1Δ background using the strong tetO promoter, or by deleting negative regulators of filamentation (Fig 6A and S6 Fig). Overexpression of ROB1, HSP21, IHD1, or SUR7, or deletion of CUP9, did not restore the ability of the mfg1Δ/mfg1Δ mutant to filament (S6A Fig and S6B Fig), suggesting that none of these effectors alone are sufficient to modulate Mfg1-mediated filamentation. As previously reported, overexpression of the transcription factor encoded by TEC1 in an otherwise wild-type background resulted in robust filamentation, even in the absence of an inducing cue [35] (Fig 6A and S6C Fig). Strikingly, overexpression of TEC1 was sufficient to induce robust filamentation in the absence of Mfg1 (Fig 6A and S6C Fig), suggesting that TEC1 may be a key regulator of filamentation downstream of Mfg1. Interestingly, TEC1 overexpression also resulted in robust filamentation in the absence of Flo8 (Fig 6A and S6C Fig), suggesting that it acts downstream of both regulators.

To determine if Flo8 and Mfg1 cooperatively regulate TEC1 expression, we then examined binding of Flo8 and Mfg1 to the promoter of TEC1 in the absence of the other regulator. By ChIP-qPCR, we observed increased binding compared to the untagged control of both Flo8 and Mfg1 to the TEC1 promoter, especially in the presence of serum at 37˚C for 1 hour, which was significantly reduced in the absence of the other regulator (Fig 6B). This suggests a functional relationship between Flo8 and Mfg1 at the TEC1 promoter. To further explore this relationship, we measured transcript levels of FLO8 and MFG1, and protein levels of Flo8-TAP and Mfg1-TAP, in the absence of the other regulator. While deletion of Flo8 or Mfg1 had no effect on transcript level of the other regulator (S7A Fig), deletion of FLO8 resulted in an approximately 4-fold decrease in Mfg1-TAP levels (S7B Fig). This decrease in Mfg1-TAP protein levels may contribute to the decreased binding observed at the TEC1 promoter in the absence of FLO8/TAP (Fig 6B). Nevertheless, Flo8-TAP binding was reduced in the absence of Mfg1 despite the protein levels remaining stable (Fig 6B and S7B Fig), demonstrating a dependency of Flo8 on Mfg1 for binding to target promoters. We further confirmed this observation with another key regulator of filamentation, BRG1, where we observed that deletion of either MFG1 or FLO8 significantly reduced binding of the other regulator at the BRG1 promoter (S8A Fig). In addition, we observed that TEC1 and BRG1 expression were decreased in the absence of either Flo8 or Mfg1 (Fig 6C and S8B Fig), illustrating that these regulators both bind to the promoters and regulate expression of a subset of key regulators of filamentation.
Divergence of a regulatory complex governing fungal filamentation

A) Wild type  mfg1Δ/mfg1Δ  flo8Δ/flo8Δ  tetO-TEC1/tetO-TEC1

YPD, 30°C  Mfg1 -  Mfg1 -  -  Mfg1 -

10% serum, 37°C  Mfg1 -  Mfg1 -  -  Mfg1 -

B) TEC1 promoter

| TEC1 promoter | Relative Recovery of Input |
|---------------|---------------------------|
| Flo8-TAP      | 20 ± 3                   |
| Flo8-TAP mfg1Δ/mfg1Δ | 15 ± 2               |
| TEC1 promoter | 30 ± 5                   |
| TEC1 promoter | 25 ± 4                   |

C) Relative Expression of TEC1

| Relative Expression of TEC1 | Untreated  | 10% serum, 37°C |
|-----------------------------|------------|-----------------|
| Wild type                   | 1.5        | 1.0             |
| flo8Δ/flo8Δ                 | 0.5        | 0.3             |
| mfg1Δ/mfg1Δ                 | 0.2        | 0.1             |

D) Relative Expression of TEC1 (Normalized to PMA1 and RIP1)

| Relative Expression of TEC1 | Untreated  | 10% serum, 37°C |
|-----------------------------|------------|-----------------|
| Wild type                   | 5.0        | 4.0             |
| tetO-FLO8/FLO8              | 2.5        | 2.0             |
| mfg1Δ/mfg1Δ                 | 1.0        | 0.5             |
| mfg1Δ/mfg1Δ                 | 0.5        | 0.2             |
Fig 6. Mfg1 and Flo8 bind to the promoter of TEC1 to regulate its expression, and overexpression of FLO8 drives TEC1 expression, enabling filamentation in the absence of Mfg1. A) Overexpression of TEC1 results in filamentous growth in the absence of MFG1 or FLO8. Cells were grown in YPD at 30˚C or in YPD with 10% serum at 37˚C for 5 hours. Scale bar is 20 µm. B) Binding of Flo8-TAP and Mfg1-TAP to the promoter of TEC1 was assessed using ChIP-qPCR. Shown is the fold-enrichment over the untagged parental strain, which is set at 1. Asterisks indicate P < 0.01 (**) or P < 0.05 (*), relative to the wild-type parent in the respective condition (two-tailed unpaired t-test). Error bars represent technical triplicates. Assays were performed in biological duplicate. C) TEC1 expression is reduced in the absence of Flo8 or Mfg1. Cells were grown in YPD at 30˚C for 3.5 hours, pelleted and washed, and then transferred to YPD at 30˚C or to YPD at 37˚C with 10% serum for 1 hour. Transcript levels were monitored using qRT-PCR and normalized to TEF1 and RIP1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.001 (***) relative to the wild-type strain in each respective condition (two-way ANOVA, Bonferroni Multiple Comparison Test). D) TEC1 expression is increased in strains overexpressing FLO8. Cells were grown in YPD at 30˚C for 4.5 hours. Transcript levels were monitored using qRT-PCR and normalized to TEF1 and RIP1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.0001 (****), relative to the respective parental strain, or as indicated (one-way ANOVA, Bonferroni Multiple Comparison Test).

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Considering the key role for Tec1 in regulating filamentation, we further explored its relationship to Flo8 and Mfg1 by examining TEC1 expression in the tetO-FLO8/FLO8 overexpression strain. Interestingly, overexpression of FLO8 drives an approximately 4-fold increase in TEC1 expression relative to the wild type, which is decreased 2-fold in the absence of Mfg1 (Fig 6D). This is consistent with the fact that overexpression of FLO8 drives filamentation in the absence of an inducing cue, and to a lesser extent in the absence of MFG1 (Fig 2C and 2D). We further confirmed this observation by examining BRG1 expression in the tetO-FLO8/FLO8 strain, and similarly observed an increase in BRG1 expression upon overexpression of FLO8, which is reduced in the absence of Mfg1 (S8C Fig). Together, this suggests that Flo8 and Mfg1 cooperatively bind to the promoters of key regulators of filamentation, and that overexpression of FLO8 is sufficient to drive expression of these regulators and induce morphogenesis even in the absence of Mfg1.

Aneuploidy of chromosome 6 restores filamentous growth in an mfg1Δ/mfg1Δ mutant

To identify additional circuitry downstream of Mfg1 important for C. albicans filamentation, we turned to an alternative, unbiased approach and employed a novel selection strategy to evolve mutants capable of filamenting in a strain lacking MFG1. Our approach employed a dominant nourseothricin (NAT) resistance marker under the control of a filament-specific promoter (HWP1p), such that the expression of NAT occurs only when cells are undergoing filamentous growth, thereby enabling selection for filamentation on plates containing NAT. We introduced this system into an mfg1Δ/mfg1Δ mutant, and plated cells on filament-inducing conditions of rich medium containing 10% serum with a high concentration of NAT, with incubation at 37˚C for two days. We evolved three independent lineages in the mfg1Δ/mfg1Δ background with a restored capacity to undergo robust filamentation in liquid medium containing serum as observed after six hours of growth (Fig 7A). Notably, this filamentation phenotype was largely lost after growth in serum for 24 hours, whereas wild-type cells were still filamentous, suggesting that filamentation could not be maintained in the absence of MFG1 (S9A Fig). This demonstrates that our HWP1p-NAT selection strategy provides adequate selective pressure to evolve the capacity for morphogenesis in filamentation-defective mutants.

To determine the genetic basis for this restoration of filamentation in the absence of MFG1, we performed whole genome sequencing of the three independently evolved mutants. Although no single nucleotide variants or small insertions or deletions in coding regions were identified that were common to all of the evolved lineages, we did observe a common decrease in copy number of chromosome 4 and an increase in copy number of chromosome 6 relative to the parent (Fig 7B). Upon closer examination, the apparent loss of chromosome 4 was due to a chromosome 4 trisomy in the parental strain, resulting in all evolved lineages having two
copies of this chromosome. We therefore focused our analysis on the increased copy number of chromosome 6. While many genes involved in filamentation are located on chromosome 6, a likely candidate responsible for restoring filamentous growth was \textit{FLO8}, as we previously observed that overexpression of \textit{FLO8} was sufficient to promote morphogenesis in the absence of \textit{MFG1} (Fig 2D). Increased expression of \textit{FLO8} in the evolved strains was verified by qRT-PCR (S9B Fig). To determine if the increase in \textit{FLO8} copy number was responsible for restoring filamentation of the \textit{mfg1Δ/mfg1Δ} mutant, we deleted one allele of \textit{FLO8} from one of the evolved filamentous mutants and observed a corresponding decrease in ability of this strain to filament in response to serum (Fig 7C). This suggests that the chromosome 6 amplification may have enabled filamentation in response to serum by increasing the copy number of \textit{FLO8}. Furthermore, we examined \textit{TEC1} and \textit{BRG1} expression in this evolved strain, as we had observed that \textit{FLO8} overexpression results in increased expression of these important regulators of filamentation, even in the absence of \textit{Mfg1} (Fig 6D and S8C Fig). Indeed, when grown in filament-inducing conditions, we observe a small but significant increase in both \textit{TEC1} and \textit{BRG1} transcript levels in an \textit{mfg1Δ/mfg1Δ} evolved strain with a chromosome 6 amplification (S9C Fig). Notably, we also attempted to evolve filament-capable mutants in the \textit{flo8Δ/flo8Δ} background and the \textit{mfg1Δ/mfg1Δ flo8Δ/flo8Δ mss11Δ/mss11Δ} triple deletion mutant, yet were unable to obtain \textit{NAT}-resistant colonies when plating cells on medium containing serum and a high concentration of \textit{NAT} (S9D Fig), confirming that \textit{Flo8} is critical for morphogenesis in this experimental context. Together this suggests that \textit{Flo8} plays a pivotal role in enabling filamentation even in the absence of \textit{MFG1}, and may do so by driving expression of key regulators of filamentation, including \textit{TEC1} and \textit{BRG1}.

To further explore if \textit{FLO8} amplification underpins this adaptive mechanism to restore filamentation by chromosome 6 amplification, we engineered a strain in which both alleles of \textit{FLO8} were deleted from the endogenous location on chromosome 6 and integrated at a distinct location in the genome, on the right arm of chromosome 5. To confirm that \textit{FLO8} in a new chromosomal context is functional, we verified expression of two genes whose expression increase in the \textit{flo8Δ/flo8Δ} null and \textit{mfg1Δ/mfg1Δ flo8Δ/flo8Δ} double mutant (S2 Table), but are restored to wild-type levels when \textit{FLO8} is integrated on chromosome 5 (S10A Fig). Using our established selection regime, we successfully evolved five lineages in the \textit{mfg1Δ/mfg1Δ} background with \textit{FLO8} located on chromosome 5 that were capable of undergoing filamentation upon growth in liquid medium containing serum (Fig 8A), with many but not all reverting to yeast form growth after 24 hours (S10B Fig). Whole genome sequencing revealed that none of the newly evolved lineages possessed an aneuploidy of chromosome 6 (Fig 8B), providing evidence that the presence of \textit{FLO8} on chromosome 6 was an important determinant of aneuploidy formation in the original lineages (Fig 7). Consistent with the importance of \textit{FLO8} in aneuploidy formation, one of the newly evolved lineages acquired an amplification of the right arm of chromosome 5 (Evo #C1-1), on which \textit{FLO8} was located in this background (Fig 8B). A significant increase in \textit{FLO8} expression in this evolved lineage was verified by qRT-PCR (S10C Fig). Intriguingly, the other newly evolved lineages acquired distinct chromosomal alterations that were independent of \textit{FLO8} copy number, including amplification of the right...
portion of chromosome 1 (Evo #C1-2), amplification of the left portion of chromosome 7 (Evo #C1-1 and Evo #C1-2), and loss of the right portion of chromosome 7 (Evo #C1-2) (Fig 8B). Interestingly, NRG1 is located on the right arm of chromosome 7, and deletion of NRG1 allows filamentous growth in an mfg1Δ/mfg1Δ mutant (Fig 1B), suggesting that reduced NRG1 dosage might be an adaptive mechanism to promote filamentation in this evolved strain. In addition to chromosomal alterations, a few SNPs were identified in the evolved lineages, including in

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Fig 8. Evolution of filament-capable mfg1Δ/mfg1Δ mutants with FLO8 at a non-native locus is not accompanied by chromosome 6 triplication. A) An mfg1Δ/mfg1Δ mutant with FLO8 located on chromosome 5 (at the HIS1 locus) and expressing the NAT (nourseothricin) resistance marker under the control of the HWP1 promoter evolved the ability to filament upon selection on NAT and serum. Mutants Evo #C1-1 and Evo #C1-2 arose from the same overnight culture but showed distinct phenotypes in their ability to filament at later time points. Mutants Evo #C1, C2, C3, and C4 are all independently generated. Cells were grown in YPD at 30˚C or in YPD with 10% serum at 37˚C for 6 hours. Scale bar is 20 μm. B) The genomes of five evolved filamentous mutants in the mfg1Δ/mfg1Δ background were sequenced and profiled for copy number variants using YMAP.
the predicted kinase gene YAK1 and upstream of the putative transcription factor gene HOT1 (S5 Table). Together, these results suggest that amplification of FLO8 is a major driving force of aneuploidy formation in order to restore filamentation in the absence of MFG1, and emphasizes that additional factors remain to be discovered.

Discussion

Fungal species rely on morphogenetic transitions for diverse biological processes, including reproduction, stress adaptation and virulence [2,3]. The transcriptional regulators Flo8, Mfg1 and Mss11 work as a complex in the model yeast S. cerevisiae to control the expression of genes that enable filamentous growth [26]. Although these regulators are conserved in C. albicans, as is their physical interaction under basal conditions, we established here that Flo8, Mfg1 and Mss11 possess divergent roles in regulating C. albicans filamentation (Fig 1 and Fig 2). Genome scale analyses supported the model that although Flo8 and Mfg1 are capable of acting in concert to regulate gene expression, the majority of their transcriptional targets are distinct and environmentally contingent (Fig 5 and S5 Fig). Moreover, we described a novel approach for mapping genetic circuitry important for C. albicans filamentation, and discovered that increasing FLO8 copy number can drive aneuploidy formation to restore morphogenesis in the absence of MFG1 (Fig 7). Thus, we reveal complex genetic circuitry through which Flo8, Mfg1 and Mss11 regulate filamentation, and implicate a new facet of genomic plasticity in pathogen adaptation.

A global analysis exploring regulators of filamentation in S. cerevisiae and C. albicans identified the previously uncharacterized Mfg1 as a core regulator of morphogenesis in both species, and showed that it physically interacts with transcriptional regulators Flo8 and Mss11 [26]. In S. cerevisiae, these three proteins act in concert to enable pseudohyphal growth, biofilm formation and invasive growth through transcriptional regulation of target genes [26]. Genome-scale analyses demonstrated that deletion of any component of this complex largely abrogated binding of other complex members to target genes [26], highlighting the functional interdependence of these transcriptional regulators. In contrast, we identified considerable divergence in complex member function in C. albicans. Although both Flo8 and Mfg1 were required for filamentous growth, Mss11 was dispensable for hyphal formation in response to any cue that we tested, and its physical interaction with Mfg1 was reduced in filament-inducing conditions (Fig 1 and Fig 5B). Although Flo8 and Mfg1 target largely different genes, they do cooperatively bind to a subset of key regulators of filamentation, including TEC1 and BRG1. Mfg1 and Flo8 binding to these promoters is reduced in the absence of the other regulator (Fig 6B, S8A and S10D Figs), leading to reduced expression of targets such as TEC1 and BRG1 (Fig 6C and S8B Fig). However, overexpression of FLO8 was sufficient to result in increased expression of TEC1 and BRG1, resulting in filamentous growth even in the absence of Mfg1 (Fig 2, Fig 6D, S8C Fig, and S10D Fig), and suggesting that Mfg1 is dispensable for Flo8 binding to at least a subset of key targets or that overexpression of FLO8 can compensate for the loss of Mfg1. Although Flo8 was required for filamentation in response to loss of the filamentation repressors Nrg1 and Lrg1, Mfg1 was dispensable, highlighting another distinct facet of their consequences on morphogenesis. Interestingly, mfg1Δ/mfg1Δ mutants resembled mutants lacking Eed1, a regulator of hyphal extension, which form germ tubes after a short exposure to filament-inducing cues, but revert to yeast after prolonged exposure to the inducing cue [36]. This suggests that Mfg1 may be a key regulator of hyphal extension as opposed to initiation. Together, these findings support evolutionary rewiring of complex member function compared with that observed in S. cerevisiae.
Experimental evolution has been used extensively to investigate circuitry governing microbial drug resistance, virulence and filamentation. Such studies have revealed that aneuploidies are a prevalent adaptive mechanism in fungi, involving ploidy gain or reduction, upon exposure to stressful environmental conditions [37]. The acquisition of aneuploidies in *C. albicans* upon exposure to azoles, the most widely deployed class of antifungal, occurs readily in both *in vitro* experimental evolution experiments, as well as in clinical settings [38–40]. Most common is the formation of an isochromosome on the left arm of chromosome 5 (i(5L)), which increases the copy number of the azole drug target gene *ERG11*, as well as a gene encoding a transcriptional regulator of an efflux pump, *TAC1* [41]. Experimental evolution has also identified adaptations that enable filamentation of non-filamentous *Candida* within mammalian macrophages, where filamentation is favoured as it enables host cell escape [42,43]. A single nucleotide polymorphism in the chitin synthase gene *CHS2* was found to restore filamentation in *Candida glabrata* [43], and a mutation in *SSN3*, encoding a component of the Mediator complex, facilitated filamentation in a non-filamentous *C. albicans* strain [42]. We employed a novel selection method to restore morphogenesis in non-filamentous *C. albicans* mutants by placing a dominant NAT resistance cassette under the control of the filament-specific *HWP1* promoter. A key advantage of this strategy is that it can be easily applied to diverse genetic backgrounds and environmental conditions. With this approach, we restored filamentous growth in an *mfg1Δ/mfg1Δ* mutant and identified an increase in chromosome 6 copy number as a mechanism to enable filamentation (Fig 7). Notably, recent findings identified a chromosome 6 trisomy in a parasexual isolate with increased virulence and capacity to filament [44]. Furthermore, preferential amplification of *C. albicans* chromosome 6 has been identified upon passage in a murine model of oropharyngeal candidiasis [45], suggesting that amplification of chromosome 6 is also important for adaptation *in vivo*. Analogous adaptive strategies have been reported in *S. cerevisiae*, where the gain of a single chromosome was sufficient to transition between smooth and fluffy colony morphologies [46]. Although genomic plasticity has been associated with altered morphologies in fungal pathogens [45,47–52], our work provides the first example of aneuploidy formation as a causative mechanism enabling filamentous growth in the context of *C. albicans* experimental evolution.

As the frequency and severity of fungal infections continue to climb, the necessity for novel therapeutic interventions is of utmost importance. Given the limited number of antifungal classes uncovered by traditional approaches of targeting essential proteins, targeting regulators of pathogen-specific virulence traits as a therapeutic strategy is attracting increasing interest [15]. For *C. albicans*, the transition between yeast and hyphae is of particular relevance, as both forms are required for virulence. Many small molecules with antifungal properties also have profound effects on fungal dimorphism, including the azole class of antifungals [53], compounds that target the molecular chaperone Hsp90 [54], the promiscuous protein kinase inhibitor staurosporine [55], the natural product beauvericin [56], and the broad-spectrum metal chelator DTPA [57]. Various other small molecules have also been described as able to modulate the transition between yeast and hyphae [58]. Although large-scale chemical biology screens typically monitor pathogen viability to reveal bioactive molecules, recently a screen to identify inhibitors of *C. albicans* adhesion uncovered filastatin, an inhibitor of hyphal formation [59]. Analogous approaches using expanded libraries of chemical matter have the potential to reveal additional small molecules capable of attenuating fungal virulence. Intriguingly, targeting specific aneuploidies with small molecules has also been described as a potential strategy, specifically to treat azole-resistant *C. albicans* harbouring (i(5L)) [60]. Thus, exploring circuitry governing *C. albicans* filamentation has the potential to reveal new strategies to cripple fungal pathogens and also to uncover fascinating biological insights into adaptive mechanisms governing key virulence traits.
Materials and methods

Strains and culture conditions

Archives of *C. albicans* and *S. cerevisiae* strains were maintained at −80°C in rich medium (YPD) or in synthetic defined (SD) medium with 25% glycerol. YPD was prepared as follows: 1% yeast extract, 2% bactopeptone, 2% glucose, with 2% agar for solid medium. SD was prepared as follows: 6.7 g/L yeast nitrogen base, 2% glucose, supplemented with amino acids as necessary. Strains were propagated in YPD or SD medium as required. Spider medium was prepared as previously described [61]. RPMI was prepared as follows: 10.4 g/L RPMI-1640, 3.5% MOPS, 2% glucose, supplemented with an additional 5 mg/mL histidine as required, pH 7. Synthetic low ammonia dextrose (SLAD) solid medium plates supplemented with 15 μM leucine and 10 μM histidine were prepared as previously described [26,62]. Geldanamycin was obtained from LC laboratories, G-4500, and was dissolved in DMSO. Heat-inactivated newborn calf serum (Gibco) was used in YPD at 10%. All strains used in this study are listed in S5 Table and construction is described in S1 Text. To select for nourseothricin (NAT)-resistant mutants, NAT (Jena Bioscience) stock solution was prepared in water at a concentration of 250 μg/mL and YPD plates were supplemented with 150 μg/mL NAT. The SAP2 promoter was induced to drive expression of the FLP recombinase to excise the NAT marker cassette [63,64]. All plasmids used in this study are listed in S6 Table and all oligonucleotide sequences used in this study are included in S7 Table.

Microarray profiling

Each microarray experiment was conducted in triplicate. Cells were grown overnight in YPD at 30°C, diluted to OD_{600} of 0.2, and grown to mid-log phase, at which point 10% serum was added and cultures were moved to 37°C for 1 or 3 hours. Cultures were pelleted at 3000 rpm for 5 min and frozen overnight at −80°C. RNA was isolated using the QIAGEN RNeasy kit and RNasefree DNase (QIAGEN). Microarray experiments were performed essentially as described, using a high-density tiling array containing 240,798 unique 60-mer probes [65,66]. Briefly, 20 μg of RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and oligo(dT)21 in the presence of Cy3- or Cy5-dCTP (Invitrogen). Template RNA was degraded using 2.5 units RNase H (USB) and 1 μg RNase A (Pharmacia), incubated at 37°C for 15 min. Labeled cDNA was purified with a QIAquick PCR Purification Kit (QIAGEN). The hybridization was carried out with DIG Easy Hyb Solution (Roche Diagnostics) containing 0.45% salmon sperm DNA and 0.45% yeast tRNA at 42°C for 24 hours in a Slide-Booster Hyb chamber SB 800 (Advalytix, Brunthtal, Germany) with regular microagitation. The slides were washed once in 1.0% SSC (0.15 M NaCl and 0.015 M sodium citrate) with 0.2% SDS at 42°C for 5 min; twice in 0.1% SSC with 0.2% SDS at 42°C for 5 min; and once in 0.1% SSC at 24°C for 5 min, followed by four rinses in 0.1% SSC. The microarray slides were air dried before being scanned using a ScanArray Lite microarray scanner (Perkin Elmer). Microarray data were analyzed with GeneSpring GX v7.3 (Agilent Technologies), and genes with statistically significant \( P < 0.05 \) changes in transcript abundance of ≥1.5-fold were identified with a volcano plot and compared to other lists of significantly modulated genes. Data are accessioned at NCBI GEO GSE117477.

Whole-genome location profiling by ChIP-chip

ChIP-chip was performed as previously described [65,66]. In brief, binding locations were determined in duplicate ChIP-chip experiments using a high density tiling array containing 240,798 unique 60-mer probes. Fluorescence intensities were quantified using ImaGene.
software 9.0 (BioDiscovery Inc.), background corrected, and normalized for signal intensity (Lowess normalization). The significance cut-off was determined using the distribution of log-ratios for each factor. It was set at two standard deviations from the mean of log transformed fold enrichments (cut off log ratio of 0.4). ORFs with a binding peak within the 1500 bp 5' region were identified as targets. Values shown are an average of two biological replicates of tagged and mock constructs. Data are accessioned at NCBI GEO GSE117477.

**Whole genome sequencing**

To select for filamentous mutants, 4x10^7 cells from independent overnights were plated on YPD+10% serum +250 μg/mL NAT. NAT resistant colonies appeared after 2 days at 37˚C. Genomic DNA was isolated with phenol chloroform, as described previously [67]. Libraries were prepared using the NexteraXT DNA Sample Preparation Kit following the manufacturer’s instructions (Illumina). Libraries were purified with AMPure XP beads (Agencourt) and library concentration was quantified using a Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies) and a Qubit High Sensitivity dsDNA fluorometric quantification kit (Life Technologies). DNA Libraries were sequenced using paired end 2x250 flow cells on an Illumina MiSeq (Creighton University). Copy number variation was visualized using Y

MAP [68]. The sequence data is publicly available on the NCBI Sequence Read Archive with accession number SRP124459.

**ChIP-qPCR**

Flo8-TAP and Mfg1-TAP binding at the \textit{BRG1} and \textit{TEC1} promoters were analyzed by ChIP-qPCR, as described in reference [69], with the following modifications. Cells were grown in YPD to middle-late log phase (OD_{600} 4–5), collected by centrifugation and reinoculated to OD_{600} of 1 in 50 mL pre-warmed 30˚C YPD or 75 mL 37˚C YPD with 10% new-born calf serum (Gibco) and grown for 1 hour before fixation with 1% formaldehyde for 20 minutes. Cells were lysed by bead beating for 5 x 1 minute, and chromatin was sheared by probe sonication (40% amplitude) for 4 x 16 seconds and in a Bio-disruptor water bath sonicator (high setting; 30 seconds on/ 30 seconds off) for 4 x 5 minute intervals. 300 μL of sonicated cell lysates were incubated with 20 μL rabbit IgG agarose beads (Sigma) at 4˚C overnight. Beads were extensively washed with wash buffer, deoxycholate buffer and TE. Final ChIP products were eluted and reverse-crosslinked in TE-SDS buffer and purified by PCR purification column (Qiagen). An untagged parental strain (SN95) was tested in parallel as a control, where the tagged cells grown in untreated or hyphal-inducing conditions were normalized to the untagged parents grown in either untreated or hyphal-inducing conditions, respectively. Data analyses were performed similarly as described in reference [69]. Binding was assessed by qPCR using primers oLC7051/oLC7054 (\textit{BRG1}) and oLC7371/oLC7372 (\textit{TEC1}).

**Immunoblotting**

Immunoblotting to monitor Flo8-TAP and Mfg1-TAP protein levels was performed as described in reference [69]. Crude cell lysates were resolved on 6% SDS-PAGE and probed by a rabbit polyclonal TAP antibody (Invitrogen; CAB1001). A gel slice that does not contain immunoblotting signals was stained by Simple Blue Safe (Invitrogen) as a loading control.

**Affinity purification mass spectrometry**

Cell preparation and protein extraction. GFP-tagged Mfg1 or GFP-tagged Enol1 (negative control) strains were grown in YPD overnight at 30˚C. Cells were diluted to an OD_{600} of
0.1 in 500 mL of YPD (2 x 500 mL for untreated cells) and grown to an OD of 0.6–0.8 at 30˚C, at which point untreated cells were harvested at 4000 rpm for 20 minutes. Cells to be treated with serum were washed with PBS and grown for an additional 1 hour or 3 hours in the presence of 10% heat-inactivated new-born calf serum (Gibco), at which point cells were harvested as before.

To extract proteins, lysis buffer (50 mM HEPES-Na (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM DTT, 0.1% NP-40, 1x ROCHE protease inhibitors) was added 1:1 to the cell pellets (g: mL), and vortexed at 4˚C with an equivalent volume of glass beads (0.5 mm) for 4 x 1 minute, with 1 minute on ice in between. A needle (27 ½) was used to poke a hole in the tube, which was then spun at 1000 rpm for 1–2 minutes at 4˚C. This lysate was sonicated (Q SONICA, probe CL-18, power 30%) at 4˚C for 3 x 10 seconds with 3 seconds between pulses. Twenty-five units of Benzonase Nuclease (EMD Millipore) was added and samples were rotated at 4˚C for 30 minutes, before centrifugation at 14,000 rpm for 20 minutes at 4˚C.

GFP-tagged proteins were affinity-purified using the GFP-Trap resin (Chromotek). Resin was equilibrated three times with 1 mL of lysis buffer, and 25 μL of resin was used per 1 L of culture. Extracted proteins were added to resin and incubated at 4˚C for 2 hours with rotation. Beads were washed with 1 mL of lysis buffer, followed by 1 mL of wash buffer (20 mM Tris-HCl (pH 8.0), 2 mM CaCl₂). Beads were then washed with 20 mM Tris-HCl (pH 8.0), and incubated with 1 μg of trypsin (Sigma) at 37˚C for 4 hours with rotation. Beads were removed magnetically and 0.5 μg of trypsin was added for incubation at 37˚C without rotation overnight. Formic acid was then added to a final concentration of 0.2%, and dried. Peptides were stored at -80˚C until analysis.

**MS/MS.** Affinity purified and digested peptides were analyzed using nano-HPLC (High-performance liquid chromatography) coupled to MS on the AB SCIEX 5600 TripleTOF in data-dependent acquisition (DDA) mode. Nano-spray emitters were generated from fused silica capillary tubing, with 75 μm internal diameter, 365 μm outer diameter and 5–8 μm tip opening, using a laser puller (Sutter Instrument Co., model P-2000, with parameters set as heat: 280, FIL = 0, VEL = 18, DEL = 2000). Nano-spray emitters were packed with C18 reversed-phase material (Reprosil-Pur 120 C18-AQ, 3 μm) resuspended in methanol using a pressure injection cell. 5 μL of each sample was directly loaded at 400 nl/min for 10 min onto a 75 μmx10 cm nano-spray emitter. Peptides were eluted from the column with an acetonitrile gradient generated by an Eksigent NanoLC-Ultra 1D plus, and analyzed on a TripleTOF 5600 instrument (AB SCIEX, Concord, Ontario, Canada). The gradient was delivered at 200 nl/min from 2% acetonitrile with 0.1% formic acid to 35% acetonitrile with 0.1% formic acid using a linear gradient of 90 min. This was followed by a 5 min wash with 80% acetonitrile with 0.1% formic acid, return to 2% acetonitrile over 5 min and equilibration for another 15 min at 2% acetonitrile with 0.1% formic acid. The total DDA protocol is 120 min. The first DDA scan had an accumulation time of 250ms within a mass range of 400–1250 Da. This was followed by up to 20 MS/MS scans, with accumulation time of 100 ms for each MS/MS scan. Each candidate ion was required to have a charge state from 2–4 and a minimum threshold of 250 counts per second, isolated using a window of 50 mDa. Previously analyzed candidate ions were dynamically excluded for 15 seconds.

**AP-MS data analysis and visualization.** Mass spectrometry data generated were stored, searched and analyzed using ProHits laboratory information management system (LIMS) platform [70]. Within ProHits, WIFF files were converted to an MGF format using the WIFF2MGF converter and to an mzML format using ProteoWizard (V3.0.10702) and the AB SCIEX MS Data Converter (V1.3 beta). The data was then searched using Mascot (V2.3.02) [71] and Comet (V2016.01 rev.2) [72]. The spectra were searched with the C. albicans sequences in the RefSeq database (txid5476[Organism:exp]) acquired from NCBI, supplemented with...
“common contaminants” from the Global Proteome Machine (GPM; ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta) and forward&reverse sequences (labeled “DECOY”) for a total of 30,018 entries. Database parameters were set to search for tryptic cleavages, allowing up to 2 missed cleavages sites per peptide with a mass tolerance of 35ppm for precursors with charges of 2+ to 4+ and a tolerance of 0.15 amu for fragment ions. Variable modifications were selected for deamidated asparagine and glutamine and oxidized methionine. Results from each search engine were analyzed through TPP (the Trans-Proteomic Pipeline, v.4.7 POLAR VORTEX rev 1) via the iProphet pipeline [73].

SAINTexpress version 3.6.1 was used as a statistical tool to calculate the probability of potential protein-protein interactions from background contaminants using default parameters [74]. SAINT analysis was performed using two biological replicates per bait. Six negative control experiments were conducted using GFP-Eno1 as bait. Eno1-GFP cells were grown and treated identically to the GFP-Mfg1 cells. Controls were compressed to 2 samples and two unique peptide ions and a minimum iProphet probability of 0.95 were required for protein identification. SAINT probabilities were calculated independently for each sample, averaged (AvgP) across biological replicates and reported as the final SAINT score. Only SAINT scores with a false discovery rate (FDR) ≤ 1% were considered high-confidence protein interactions. Dot plots were generated using the “Dot plot generator” tool in ProHits-viz [75] using SAINTexpress file generated from ProHits. Parameters set in the tool were: primary FDR cutoff = 0.01, secondary FDR cutoff = 0.05 and maximum spectral count = 50. Hierarchical clustering was performed using Pearson correlation and average linkage.

All MS files used in this study were also deposited as a complete submission to the MassIVE repository (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp) and assigned the accession number MSV000083218. The ProteomeXchange accession is PXD012004.

Quantitative reverse transcription-PCR (qRT-PCR)

Cells were grown overnight in YPD at 30˚C, diluted to an OD_600 of 0.1, and grown at 30˚C to mid-log phase. To verify overexpression of various genes in the mfg1Δ/mfg1Δ background, cells were subcultured to an OD_600 of 0.1 in YPD in the presence of 10% serum and grown at 37˚C to mid-log phase. To examine expression of TPK2, cells were grown as above, but subcultured to OD_600 of 0.1 and grown at 30˚C or 34˚C to mid-log phase. Cultures were pelleted and frozen at -80˚C. RNA extraction, complementary DNA synthesis and PCR were performed as previously described [18]. Reactions were performed in triplicate, for two biological replicates and data were analyzed using the BioRad CFX Manager 3.1. Transcript levels were examined using the primers, oLC1988/oLC1989 (TEF1), oLC2285/oLC2286 (ACT1), oLC3796/oLC751 (HWP1), oLC5038/oLC5320 (MFG1), oLC5040/oLC5323 (MS11), oLC5036/oLC5322 (FLO8), oLC4839/oLC4840 (SUR7), oLC4831/oLC4832 (HSP21), oLC2637/oLC2638 (ROB1), oLC5699/oLC829 (TPK2), oLC6472/oLC6473 (PMA1), oLC6476/oLC6477 (RIP1), oLC6739 (orf19.3897), oLC6736/oLC6737 (PGA26), oLC1456/oLC1457 (IHD1), oLC6718/oLC6719 (TEC1), and oLC2635/oLC2636 (BRG1). All oligonucleotide sequences are listed in S7 Table.

Microscopy and imaging

Pseudohyphal growth of diploid S. cerevisiae cells was assayed on SLAD solid medium supplemented with 15 μM leucine and 10 μM histidine as previously described [26,62]. Plates were incubated at 30˚C for 11 days. Images of single colonies were taken on a Zeiss Axio Observer. Z1 (Carl Zeiss). Invasive growth of haploid S. cerevisiae was assayed by spotting equal cell dilutions on YPD plates with 2% agar. Plates were incubated at 30˚C for 4 days before washing.
with water to remove non-invasive cells. Images were taken before and after washing with a Canon Power Shot A610. To image *C. albicans*, cells were grown overnight in YPD at 30°C. Cultures were diluted to an OD$_{600}$ of 0.1 and grown in the indicated conditions. Cells were imaged using differential interference contrast (DIC) microscopy using a Zeiss Axio Imager MI (Carl Zeiss). All images are representative of multiple fields of view from at least biological duplicate experiments.

For nuclear staining and microscopic analysis of cell morphology, cells were harvested, washed once with PBS and resuspended in 1 mL PBS containing 5 μg/mL Hoechst 33342. Following an incubation of 15 min in the dark at room temperature, cells were spun down and resuspended in 50 μL of PBS and imaged. Imaging was performed on a Zeiss Imager M1 upright microscope at 40X magnification on the green fluorescent protein (GFP) channel for GFP tagged proteins, the DAPI (4',6-diamidino-2-phenylindole) channel for nuclei stained with Hoechst 33342 and the DIC channel. At least three fields were imaged for each strain, in at least two biological replicates.

Supporting information

**S1 Text.** Details of strain and plasmid construction. (DOCX)

**S1 Fig.** Complementation of the flo8Δ/flo8Δ mutant with a wild-type allele of FLO8 restores its ability to filament. Cells were grown in the conditions indicated and imaged after 3.5 hours. Scale bar is 20 μm. (TIFF)

**S2 Fig.** Deletion of MSS11 does not block filamentous growth in a variety of inducing cues. Two independently generated mss11Δ/mss11Δ mutants, M1 from Fig 1 and a second mutant M2, show no defect in filamentation in response to any cue tested. Cells were grown in the conditions indicated, and were imaged after 3.5 hours, except for those grown in the presence of GdA, which were imaged after 24 hours. Scale bar is 20 μm. (TIFF)

**S3 Fig.** Replacing the native promoter of FLO8 or MSS11 with a tetracycline-repressible promoter in an mfg1Δ/mfg1Δ mutant results in overexpression of these regulators. Quantification of overexpression of FLO8 or MSS11 in the mfg1Δ/mfg1Δ mutant by qRT-PCR. Cells were grown in YPD at 30°C for 3 hours. Transcript levels were normalized to ACT1 and TEF1 and error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.0001 (***') relative to parental strain (two-tailed unpaired t-test). (TIFF)

**S4 Fig.** Flo8 and Mfg1 localize to the nucleus, and tagged proteins are functional. A) Cells expressing Mfg1-GFP or Flo8-GFP were grown in either YPD at 30°C or YPD with 10% serum at 37°C for 1 hour. Cells were treated with Hoechst 33342 dye to monitor nuclear localization. B) Cells expressing Flo8-TAP or Mfg1-TAP were grown in YPD with 10% serum at 37°C for 6 hours. Scale bar is 20 μm. C) Mfg1-GFP cells were grown in YPD at 30°C or in YPD with 10% serum at 37°C for 2 hours. Scale bar is 20 μm. (TIFF)

**S5 Fig.** ChIP-chip and transcriptional analyses reveal a core set of genes bound by both Flo8 and Mfg1, but also many condition-specific and temporal differences. A) Cells for ChIP-chip were grown in YPD at 30°C until mid-log phase (basal), and then treated for 1 hour...
or 3 hours in 10% serum at 37˚C. Genes were clustered using Cluster Gene 3.0 and visualized using JavaTreeView, where green represents an increased binding intensity. B) ChIP-chip analysis reveals a core set of genes bound by both Flo8 and Mfg1 under basal (untreated 30˚C) and filament-inducing (serum 37˚C) conditions (top Venn diagram). Our analysis also identified many unique targets under all environmental conditions, as well as substantial changes in promoter binding upon exposure to serum (bottom Venn diagrams). C) Wild-type, flo8Δ/mfg1Δ, mfg1Δ/mfg1Δ, or flo8Δ/flo8Δ mfg1Δ/mfg1Δ strains were grown as in A) for transcriptional analysis by microarray. Heat map was generated as in A. Plotted are the log\textsubscript{2} fold-change in expression of the mutant strain relative to wild type. D) Microarray analysis reveals that Flo8 and Mfg1 have distinct effects on gene expression (DEG: differentially expressed gene) and identifies temporal transcriptional changes that occur in response to serum. (TIFF)

**S6 Fig.** Overexpression or deletion of specific target genes downstream of Mfg1 do not restore the ability of an mfg1Δ/mfg1Δ mutant to filament. A) Select genes both bound and transcriptionally regulated by Mfg1 upon exposure to serum were overexpressed (positive regulators of filamentation), or deleted (negative regulator of filamentation) in an mfg1Δ/mfg1Δ mutant. Cells were grown in YPD at 30˚C, or in YPD with 10% serum at 37˚C for 5 hours. Scale bar is 20 μm. B) Quantification of overexpression of target genes by qRT-PCR. Overexpression of each target open reading frame was achieved by replacing the native promoter of one or both alleles for each gene with a tetracycline-repressible promoter, tetO. Cells were grown in YPD in the presence of 10% serum at 37˚C for 6 hours. Transcript levels were monitored using qRT-PCR and normalized to ACT1 and TEF1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate \( P < 0.0001 \) (***) relative to the parental strain (one-way ANOVA, Bonferroni Multiple Comparison Test). C) Replacing both native TEC1 promoters with the strong tetO promoter results in increased TEC1 expression. Cells were grown in YPD at 30˚C for 4 hours. Transcript levels were monitored using qRT-PCR and normalized to ACT1 and TEF1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate \( P < 0.0001 \) (***) relative to the parental strain (one-way ANOVA, Bonferroni Multiple Comparison Test).

(TIFF)

**S7 Fig.** Deletion of either FLO8 or MFG1 does not affect transcript levels of the other regulator, but Mfg1 protein levels are reduced in the absence of Flo8. A) Deletion of FLO8 or MFG1 does not alter the expression of the other regulator. Cells were grown in YPD at 30˚C for 4 hours. Transcript levels were monitored using qRT-PCR and normalized to ACT1 and TEF1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate \( P < 0.0001 \) (***) relative to the wild type (two-tailed unpaired t-test). B) Protein levels of Mfg1-TAP and Flo8-TAP in wild-type cells or cells lacking the other regulator were monitored with immunoblotting. Cells were grown in YPD at 30˚C for 5 hours. Staining with Simple Blue Safe (SBS) was used as a loading control. ‘Loading’ indicates relative amount of lysate loaded.

(TIFF)

**S8 Fig.** Mfg1 and Flo8 bind to the promoter of BRG1, whose expression increases upon FLO8 overexpression. A) Binding of Flo8-TAP and Mfg1-TAP to the promoter of BRG1 was assessed using ChIP-qPCR. Shown is the fold-enrichment over the untagged parental strain, which is set at 1. Asterisks indicate \( P < 0.01 \) (**) or \( P < 0.001 \) (***) relative to the wild-type parent in the respective condition (two-tailed unpaired t-test). Error bars represent the
standard deviation of technical triplicates. Assays were performed in biological duplicate. B) BRG1 expression is decreased in the absence of Flo8 or Mfg1. Cells were grown in YPD at 30°C for 3.5 hours, and then transferred to 37°C YPD with 10% serum for 1 hour. Transcript levels were monitored using qRT-PCR and normalized to PMA1 and RIP1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.001 (***) or P < 0.05 (*), relative to the wild-type strain in each respective condition (two-way ANOVA, Bonferroni Multiple Comparison Test). C) BRG1 expression is increased in strains overexpressing FLO8. Cells were grown in YPD at 30°C for 4.5 hours. Transcript levels were monitored using qRT-PCR and normalized to PMA1 and RIP1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.0001 (****, relative to the respective parental strain, or as indicated (one-way ANOVA, Bonferroni Multiple Comparison Test).

**S9 Fig.** Expression of FLO8 is increased in evolved lineages with increased FLO8 copy number, and mutants revert to yeast form growth after 24 hours in serum. A) Evolved mutants revert to yeast form after 24 hours of growth in serum. Cells were grown in YPD at 30°C or in YPD with 10% serum at 37°C for 24 hours. Scale bar represents 20 μm. B) Expression of FLO8 is increased in evolved lineages with increased FLO8 copy number. Cells were grown in YPD at 30°C for 3.5 hours. Transcript levels were monitored using qRT-PCR and normalized to PMA1 and RIP1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.001 (***) and P < 0.01 (**), relative to the parent (two-tailed unpaired t-test). C) Expression of BRG1 and TEC1 are increased in the evolved lineage Evo #A2 in the presence of 10% serum at 37°C. Cells were grown in YPD at 30°C for 3.5 hours, before being transferred to 37°C YPD with 10% serum for 1 hour. Transcript levels were monitored using qRT-PCR and normalized to PMA1 and RIP1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.05 (*), relative to the parental strain (two-tailed unpaired t-test). D) NAT-resistant colonies were not obtained when the flo8Δ/flo8Δ mutant or the mfg1Δ/mfg1Δ flo8Δ/flo8Δ mss11Δ/mss11Δ triple mutant were plated on 10% serum and 250 μg/mL of NAT. 4 x 10⁷ cells were plated in triplicate, and incubated for 72 hours.

**S10 Fig.** FLO8 at the HIS1 locus is functional, and FLO8 expression increases with FLO8 copy number. A) Expression of Flo8-repressed genes does not increase in the strain in which FLO8 is at the HIS1 locus on chromosome 5. Cells were grown in YPD at 30°C for 4 hours or in YPD with 10% serum at 37°C for 3 hours. Transcript levels were monitored using qRT-PCR and normalized to PMA1 and TEF1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.001 (**), relative to the wild type (two-way ANOVA, Bonferroni correction). B) Most filament-capable mfg1Δ/mfg1Δ mutants with FLO8 at a non-native locus revert to yeast form growth after 24 hours in serum. Cells were grown in YPD at 30°C or in YPD with 10% serum at 37°C for 24 hours. Mutants Evo #C1-1 and Evo #C1-2 arose from the same overnight culture but showed distinct phenotypes in terms of their ability to filament at a later time point. Mutants Evo #C1, C2, C3, and C4 are all independently generated. Scale bar is 20 μm. C) Expression of FLO8 is most increased in the evolved lineage with increased FLO8 copy number. Cells were grown in YPD at 30°C for 3.5 hours. Transcript levels were monitored using qRT-PCR and normalized to PMA1 and TEF1. Error bars represent standard error of technical triplicates. Assays were performed in biological duplicate. Asterisks indicate P < 0.001 (**), P < 0.01 (**), or P < 0.05 (*) relative to the parent (two-tailed unpaired t-test). D) A model depicting the roles of Mfg1 and
Flo8 in governing expression of key regulators of filamentation. Mfg1 and Flo8 are both required for optimal binding to the promoters of BRG1 and TEC1, however, overexpression of FLO8 in the absence of Mfg1 is sufficient to drive filamentation and expression of BRG1 and TEC1.

**S1 Table. ChIP-chip analysis.** List of targets bound by Mfg1 or Flo8 under basal conditions (30°C) or filament-inducing conditions (10% serum for one hour or 3 hours, 37°C).

**S2 Table. Microarray analysis.** List of targets whose expression is altered in the mfg1Δ/mfg1Δ, flo8Δ/flo8Δ, or mfg1Δ/mfg1Δ flo8Δ/flo8Δ double mutant, under basal conditions (30°C) or filament-inducing conditions (10% serum for one hour or 3 hours, 37°C).

**S3 Table. Genes bound and regulated by Mfg1 or Flo8.** List of targets bound by Mfg1 or Flo8 and whose expression is altered in the mfg1Δ/mfg1Δ or flo8Δ/flo8Δ mutants respectively.

**S4 Table. AP-MS MassIVE output files.** MassIVE Table 1: Sample description table. Baits are Mfg1-GFP grown in YPD at 30°C (MFG1_untreat), or in the presence of 10% serum at 37°C for 1 hour (MFG1_1) or for 3 hours (MFG1_3). Eno1-GFP was used as the negative control, grown in the same conditions. MassIVE Table 2: Protein identification evidence. MassIVE Table 3: MassIVE SAINTexpress v.3.6.1 output. Prey Accession is the NCBI protein accession number; Prey Gene is as per NCBI Entrez Gene. Spectral counts for the prey (column D, separated by '" delimiter; column E, summed, column F, averaged), number of replicates performed (column G), spectral counts for the prey across all negative controls (column H), Averaged probability across replicates (column I), maximal probability (column J), log Odds score (column K), Fold Change (counts in the purification divided by counts in the controls plus small factor to prevent division by 0; column L) and Bayesian FDR (column M) are listed for each bait-prey relationship and are directly from the SAINTexpress output. Columns N-P are the unique prey peptides as calculated through ProHits. Column Q is the prey protein length and column R is the UniProt accession number. The values for Mfg1, Flo8, and Mss11 are highlighted in yellow.

**S5 Table. Strains used in this study, including SNPs in the evolved lineages identified by whole genome sequencing.**

**S6 Table. Plasmids used in this study.**

**S7 Table. Oligonucleotides used in this study.**

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