Recording of DNA-binding events reveals the importance of a repurposed \textit{Candida albicans} regulatory network for gut commensalism

\textbf{Highlights}

- Eight regulators of \textit{C. albicans} mating also control its fitness as a gut commensal
- Calling Card-seq revealed DNA-binding activity of these regulators in colonized hosts
- Analysis of highly regulated target genes reveals effectors of fungal commensalism

\textbf{In brief}

\textit{Candida albicans} is a fungal pathobiont of the mammalian gut. Witchley, Basso, et al. show that transcription factors (TFs) that control fungal mating also control commensalism. Using Calling Card-seq and RNA-seq to determine TF DNA-binding and regulatory activity during host colonization, they identify chitinase Cht2 as a commensalism effector.
Recording of DNA-binding events reveals the importance of a repurposed *Candida albicans* regulatory network for gut commensalism

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**SUMMARY**

*Candida albicans* is a fungal component of the human gut microbiota and an opportunistic pathogen. *C. albicans* transcription factors (TFs), Wor1 and Efg1, are master regulators of an epigenetic switch required for fungal mating that also control colonization of the mammalian gut. We show that additional mating regulators, WOR2, WOR3, WOR4, AHR1, CZF1, and SSN6, also influence gut commensalism. Using Card-seq to record *Candida* TF DNA-binding events in the host, we examine the role and relationships of these regulators during murine gut colonization. By comparing in-host transcriptomes of regulatory mutants with enhanced versus diminished commensal fitness, we also identify a set of candidate commensalism effectors. These include Cht2, a GPI-linked chitinase whose gene is bound by Wor1, Czf1, and Efg1 in vivo, that we show promotes commensalism. Thus, the network required for a *C. albicans* sexual switch is biochemically active in the host intestine and repurposed to direct commensalism.

**INTRODUCTION**

*Candida albicans* is the major fungal commensal and opportunistic pathogen of humans. As a pathogen, *C. albicans* is responsible for syndromes ranging from simple vaginitis and oral thrush to devastating infections of the blood and internal organs. Life-threatening disease is restricted primarily to patients with underlying risk factors (Odds, 1987; Pfaller and Diekema, 2007), whereas virtually all humans encounter commensal *C. albicans* as part of normal gut, skin, and genitourinary microbiota (Drell et al., 2013; Hoffmann et al., 2013; Odds, 1988). Observations in gnotobiotic mice suggest that gut colonization confers immune benefits to the host by triggering the expansion of CD4+Th17 cells, a critical defense at mucosal barriers (Atarashi et al., 2013; Hoffmann et al., 2013; Odds, 1988). Likewise, analysis of human CD4+Th17 cells suggests that commensal *C. albicans* may entrain host immune responses to exogenous fungal pathogens (Bacher et al., 2019). Given the fundamental roles played by this pathobiont in human health and disease, it is important to understand the mechanisms that underpin its interactions with mammalian hosts.

Our group previously identified two *C. albicans* transcription factors (TFs), Efg1 and Wor1, with opposing roles in the regulation of gut commensalism (Figure 1A) (Pande et al., 2013). Efg1 inhibits commensalism, such that an efg1 null mutant strongly outcompetes wild-type (WT) *C. albicans* in a mouse model of gut colonization (Pande et al., 2013; Pierce and Kumamoto, 2012; Witchley et al., 2019; Liang et al., 2019). In contrast, Wor1 promotes commensalism, and a wor1 mutant is rapidly outcompeted by WT (Pande et al., 2013). Consistent with a special role for Wor1 in the gastrointestinal niche, WOR1 gene expression—which is barely detectable under *in vitro* conditions—is upregulated ~10,000-fold in the host digestive tract (Pande et al., 2013). Furthermore, forced expression of WOR1 (WOR1OE) via a heterologous promoter confers enhanced commensal fitness (Pande et al., 2013). Interestingly, the fitness advantage of WOR1OE does not emerge until after 5 to 10 days of host colonization, and it coincides with a morphological transition from the standard, oval-shaped “white” cell type to a larger, elongated gastrointestinal induced transition (“GUT”) cell type (Pande et al., 2013). Compared with white cells, the metabolism of GUT cells is optimized for nutrients available in the mammalian digestive tract. We hypothesize that WT *C. albicans* undergoes an analogous transition while in the host, but reverts to white morphology when excreted because of the loss of signals required for WOR1 expression. Besides their roles in commensalism, Efg1 and Wor1 are also “master regulators” of *C. albicans* mating (Huang et al., 2006; Srikantha et al., 2006; Zordan et al., 2006, 2007). Wor1 promotes and Efg1 opposes the white-to-“opaque” morphological transition, an epigenetic switch that confers sexual competency in this species. Most *C. albicans* strains are incapable of mating because they express a1→2, a potent transcriptional inhibitor of WOR1 that is jointly encoded by two heterologous alleles of
Figure 1. C. albicans sexual regulators control commensal fitness

(A) Regulation of gut commensalism. Efg1 inhibits (bar) and Wor1 promotes (arrow) fungal commensalism (Pande et al., 2013). Wor1 promotes the white-to-GUT developmental switch that confers enhanced fitness in the mammalian digestive tract.

(B) Regulation of mating. Efg1 is the central inhibitor and Wor1 the central activator of mating and the white-to-opaque switch. Six additional transcription factors interact with Efg1 and Wor1 via the depicted regulatory circuit (Hernday et al., 2013, 2016, 2010; Huang et al., 2006; Lohse et al., 2010; Tuch et al., 2010; Wang et al., 2011b; Zordan et al., 2006, 2007).

(C) WOR2, WOR3, CZF1, AHR1, and SSN6 regulate the commensal fitness of C. albicans. Each of the indicated null mutants was tested in 1:1 competitions with WT in a mouse model of stable gut colonization. Mutants with loss-of-fitness phenotypes are shown in blue, and those with gain-of-fitness in white (with black outline). Statistical comparisons were performed using the paired Student’s t test; ns, not significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Results for independent isolates of these mutants are presented in Figure S2.

(D) Comparison of the roles of eight fungal transcription factors in mating versus commensalism.
the mating type-like locus, MTLa and MTLz. To mate, diploid a/α white cells must lose one allele of MTL and switch to the opaque cell type, before opaque a and α cells fuse to generate tetraploid a/α progeny. In addition to Wor1 and Efg1, other TFs control sexual competency through a complex, self-reinforcing regulatory circuit that has been deduced from genetic epistasis experiments (Figure 1B; Du et al., 2015; Hernday et al., 2013, 2016; Lohse and Johnson, 2016; Lohse et al., 2010). Furthermore, chromatin immunoprecipitation (ChIP) experiments indicate that Wor1, Efg1, Wor2, Wor3, Wor4, Czf1, Ahr1, and Ssn6 engage in extensive cross-regulation, with each TF binding to its own promoter and to those for most or all of the other regulators in the circuit (Figures S1A and S1B; Hernday et al., 2013, 2016; Lohse et al., 2013, 2010; Lohse and Johnson, 2016; Srikantha et al., 2006; Vinces and Kumamoto, 2007; Wang et al., 2011a, 2011b; Zordan et al., 2006, 2007).

Given that an antagonistic relationship between Wor1 and Efg1 is conserved between mating and commensalism, we hypothesized that additional components of the mating circuit may participate in the regulation of commensalism. To test this idea, we performed a systematic analysis of wor2, wor3, wor4, czf1, ahr1, and ssn6 null mutants in a murine gut colonization model. Remarkably, all six TFs exert significant effects on commensal fitness, although the activities of several regulators differ between sex and commensalism. To validate these relationships in Candida colonizing the murine gut, we adapted a transposon-based Calling Card-seq method to record the in-host regulatory targets of DNA-binding proteins. Finally, by comparing the transcriptomes of regulatory mutants with enhanced versus diminished commensal fitness, we identified a set of candidate commensalism effectors. To determine whether sexual regulators besides Wor1 and Efg1 play roles in commensalism, we generated null mutants affecting CZF1, WOR2, WOR3, WOR4, AHR1, and SSN6. Homozygous disruptants of each open reading frame (ORF) were created in SN152, the same genetic background used for our previous observations of efg1 null mutant (note that the C. albicans genome is diploid). As shown in Figure 2B, efg1wor1 outcompetes WT in the gut colonization model. Nevertheless, it seemed to us that the enhanced fitness of efg1wor1 is less pronounced than our previous observations of efg1 single mutants (Pande et al., 2013; Witchley et al., 2019). To clarify the relative fitness of efg1 and efg1wor1, we compared the two strains directly in the same animals. As shown in Figures 2C and S2H, efg1 consistently outcompetes efg1wor1 in direct competition. The finding that an efg1wor1 double mutant manifests an intermediate phenotype between the strong gain-of-fitness phenotype of efg1 and the moderate loss-of-fitness phenotype of wor1 suggests that Wor1 and Efg1 regulate commensalism by a nonlinear pathway.

Genetic epistasis reveals functional relationships among commensal regulators
To clarify the relationships among selected sexual regulators in the control of commensalism, we turned to genetic epistasis analysis. This classical technique is used to infer the nature of regulatory pathways based on the phenotypes of strains that combine two mutations whose individual phenotypes are distinct. For example, imagine a process that is promoted by factor A and inhibited by factor B. If A and B participate in a linear regulatory pathway (Figure 2A, linear), then the loss-of-function phenotype of the more downstream regulator will predominate (be epistatic) in a mutant that lacks both factors. Conversely, if A and B are related by parallel or branched regulatory pathways (Figure 2A, parallel and branched), then the phenotype of a double mutant typically differs from (often assuming an intermediate phenotype between) those of either single mutant.

We began with Efg1 (a commensalism inhibitor) and Wor1 (a commensalism promoter). To create an efg1wor1 double mutant, both copies of the WOR1 ORF were disrupted in an existing efg1 null mutant (note that the C. albicans genome is diploid). As shown in Figure 2B, efg1wor1 outcompetes WT in the gut colonization model. Nevertheless, it seemed to us that the enhanced fitness of efg1wor1 is less pronounced than our previous observations of efg1 single mutants (Pande et al., 2013; Witchley et al., 2019). To clarify the relative fitness of efg1 and efg1wor1, we compared the two strains directly in the same animals. As shown in Figures 2C and S2H, efg1 consistently outcompetes efg1wor1 in direct competition. The finding that an efg1wor1 double mutant manifests an intermediate phenotype between the strong gain-of-fitness phenotype of efg1 and the moderate loss-of-fitness phenotype of wor1 suggests that Wor1 and Efg1 regulate commensalism by a nonlinear pathway.
Figure 2. Epistasis analysis suggests a branched regulatory pathway

(A) Examples of linear, parallel, and branched regulatory pathways. If factor A and factor B play opposite roles in a linear regulatory pathway, the double mutant $ab$ will exhibit the same phenotype as a null mutant affecting the more downstream regulator (mutant $a$, in this example). If factor A and factor B participate in a parallel or branched regulatory pathway, then the phenotype of the double mutant is likely to be intermediate between those of (A) and (B).

(B–G) Competitive fitness of double mutants in the gut colonization model. B. WT (ySN425) v. $efg1wor1$ (ySN1126).

(C) $efg1$ (ySN1338) v. $efg1wor1$ (ySN1126).

(legend continued on next page)
We next turned to Efg1 and Czf1 (a commensalism promoter). As shown in Figures 2D and S2I, an efg1czf1 double mutant is hypercompetitive in the gut colonization model, similar to the phenotype of efg1. However, unlike the previous result with efg1-wor1, efg1czf1 and efg1 exhibit identical commensal fitness in a direct competition experiment (Figure 2E). EFG1 is thus epistatic to CZF1, which suggests that Efg1 lies downstream of Czf1 in a linear regulatory pathway.

Because Wor1 and Czf1 both promote commensalism and share similar (loss-of-function) null mutant phenotypes, we paired a WOR1OE gain-of-fitness allele with a czf1 null allele for epistasis analysis. In competition with WT, czf1WOR1OE exhibits reduced commensal fitness (Figure 2F), similar to the phenotype of czf1 (Figure 1C). These results would seem to place CZF1 downstream of WOR1 in a linear regulatory pathway. However, as described in the introduction, Wor1 promotes commensalism by fostering the white-to-GUT switch after 5 to 10 days of exposure to the host digestive tract (Pande et al., 2013). Because czf1WOR1OE is outcompeted within 5 days (Figure 2F), there may be insufficient time to manifest any fitness advantage conferred by WOR1OE. To determine whether czf1WOR1OE white cells are able to undergo the white-to-GUT switch, we gavaged three animals with czf1WOR1OE white cells. Despite successful colonization of the murine GI tract (median titer of ~5 × 10^7 CFUs/g feces), czf1WOR1OE failed to produce any fully GUT colonies during 30 days of observation. However, interestingly, when homozygous czf1 deletions are introduced into a WOR1OE GUT strain, the GUT phenotype is maintained. Moreover, these czf1WOR1OE GUT cells are able to outcompete WT white cells in the gut colonization model (Figure 2G), albeit less robustly than a WOR1OE GUT strain (Pande et al., 2013). Taken together, these results suggest that Czf1 promotes the white-to-GUT switch but is not required for maintenance of the GUT phenotype and that Czf1 and Wor1 regulate commensalism by a nonlinear pathway.

**Calling Card-seq records TF binding events in *C. albicans***

To further explore the commensal function of these regulators, we sought a method to determine their DNA-binding activities during active colonization of the host. ChIP is a commonly used biochemical technique for identifying the binding sites of TFs. ChIP relies on extracts prepared from cells that are typically propagated under *in vitro* conditions that foster the activity of the relevant TF. Because there are no known laboratory conditions that accurately mimic the mammalian gastrointestinal tract, where commensalism occurs, we developed an alternative strategy that can be performed in cells that are propagated in a host.

Originally developed for use in *S. cerevisiae* and later in mammalian cells, the Calling Card-seq technique utilizes PiggyBac transposase (PBase) fused to a TF of interest to deposit PiggyBac transposon (PB) into a TTAA sequence near the binding site of the TF (Wang et al., 2011a, 2012). Transposition is mediated in living cells, and the binding sites are determined by subsequent DNA sequencing of transposon insertion sites across a cell population. Multiple modifications were required to adapt the technique for use in *C. albicans* (Figures S3A and S3 and STAR methods). First, until recently (Bijlani et al., 2019), there have been no stable, autonomously replicating plasmids available for use in *C. albicans*. Therefore, we integrated one or two copies of PB into a neutral locus in the *C. albicans* genome. Second, because *C. albicans* uses a non-universal genetic code (CTG encodes serine rather than leucine in this species; Santos et al., 2011), we synthesized a codon-optimized version of PBase. Finally, we created a custom *C. albicans* strain to facilitate the detection and selection of transposon excision and reinsertion events. Starting with Arg’His’Leu’ reference strain SN152, a copy of PB flanked by 5’ and 3’ halves of the ARG4 gene was inserted into a neutral chromosomal locus. Precise excision of PB from the donor site results in prototrophy for arginine. In addition, an intact copy of HIS1 was embedded within PB itself, such that cells that have undergone transposon excision followed by reinsertion elsewhere in the genome are prototrophic for both arginine and histidine. For experiments in animals, we created a second Calling Card strain that contains two copies of PB, one marked with HIS1 and the second marked with LEU2.

To validate the modified Calling Card system, we deployed it to map the binding sites of Efg1-PBase under *in vitro* conditions. A strain expressing untethered PBase (not fused to a TF) under the regulation of the EFG1 promoter was used as a control; note that PBase contains its own nuclear localization signal (Keith et al., 2008). Transposition was permitted for seven days, while the strains were propagated on solid culture medium (Lees 2% GlcNAc/agar pH 6.8) at 25°C. Transposition reintegrants were then selected by replica-plating onto medium lacking arginine and histidine. Genomic DNA from Arg’His’ colonies was used for inverse PCR and sequencing library preparation, as described in the STAR methods. After mapping of transposon insertion sites to the genome, background signal associated with untethered PBase was subtracted from the Efg1-PBase signal. Finally, high confidence Efg1-PBase binding sites were defined as promoters with at least 5 independent transposon insertion events (i.e., at different TTAA sequences) within a 1,000 bp window and p < 0.05 using a Poisson or hypergeometric test.

The modified Calling Card-seq technique identified 455 high confidence DNA-binding targets for Efg1-PBase (Table S1). Two groups have previously used ChIP to identify Efg1 binding targets in a/x strains. Lassak et al. reported 84 Efg1 binding sites in *C. albicans* yeast propagated in liquid YPD medium at 30°C (Lassak et al., 2011), and Nobile et al. reported 435 Efg1 binding sites in *C. albicans* biofilms propagated in Spider medium at 37°C (Nobile et al., 2012). Notably, the set of targets identified using Calling Card-seq exhibits significant overlap with both sets of ChIP-defined targets (63% in common with the Lassak dataset, p < 2.2 × 10^−16, and 222 in common with the Nobile
upregulation in the gut and yellow ≥2-fold downregulation (adjusted p < 0.05 using a linear fit model).

(E) Schematic of cross regulation among Efg1, Wor1, and Czf1 within the host digestive tract. Results obtained with in vivo CCS suggest that, unlike under in vitro conditions, a/x cells express all three regulators within the mammalian gut.

Moreover, the significance of overlap between Calling Card-defined targets and either set of ChiP-defined targets is approximately the same as those of ChiP-defined targets with each other (59 common targets, p < 2.22 × 10^-16) (Figure 3B). GO-term analysis of the 55 targets identified by all three studies reveals significant enrichment for processes known to be regulated by Efg1, including “regulation of filamentous growth” (p = 1.2 × 10^-5), “cell adhesion” (p = 2.6 × 10^-5), “regulation of growth” (p = 2.9 × 10^-5), “regulation of single-species biofilm formation” (p = 2.9 × 10^-5), and “regulation of phenotypic switching” (p = 4.2 × 10^-5). Moreover, the three studies reveal similar “footprints” of multiple Efg1 binding events at some large promoters, including the ~10-kb promoter of EFG1 itself (Figure 3C; Lassak et al., 2011; Nobile et al., 2012).

In addition to targets that were also detected using ChiP, Calling Card-seq identified 227 potential Efg1 binding targets that have not previously been described (Table S1). GO-term analysis of this gene set indicates enrichment of terms such as “interspecies interaction between organisms” (p = 8.3 × 10^-25, FDR = 2%), “septum digestion after cytokinesis” (p = 0.021, FDR = 1%), and “symbiotic process” (p = 0.027, FDR = 0.67%). Consistent with a role for Efg1 in the regulation of at least some of these targets, 8 of the Calling Card-seq-specific targets (RBE1, EAPl, RCT1, PGA54, HWP2, CHT2, SUN41, and PTP3) have previously been reported to exhibit abnormal expression in efg1 mutants (Harcus et al., 2004; Li and Palecek, 2003; Nobile et al., 2012; Sohn et al., 2003).

Recording of TF binding events during mammalian gut commensalism

To determine the activities of Efg1, Wor1, and Czf1 during commensalism, we utilized strains containing Efg1-PBase, Wor1-PBase, or Czf1-PBase. Each strain was introduced into an independent group of BALB/c mice (n = 4–5 animals per strain),
and colonization was allowed to progress for 15 (Efg1-PBase and Czf1-PBase) or 20 (Wor1-PBase) days; the Wor1-PBase strain was provided 5 extra days because of our previous observation that the pro-commensalism activity of WOR1 (in a WOR1<sup>OE</sup> strain) begins after ~5 days of host colonization (Pande et al., 2013). Animals were euthanized periodically over the experimental time course, and yeasts recovered from ceca (the gut compartment containing the highest burden of <i>C. albicans</i>) were plated onto medium lacking arginine, histidine, and leucine to select for transposon excision and reintegration events. A strain that expresses untethered PBase (PBase<sup>OE</sup>) was propagated in vitro as a negative control. Genomic DNA extraction, inverse PCR, and preparation of sequencing libraries were performed as described in the STAR methods.

After subtraction of background signal (signal from untethered PBase) and after thresholding for high confidence hits, Calling Card-seq revealed 2,745 <i>C. albicans</i> genes that are targeted by at least one of the TFs in commensally propagated cells (Table S2). Direct binding targets Wor1, Czf1, and Efg1 are depicted as nodes in the graphic presented in Figure 3D. Notably, each of the three TFs binds to its own promoter, and those of the other two profiled TFs during host colonization (Figures 3D and 3E). Similar results for Efg1 have been reported by Nobile et al., based on ChIP analysis of <i>in vitro</i>-propagated cells (Nobile et al., 2012); however, the DNA-binding activities of Wor1 and Czf1 have not previously been studied in a<sub>a</sub> cells. Indeed, it would be difficult to analyze Wor1 using ChIP because of its very low abundance in this cell type under <i>in vitro</i> conditions. 178 genes are targeted by all three DNA-binding proteins (Figure 3D; Table S2); this set of coregulated genes includes ones encoding 24 TFs, in addition to Wor1, Czf1, and Efg1. Importantly, eight of the additional TFs play demonstrated roles in commensal regulation: Wor2, Wor3, Wor4, and Ahr1 (shown in Figure 1C) and Tec1, Brg1, and Ume6 (Witchley et al., 2019) inhibit gut colonization, whereas Crz2 has been reported to enhance the establishment of colonization (Znaidi et al., 2018).

In addition to direct binding targets, the graphic in Figure 3D includes transcriptomics (RNA-seq) information from a previous comparison of WT <i>C. albicans</i> propagated in the murine digestive tract versus under standard <i>in vitro</i> culture conditions (YPD, 30°C; Witchley et al., 2019). For genes exhibiting differences of ≥2-fold between the two conditions (<i>p</i> < 0.05 using a linear fit model; Ritchie et al., 2015), node size reflects the relative magnitude of regulation, and node color reflects whether expression is increased (blue) or decreased (yellow) in the gut. Overall, Wor1, Czf1, and Efg1 directly bind to almost half of the 2,201 genes with gut-associated expression changes.

**Comparative transcriptomics of circuit-altered strains uncovers commensalism effectors**

Our previous unbiased genetic screens for commensalism factors have exclusively identified transcriptional regulators (Pande et al., 2013; Witchley et al., 2019), likely reflecting the outsized effects of regulatory proteins that control the expression of hundreds or thousands of genes. We hypothesized that TF mutants with contrasting commensal phenotypes might be useful tools for the discovery of commensalism effectors. In particular, we predicted that comparing the transcriptomes of mutants with enhanced versus diminished commensal fitness would reveal effectors of commensal interactions with the host.

To test this hypothesis, we used RNA-seq to profile RNA expression in <i>efg1</i>, <i>efg1wor1</i>, WOR1<sup>OE</sup> GUT cells, <i>wor1</i>, <i>czf1</i>, and isogenic WT controls. To capture gene expression under commensal conditions, each strain was gavaged into a separate group of BALB/c mice (<i>n</i> = 5–6 per strain). After 10 days of colonization, strains were recovered from host large intestines, and RNAs were extracted for RNA-seq. For comparison, the strains were also profiled under <i>in vitro</i> conditions (liquid YPD, 30°C).

Shown in Figure 4A is a volcano plot of gene expression in commensally propagated hyperfit strains (efg1, WOR1<sup>OE</sup> GUT cells, <i>efg1wor1</i>) versus strains with reduced commensal fitness (<i>wor1</i>, <i>czf1</i>). For each gene, relative RNA abundance (log2 abundance in hyperfit/abundance in hypofit) is plotted on the x axis, and significance (−log10 [<i>p</i> value]) is plotted on the y axis; all RNA-seq results are presented in Table S3. In total, 312 genes were significantly (<i>p</i> < 0.05) upregulated by at least 2-fold in the hyperfit strains, whereas 590 were downregulated. We hypothesized that genes that inhibit commensalism would be downregulated in hyperfit strains. In support of this idea, GO-term analysis of the downregulated gene set reveals significant enrichment of hypha-associated genes (hyphal cell wall, <i>p</i> = 6.48 × 10<sup>−7</sup>). In previous work, we have shown that hypha-specific cell wall and secreted factors diminish the fitness of <i>C. albicans</i> in the gut colonization model, likely because of host restriction of this invasive cell type (Witchley et al., 2019).

We hypothesized that genes encoding positive effectors of commensalism would be upregulated in the hyperfit strains. GO-term analysis of this gene set revealed enrichment of genes associated with translation (ribosome, <i>p</i> = 4.51 × 10<sup>−2</sup>) and sugar transport (monosaccharide transmembrane transporter activity, <i>p</i> = 6.48 × 10<sup>−7</sup>). To test this hypothesis, we used Calling Card-seq to identify genes that would be upregulated in hyperfit strains. In support of this idea, GO-term analysis of the derived Calling Card-seq (Figure 4B; Table S2) and was also identified by <i>in vitro</i> Calling Card-seq analysis of Efg1-PBase (Table S1) but not by previous Efg1 ChIP studies (Lassak et al., 2011; Nobile et al., 2012).

To determine whether CHT2 plays a role in <i>C. albicans</i> commensalism, we generated two independent isolates of a <i>cht2</i> null mutant. As shown in Figure S4, both isolates of <i>cht2</i> resemble WT under standard <i>in vitro</i> conditions (YPD agar, Figure S4A), under cell wall stress-inducing conditions (Congo red, Calcifluor white, 0.04% SDS, Figure S4A), and under microaerophilic and anaerobic conditions (Figure S4B). In contrast, <i>cht2</i> exhibits a striking competitive deficit in the gut colonization model (Figures 4C and S4C). Restoration of one copy of <i>cht2</i> to the null mutant suppresses the commensalism defect (Figure 4D), supporting linkage between <i>cht2</i> and commensal fitness. Together, these results suggest that Cht2 is a positive effector of commensalism.
Cht2 chitinase activity promotes commensalism

Fungal chitinases degrade chitin, a linear polymer of β(1,4)-N-acetyl-D-glucosamine (GlcNAc) that provides tensile strength to the fungal cell wall. Chitinases have previously been implicated in cell wall remodeling, autolysis, nutrient acquisition, and virulence (Yang and Zhang, 2019), but not in commensalism. Among the four chitinase genes in the *C. albicans* genome (*CHT1, CHT2, CHT3, CHT4*; Dunkler et al., 2005), CHT2 is the only one to be induced in commensally hyperfit strains (Table S3).

To determine whether chitinase activity is required for the commensal role of Cht2, we generated a series of mutant *CHT2* alleles that substitute alanine (A) for conserved residues that are critical for the enzymatic function of Cts1, a well-characterized homolog in *Saccharomyces cerevisiae* (Figure S4D; Dunkler et al., 2005; Hurtado-Guerrero and van Aalten, 2007). *CHT2*Δ151Δ and *CHT2*Δ151ΔΔE152A, and *CHT2*ΔY211A target aspartate (D) and/or glutamate (E) residues of the DxE motif at the predicted active site, and *CHT2*ΔW278A and *CHT2*ΔY211A and *CHT2*ΔW278A target tyrosine (Y) or tryptophan (W) residues that line the predicted chitin binding pocket. A set of isogenic strains was created by integrating one copy of each point mutant allele into its endogenous locus in a *cht2* null background. Total cellular chitinase activity was then determined for the mutants and WT, *cht2*, and *cht2*+*CHT2* controls (Figure 4E). Compared with WT, a *cht2* null strain exhibits a ~2-fold reduction in chitinase activity, with residual activity likely attributable to the remaining cellular chitinases (Cht1, Cht3, Cht4). Restoration of one copy of *CHT2* to the null mutant yields intermediate activity between that of WT (*CHT2*/*CHT2*) and *cht2*, suggesting that enzymatic activity reflects the gene copy number. By comparison, all five *CHT2* point mutants exhibit a similar level of chitinase activity to *cht2*, suggesting that the encoded proteins are enzymatically inactive.

We next tested the commensal fitness of strains bearing one copy of *CHT2*Δ151Δ or *CHT2*Δ151ΔΔE152A in competition with a strain bearing one copy of WT *CHT2*. As shown in Figures 4F and S4E, the strains expressing enzymatically inactive protein are outcompeted over the first 15 days of colonization, suggesting that chitinase activity contributes to the commensal function of each point mutant allele into its endogenous locus in a *cht2* null background. Total cellular chitinase activity was then determined for the mutants and WT, *cht2*, and *cht2*+*CHT2* controls (Figure 4E). Compared with WT, a *cht2* null strain exhibits a ~2-fold reduction in chitinase activity, with residual activity likely attributable to the remaining cellular chitinases (Cht1, Cht3, Cht4). Restoration of one copy of *CHT2* to the null mutant yields intermediate activity between that of WT (*CHT2*/*CHT2*) and *cht2*, suggesting that enzymatic activity reflects the gene copy number. By comparison, all five *CHT2* point mutants exhibit a similar level of chitinase activity to *cht2*, suggesting that the encoded proteins are enzymatically inactive.

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of Cht2. However, unlike a cht2 null mutant (Figures 4C and S4C), the CHT2 point mutants recover at subsequent time points. The superior fitness of cht2+CHT2D151A and cht2+CHT2D151A+E153A compared with cht2 suggests that Cht2 may promote commensalism using features in addition to chitinase activity.

**DIFFUSION**

In this work, we establish roles for 8 key regulators of *C. albicans* matin in colonization of the mammalian gut. Null mutants affecting WOR2, WOR3, WOR4, AHR1, CZF1, and SSN6 exhibit altered fitness in a mouse model of commensal colonization, as we have previously demonstrated for mutants affecting WOR1 and EFG1 (Pande et al., 2013). Moreover, genetic epistasis analysis of double mutants indicates that core relationships among Czf1, Efg1, and Wor1 are preserved between sex and host colonization. The repurposing of a complex regulatory pathway for growing yeasts.

Based on our analysis of *C. albicans* single and double null mutants in the murine gut, we propose a model for the regulation of commensalism that is depicted in Figure 5. CZF1 is proposed to sit atop the regulatory cascade, where it promotes commensalism by (directly or indirectly) inhibiting EFG1, a known inhibitor of commensalism (Liang et al., 2019; Pande et al., 2013; Pierce and Kumamoto, 2012; Witchley et al., 2019). The pro-commensal activity of CZF1 is deduced from the fitness defect of czf1 (Figures 1C and S2D), and the position of CZF1 upstream of EFG1 in a linear regulatory pathway is deduced from the enhanced fitness of efg1czf1, which precisely matches the phenotype of efg1 (Figures 2D and 2E). EFG1 is proposed to inhibit commensalism by a branched regulatory pathway: The first branch draws upon Efg1’s well-documented role as a transcriptional activator of hypha-associated genes (Lo et al., 1997; Stoldt et al., 1997); such genes include SAP6, which we have previously shown to inhibit commensal fitness in the gut (Witchley et al., 2019). In this study, we show that Efg1 binds to the SAP6 promoter (Table S2) and activates SAP6 expression by more than 50-fold (\(p = 2.0 \times 10^{-11}\)) (Table S3) in commensally propagated cells. The second regulatory role of Efg1 is proposed to occur by (direct or indirect) repression of WOR1 and/or the white-to-GUT switch, which are required for normal commensal fitness (Pande et al., 2013). The proposed branched regulatory network is consistent with observations that *efg1wor1* and *czf1wor1* GUT strains exhibit distinct commensal phenotypes from those of the corresponding single mutants. For example, although *efg1* exhibits a strongly enhanced fitness phenotype and *wor1* exhibits a fitness defect (Pande et al., 2013), the *efg1wor1* strain exhibits an intermediate level of enhanced fitness compared with WT (Figure 2B) and is outcompeted by *efg1* (Figures 2C and S4H). These observations can be rationalized if both forms of Efg1-mediated inhibition (activation of SAP6 and repression of WOR1 and the white-to-GUT switch) are reversed in an *efg1* mutant, but only SAP6 activation is reversed in the *efg1wor1* strain (because the white-to-GUT switch does not occur in the absence of WOR1). Finally, the observed requirement for CZF1 for the white-to-GUT switch can be rationalized if switching requires at least transient repression of EFG1.

We note that the core genetic logic of the proposed commensalism pathway resembles that of the sexual circuit (Figure 1B), with Czf1 inhibiting Efg1, Efg1 inhibiting Wor1 and/or GUT cell formation, and Wor1 promoting a cell type switch that favors the regulated process. Differences between the circuits include (1) dual inhibitory roles for Efg1 in commensalism but only one in mating and (2) differing roles for Ssn6, Wor2, Wor3, and Wor4 in activation versus inhibition of the respective processes (Figure 1D).

To gain insight into the DNA-binding activity of three core regulators during commensal colonization, we adapted Calling Card-seq (Wang et al., 2011a, 2012) for use in *C. albicans* (Figure 3A). Under *in vitro* growth conditions, the modified technique identified 455 direct binding targets of Efg1 (Figure 3B; Table S1), approximately half of which had previously been reported in ChIP-based investigations (Lassak et al., 2011; Nobile et al., 2012). The significant overlap among targets identified by Calling Card-seq and ChIP \(p < 2.2 \times 10^{-16}\) for each comparison) supports the validity of the Calling Card-seq method. Furthermore, Calling Card-seq performed in colonized animals identified 11 additional regulators of commensalism (*WOR2, WOR3, WOR4, AHR1, TEC1, BRG1, UME6, CRZ2, WOR1, CZF1, and EFG1*) (Table S3) among the common targets of Efg1, Czf1, and Wor1.

In addition to common targets, *in vitro* Calling Card-seq and the previous ChIP investigations of Efg1 each identified a number of study-specific binding targets (Table S1; Figure 3B; Lassak et al., 2011; Nobile et al., 2012). Besides random
experimentally inferred that the study of TF binding activity under physiologically relevant conditions such as in hosts. For example, the Efg1-PBase, Czf1-PBase, and Wor1-PBase strains described in this study are suitable for use in existing mouse models of C. albicans skin colonization, cellulitis, oropharyngeal infection, vaginitis, gut colonization, gut inflammation, and disseminated infection (Cassone and Sobel, 2016; Igyártó et al., 2011; Kadosh et al., 2016; Koh, 2013; Kvaal et al., 1999; MacCallum and Odds, 2005; Solis and Filler, 2012; Xie et al., 2013).

We utilized TF mutants with strong gain- or loss-of-commensal fitness phenotypes as tools for discovery of commensalism effectors. Our transcriptomic analysis of commensally propagated hyperfert (efg1, efg1wor1, WOR1GUT) and hypofert (wor1 and czf1) strains revealed consistent downregulation of hypha-associated genes in hyperfert strains, including SAP6 (Figure 4A; Table S3). Among strongly upregulated genes, we focused on CHT2, which encodes a GPI-linked chitinase that is predicted to be localized at the fungal cell surface. CHT2 was also identified in vivo Calling Card-seq as a direct binding target of Efg1, Wor1, and Czf1 (Figure 4B; Table S2) and is among the Calling Card Seq-specific Efg1 binding targets under in vitro conditions (Table S1; Lassak et al., 2011; Noble et al., 2012). Consistent with a role for Cht2 as a commensalism effector, the cht2 null mutant exhibits a substantial competitive defect (Figure 4C). Chitinase activity seems to contribute to effector activity, given that strains expressing catalytically dead alleles (CHT2D151A or CHT2D151AE153A) are defective at early times during gut colonization (Figures 4F and S4F). Chitin is a conserved structural component of the fungal cell wall that can trigger host innate immune responses (Reese et al., 2007). Although the precise mechanism by which Cht2 promotes C. albicans fitness in the gut remains to be determined, an intriguing possibility is that Cht2 may alter host detection of commensally propagating yeasts or may modulate the resulting immune response.

Mating and the white-to-opaque epigenetic switch are among the most highly studied processes in C. albicans. Nevertheless, there is little evidence for sexual exchange among naturally circulating strains (Bougnoux et al., 2008; Legrand et al., 2004), and mating is inferred to be exceedingly uncommon. In contrast, gut colonization with C. albicans is ubiquitous among humans and other mammals, and propagation within the commensal niche may provide ongoing selection for circuit components that are shared between mating and commensalism. Recently, seven of the regulators profiled in this study (Efg1, Czf1, Wor1, Wor2, Wor3, Wor4, and Ssn6) were reported to possess prior-like domains that promote the formation of phase-separated condensates within the nucleus (Frazer et al., 2020). In future, it will interesting to determine whether the ability to form distinct condensates or alternative mechanisms permit one set of transcriptional regulators to orchestrate two very different cellular outcomes (mating versus host colonization).

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2021.03.019.

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AUTHOR CONTRIBUTIONS

S.M.N. and J.N.W. conceptualized the study. J.N.W. performed commensalism experiments, Calling Card-seq experiments, and RNA-seq experiments and wrote the manuscript. P.B. performed in vitro chitinase assays and commensalism experiments. C.A.B. performed RNA-seq and commensalism experiments in vitro with in vitro chitinase assays and commensalism experiments.
experiments. N.V.A. performed commensalism experiments. S.M.N. supervised the study and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES


## STAR★METHODS

### KEY RESOURCES TABLE

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### Deposited data

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| Card-Seq data | this study | GEO: GSE150856 |

### Experimental models: organisms/strains

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**Oligonucleotides**

Oligonucleotides are described in Table S6

| Resource | N/A | N/A |

**Recombinant DNA**

- **pSFS2A**
- **pSN42** [(Noble and Johnson, 2005)]: Gene replacement with LEU2 marker
- **pSN52** [(Noble and Johnson, 2005)]: Gene replacement with HIS1 marker
- **pSN69** [(Noble and Johnson, 2005)]: Gene replacement with ARG4 marker
- **pSN209** [Pande et al., 2013]: Replacement of WOR1 promoter with (SAT1 gene and) TDH3 promoter
- **pSN377** [this study]: Replacement of WOR1 promoter with (Flipper cassette and) TDH3 promoter
- **pSN301** [this study]: Replacement of WOR1 ORF with FLP-SAT1 construct
- **pSN314** [this study]: Replacement of WOR2 promoter with (Flipper cassette and) TDH3 promoter
- **pSN311** [this study]: Replacement of WOR3 promoter with (Flipper cassette and) TDH3 promoter
- **pSN360** [this study]: Replacement of WOR4 promoter with (Flipper cassette and) TDH3 promoter
- **pSN352** [this study]: Replacement of CZF1 promoter with (Flipper cassette and) TDH3 promoter
- **pSN329** [this study]: Replacement of CZF1 ORF with FLP-SAT1 construct
- **pSN378** [this study]: Replacement of AHR1 promoter with (Flipper cassette and) TDH3 promoter
- **pSN445** [this study]: Transposition integration into C.a.LEU2 locus
- **pSN447** [this study]: Transposition integration into C.a.LEU2 locus
- **pSN427** [this study]: Plasmid that can be used to create additional plasmids with gene homology for TF-PiggyBac transposase fusions
- **pSN422** [this study]: Replacement of TDH3 ORF with PiggyBac transposase for overexpression
- **pSN459** [this study]: Fusion of PiggyBac transposase to WOR1 ORF
- **pSN467** [this study]: Fusion of PiggyBac transposase to EFG1 ORF
- **pSN465** [this study]: Fusion of PiggyBac transposase to CZF1 ORF
- **pSN503** [this study]: CHT2 allele B addback to endogenous locus

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## RESOURCE AVAILABILITY

**Lead contact**
Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Suzanne M. Noble (suzanne.noble@ucsf.edu).

**Materials availability**
*C. albicans* strains and plasmids generated in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application.

**Data and code availability**
The raw files used for mRNA-seq analysis are available through GEO accession number GSE150855. The raw data for Calling Card experiments are available through GEO accession number GSE150856. Custom code used for analysis of CCS data is available at https://github.com/jwitch/CallingCard.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Yeast strains
The yeast strains used for this study are described in Table S4, plasmids in Table S5, and primers in Table S6. All media were prepared as previously described in Homann et al. (Homann et al., 2009) for YPD, Spider, Calcofluor white, and 0.04% SDS, in Lee et al. (Lee et al., 1975) for Lee’s media, and in Bruno et al. (Bruno et al., 2006) for Congo red concentration (50 μg/mL). From glycerols, strains were streaked to YPD and grown for 2 days at 30°C. Cells were resuspended in distilled water to an OD of 1 and then diluted as appropriate for the assay. For single colony growth analysis, 100–200 CFUs were plated in appropriate media. For spot plating, strains were diluted five-fold and 3 μL of each dilution spotted to designated media.

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### Software and algorithms

- **NumPy**: 1.14.3 (Oliphant, 2015) RRID: SCR_008633
- **Pandas**: Reback et al., 2021 RRID:SCR_018214
- **SciPy**: Virtanen et al., 2020 RRID:SCR_008058
- **tabulate**: (tabulate) https://pypi.org/project/tabulate/
- **pybedtools**: Dale et al., 2011 RRID:SCR_021018
- **BedTools**: Quinlan and Hall, 2010 RRID:SCR_006646
- **Picard**: http://broadinstitute.github.io/picard/ RRID:SCR_006525
- **Htslib**: Li et al., 2009 RRID:SCR_002105
- **samtools**: Li et al., 2009 RRID:SCR_002105
- **Pysam**: https://github.com/pysam-developers/pysam RRID:SCR_021017
- **Kallisto**: Bray et al., 2016 RRID:SCR_016852
- **Limma**: Ritchie et al., 2015 RRID:SCR_010943
- **Bowtie**: (Langmead and Salzberg, 2012) RRID:SCR_005476
- **MochiView**: Homann and Johnson, 2010 RRID:SCR_000259
- **Custom code for Calling Card analysis in Candida albicans**: this study https://github.com/jwitch/CallingCard

### Other
- Sera-Mag beads in a homemade PEG solution: Thermo-Fisher [https://openwetware.org/wiki/SPRI_bead_mix]
Studies in animals
All procedures involving animals were approved by the UCSF Institutional Animal Care and Use Committee. The mouse model of C. albicans commensalism was performed as previously described (Chen et al., 2011; Pande et al., 2013) with minor modifications. Groups of 8- to 10-week-old (18–21 gram) female BALB/c mice were housed 1 to 2 per cage and treated with penicillin 1,500 un/mL and streptomycin 2 mg/mL in drinking water starting 7 days before gavage and continuously for the remainder of the experiment. Feces were plated on Sabouraud agar, LB agar, and MRS agar to monitor for fungal and bacterial growth.

For commensal competition experiments, groups of 4–8 mice were gavaged with 10^7 CFUs of a 1:1 mix of two fungal strains in 0.9% saline. C. albicans was recovered from the inoculum and host feces at specified intervals, and strain quantification was performed as described previously (Noble et al., 2010).

For mRNA-seq experiments, 5 or 6 mice were colonized with a single strain and individually housed. After 10 days, animals were humanely euthanized, and large intestines were dissected for RNA analysis.

METHOD DETAILS
RNA-seq library preparation and analysis
RNA extraction for mRNA-seq was performed as previously described (Witchley et al., 2019), with modifications. For in vitro expression comparisons, three replicates were used for each strain per condition. Luminal contents of large intestines were resuspended in a mixture of 500 μL Buffer A (200 mM NaCl and 20 mM EDTA), 500 μL acid phenol-chloroform, 168 μL 25% SDS, and ~400 μL 0.5 mm glass beads. Samples were vortexed for 5 cycles of 2 min, interrupted with 1-min rests on ice. Samples were centrifuged at max speed in a microcentrifuge for 10 min, and supernatants were extracted four times with equal volumes of acid phenol-chloroform (Ambion), followed by four rounds of extraction with 5 cycles of equal volumes of phenol-chloroform-isooamyl alcohol (Ambion). After precipitation and resuspension of pellets in RNase-free water, RNA was further purified using the MEGAclear transcription clean-up kit (Ambion) for wor1 (ySN1351) or Sera-Mag beads in a homemade PEG solution (https://openwetware.org/wiki/SPRI_bead_mix) following the RNAclean XP protocol for ySN425, efg1 (ySN1011), czf1 (ySN1145), WOR1OE GUT (ySN1045), and efg1wor1 (ySN1126). DNase I (NEB) treatment was performed on at least 5 μg RNA for 10 min at 37°C. After DNase treatment, a final acid phenol-chloroform extraction was performed. RNA was then precipitated and resuspended in RNase-free water.

NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used in combination with NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Multiplex Oligos for Illumina to generate mRNA-seq libraries. The protocol provided by NEB was followed using 1 μg total RNA and 14 cycles of PCR, with the exception that Ampure XP bead mix was replaced with Sera-Mag Speed Beads (Thermo Fisher Scientific) in a homemade PEG solution (Rohland and Reich, 2012). Library fragment size was determined using High Sensitivity DNA chips on a 2100 Bioanalyzer (Agilent). Library quantification was performed by qPCR with a library quantification kit from KAPA Biosystems (KK4824). Sequencing was performed on a HiSeq4000 device (illumina).

Sequencing reads were mapped to the current haploid C. albicans transcriptome (Assembly 22 default coding, downloaded from candidagenome.org on November 19, 2018), and transcript abundances were determined using kallisto (Bray et al., 2016). Statistical comparisons of transcript abundances between different samples were performed on estimated counts generated by kallisto using limma as previously described (Ritchie et al., 2015). Transcriptomic analysis was performed by binning RNA expression results of strains with enhanced versus diminished commensal fitness into “hyperfit” and “hypofit” groups. Voom implemented in limma was used to model the observational variance in log-counts, and this model was subsequently used in limma’s empirical Bayes method to calculate adjusted p values for differences in expression between the two groups, using condition (YEPD 30°C, YEPD+serum 37°C or large intestine) and batch (date of processing) as factors.

GO-term analysis was performed using the Candida Genome Database GO-term finder. Differentially expressed genes were considered to be those with a fold change greater than 2 and an adjusted p < 0.05. Upregulated or downregulated genes were analyzed for process, function, and component. The raw files are available through GEO accession number GSE150855.

Generation of C. albicans strains for Calling Card-seq
Because stable, autonomously replicating plasmids have not been available for use in C. albicans until quite recently (Bijlani et al., 2019), we devised a strategy to integrate recombinant Piggyback transposons, genes encoding TF-PBase fusion proteins, and codon-optimized PBASE into the genome. First, we commissioned synthesis of a PBase gene (PBASE) that is codon-optimized for C. albicans (GenScript, Piscataway, NJ). Next, we engineered a series of plasmids to target codon-optimized PBASE to the 3′ end of a TF ORF of interest; integration results in the expression of a TF-PBase fusion protein via the natural promoter for the TF. Plasmid pSN427 (which is used to create TF-specific integrating constructs) contains the following insert: Pmel restriction site—sequence encoding an 18 amino acid linker—PBASE—FLP—SAT1—Pmel restriction site. pSN427 was generated by homologous recombination in S. cerevisiae of the following three PCR products: (1) codon-optimized PBASE, amplified with primers that include linker homology and a Pmel site at the 5′ end and homology to FLP-SAT1 cassette at the 3′ end; (2) linearized prS316 vector, amplified with primers containing linker homology on one end and FLP-SAT1 cassette homology on the other; and (3) FLP-SAT1, amplified from pSFS2A using primers with homology to PBASE at the 5′ end and prS316 at the 3′ end.

To create custom integration constructs that target PBASE to the WOR1 (pSN459), EFG1 (pSN467), and CZF1 (pSN465) ORFs, homologous recombination in S. cerevisiae was used to fuse the following three inserts: (1) linker—PBASE—FLP—SAT1 fragment from Pmel-digested pSN427; (2) Pmel restriction site—terminal ~300 bp of TF ORF sequence; and (3) 300 bp of 3′ UTR for the
TF—PmeI site. To create a PBaseOE control strain, an analogous method was used to create pSN422, which targets PBASE to replace one copy of the TDH3 ORF. pSN422 contains the following insert: PmeI—~300 bp of TDH3 5’ UTR—linker—PBASE—FLP—SAT1—~300 bp of TDH3 3’ UTR—PmeI.

Inserts from the PBaseOE (pSN422) and TF-PBase plasmids (pSN459, pSN467, pSN482) were liberated with PmeI and used to transform C. albicans strains containing either one (ySN1873) or two (ySN1877) copies of PBase transposon (at the neutral leu2Δ locus). After 1 day of propagation on YPD/agar containing 200 μg/mL nourseothricin (YPD+Nat) at 30°C, Nat-resistant colonies were streaked to fresh YPD+Nat medium and incubated overnight at 30°C. Finally, single colonies were patched to YPD+Nat, SD-histidine, SD-histidine-arginine, and SD-histidine-leucine-arginine plates. 5’ and 3’ junctions of integrants were verified by colony PCR using primers described in Table S6. Glycerol stocks were prepared from patches exhibiting His+ (1 transposon) or His+Leu+ (2 transposons) and Amp’ (transposon still present at the donor site) phenotypes. In addition, we screened for Nat+ versions of the strains after overnight propagation in YPD maltose liquid culture at 30°C, which allows for spontaneous excision of the FLP-SAT1 cassette.

To validate the modified method under in vitro conditions, Efg1-PBase (SN1988) and EFG1 promoter-PBase (SN2012) strains (containing one copy of PB) were propagated for 2 days on YEPD, followed by replating of ~50,000 CFUs onto Lee’s GlcNAc pH 6.8 agar (hypha-inducing conditions where EFG1 is well-expressed) in 100 mm petri plates. Strains were incubated at 37°C for 7 days to allow for transposition to occur. Cells were then replica plated to SD-histidine-arginine and incubated for 2 days at 30°C. His+Arg+ cells were collected by washing with water, followed by extraction of genomic DNA from cell pellets.

Calling Card-seq mouse experiments
WOR1-PBASE (ySN1933), EFG1-PBASE (ySN1943), and CZF1-PBASE (ySN1941) strains containing two copies of PB were streaked from frozen glycerol stocks onto YPD plates and incubated for 16 h at 30°C. Cells were scraped from the plates, diluted to $5 \times 10^5$ CFU/mL, and 100 μL of each cell suspension was spread onto several SD-histidine-leucine plates, with incubation for 48 h at 30°C. A disposable yellow loop was used to scrape colonies into 0.9% saline. 10^5 CFUs was gavaged into each antibiotic-treated mouse. One gavage volume was plated to SD-histidine-arginine plates to monitor for transposition events that had already occurred during preparation of the inocula (these proved to be minimal; Witchley and Noble, data not shown). To estimate the background rate of nontarget transposition events, the PBaseOE strain (ySN1904) was grown in liquid YPD or YPD+100 μM BPS (iron chelator related to TFs not reported here) overnight and then plated to selection media.

After 5, 10, 15, and/or 20 days, animals were euthanized and dissected using sterile technique to recover the cecum. 1–2 animals per group were euthanized after 5, 10, or 15 days for Efg1-PBase, 10 or 15 days for Czf1-PBase, and 10, 15 and 20 days for Wor1-PBase. Each organ was homogenized in 3 mL PBS pH 7.4. 100 μL was diluted 1:10 in PBS to determine CFUs/gram cecum, and the remainder was plated 500 μL per 100 mm SD-histidine-leucine-arginine plate. Cells were incubated for 2 days at 30°C, and colonies were recovered for DNA extraction and sequencing library preparation. All reads from the same strain recovered from animals were pooled before data analysis.

Calling Card-seq library preparation and analysis
Sequencing libraries were prepared as previously described (Wang et al., 2011a, 2011b, 2012) with modifications. Genomic DNA was digested Mspl, Rsal and Taq1 α, in independent reactions. Next, the DNA was precipitated with isopropanol and 5 M ammonium acetate, washed with 70% ethanol, and resuspended in 30 μL nuclease-free water. To circularize the pooled restriction fragments, the DNA was resuspended to 2–3 ng/μL in a 400 μL ligation reaction with T4 ligase. The ligation reaction was incubated at 15°C for greater than 16 h then precipitated with ethanol and 3 M sodium acetate, washed with 70% ethanol, and resuspended in 30 μL nuclease-free water. 15 μL of ligated DNA was used in a PCR reaction with Phusion polymerase and custom read one primer SNO2980 and one of the primers described in Table S6. Glycerol stocks were prepared from patches exhibiting His+ (1 transposon) or His+Leu+ (2 transposons) and Amp’ (transposon still present at the donor site) phenotypes. In addition, we screened for Nat+ versions of the strains after overnight propagation in YPD maltose liquid culture at 30°C, which allows for spontaneous excision of the FLP-SAT1 cassette.

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or hypergeometric distribution) for at least one of the clusters upstream of the target gene. These data are presented in Figures 3B and 3D and Tables S1 and S2. The raw data are available through GEO accession number GSE150856.

For visualization in MochiView (Homann and Johnson, 2010), duplicate reads were first removed using a two-step process: duplicates in each BAM (binary sequence alignment map) file were flagged using Picard MarkDuplicates (Broad Institute, MIT); then, if the number of duplicate reads was greater than 50, a single read was kept in the BAM file as the “Unique Insertion,” else the reads were discarded. The genomeCoverageBed function of bedtools (Quinlan and Hall, 2010) was used to collect sequencing depth information in a bedGraph file format. The bedGraph files were then converted to fit the MochiView file format (Homann and Johnson, 2010). Formatted files were imported into MochiView as a Location/Data set. A bar graph style was used for visualization owing to the nature of the data and the MochiView file format. These data are presented in Figures 3C and 4B.

**Chitinase activity assay**

Overnight cultures of *C. albicans* strains were diluted in YEPD to OD$_{600}$ = 0.1 and allowed to grow to log phase (OD$_{600}$ = 1). One mL of each culture was centrifuged at 3000 rpm for 10 min. The supernatant was removed and saved, and pellets were washed and resuspended in 1 mL of 0.9% sterile saline. Ten μL of the supernatant and cell suspension were tested separately for enzymatic activity using the fluorometric chitinase activity kit (CS1030) from Sigma-Aldrich according to the manufacturer’s instructions. The plate was incubated for 1 h at 37°C. Fluorescence was measured with a Tecan plate reader with an excitation wavelength of 360 nm and an emission of 450 nm.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical parameters and statistical test type (value of n, statistical significance, dispersion, and precision measures) are reported in the figure legends and in method details, above. Significance is generally defined as p < 0.05. The Student’s t test was used to assess the results of commensal competition experiments and chitinase assays, using GraphPad Prism 9.0. Statistical analysis of RNA-seq and Calling Card-seq experiments are included with the description of the methods, above. The Fisher’s exact test was used to determine the significance of overlap between *in vitro* binding targets of Efg1 identified by CCS versus ChIP, using the built-in fisher.test function of RStudio.