Post-Transcriptional Regulation of the Sef1 Transcription Factor Controls the Virulence of Candida albicans in Its Mammalian Host

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Abstract

The yeast Candida albicans transitions between distinct lifestyles as a normal component of the human gastrointestinal microbiome and the most common agent of disseminated fungal disease. We previously identified Sef1 as a novel Cys6-Zn2 DNA binding protein that plays an essential role in C. albicans virulence by activating the transcription of iron uptake genes in iron-poor environments such as the host bloodstream and internal organs. Conversely, in the iron-replete gastrointestinal tract, persistence as a commensal requires the transcriptional repressor Sfu1, which represses SEF1 and genes for iron uptake. Here, we describe an unexpected, transcription-independent role for Sfu1 in the direct inhibition of Sef1 function through protein complex formation and localization in the cytoplasm, where Sef1 is destabilized. Under iron-limiting conditions, Sef1 forms an alternative complex with the putative kinase, Ssn3, resulting in its phosphorylation, nuclear localization, and transcriptional activity. Analysis of sfu1 and ssn3 mutants in a mammalian model of disseminated candidiasis indicates that these post-transcriptional regulatory mechanisms serve as a means for precise titration of C. albicans virulence.

Introduction

Candida albicans is a ubiquitous component of the mammalian microbiome [1] as well as the most common fungal pathogen of humans [2,3,4,5]. As this organism transits between its commensal niches (mammalian skin and gastrointestinal tract) and those of virulence (bloodstream and internal organs), it experiences profound shifts in the levels of nutrients, the physical environment, and immune surveillance. We previously demonstrated that a novel C. albicans transcriptional regulatory circuit is required for survival in at least two distinct habitats, the host bloodstream and gastrointestinal tract [6], where levels of bioavailable iron differ by more than 20 orders of magnitude [7,8]. In the bloodstream, where iron is tightly sequestered by host transferrin [7], C. albicans defends against iron deficiency through expression of Sef1, a Cys6-Zn2 transcriptional activator of iron uptake genes and an indirect suppressor of the gene for Sfu1 [6]. In the gastrointestinal tract, where iron is abundant thanks to diet and sloughed cells [8,9], C. albicans defends against iron toxicity through the expression of Sfu1 [6], a GATA family transcriptional repressor that inhibits both SEF1 and genes for iron uptake [6,10]. Remarkably, the opposing roles of Sef1 and Sfu1 in iron homeostasis extend to differing relationships with the host, with Sef1 promoting virulence and Sfu1 promoting commensalism in animal models [6]. However, the details of how these transcriptional regulators are themselves regulated by iron remain to be elucidated.

Sfu1 is broadly conserved among ascomycetes, and orthologs from multiple species have been shown to play a negative role in iron homeostasis through repression of iron uptake genes [10,11,12,13,14,15]. The best-characterized ortholog is Fep1 from Schizosaccharomyces pombe that, like Sfu1, is subject to repression at the transcriptional level when environmental iron is limiting [16,17]. In this species, protein activity is also regulated by iron, since only iron-bound Fep1 can associate with DNA [18]. By contrast, orthologs of Sef1 have not been extensively characterized, in part because the genomes of only a handful of species in the Saccharomyces and Candida lineages encode this protein [6]. Moreover, C. albicans Sef1 appears to function differently from its S. cerevisiae ortholog, since iron homeostasis in the latter species is controlled by Aft family proteins [19,20,21] and is not dependent on Sef1 [6].

Here we describe studies that reveal an unexpected, transcription-independent role of C. albicans Sfu1 in inhibiting Sef1 function, as well as a role for a predicted protein kinase, Ssn3, in Sef1 activation. Specifically, we find that, under iron-replete conditions, Sfu1 physically associates with Sef1 and sequesters it in the cytoplasm, where it is destabilized. In contrast, under iron-depleted conditions, Sef1 forms an alternative complex with Ssn3, resulting in Sef1 phosphorylation, nuclear localization, and the transcriptional activation of iron uptake genes. These post-transcriptional regulatory events are of direct consequence to C.
**Author Summary**

*Candida albicans* is a fungus that resides on the skin and in the gastrointestinal tract of humans and other mammals. However, this commensal organism is also capable of proliferating and causing disease in people who have received antibiotics, who are immunocompromised, or who have suffered injury to epithelial layers. We previously identified a novel transcription factor called Sef1 that promotes *C. albicans* virulence by activating the expression of iron uptake genes in iron-poor environments, such as the host bloodstream. However, in iron-replete environments such as the gastrointestinal niche, the *SEF1* gene is repressed by a second transcription factor called Sfu1. Here, we report our discovery of a series of post-transcriptional regulatory events that determine the intracellular localization, stability, and activity of Sef1 protein. Mutants that disrupt these post-transcriptional events alter *C. albicans* virulence in a mammalian model of disseminated infection. The existence of multiple levels of regulation speaks to the importance of Sef1 in *C. albicans* virulence and suggests that close titration of Sef1 activity is important for adaptation to distinct microenvironments within the mammalian host.

*S. pombe* Sfu1 might, like its *S. pombe* ortholog [10], require iron as a cofactor for binding to DNA and transcriptional repression.

**Sfu1 promotes Sef1 localization in the cytoplasm**

Although *SEF1* mRNA levels were normal in the *SFU1*-overexpression strain (*Figure 1a*), this strain demonstrated hypersensitivity to treatment with the iron chelator, bathophenanthrolinedisulfonic acid (BPS; *Figure S2*), suggestive of a potential defect in iron acquisition. Addition of FeCl3 to the BPS-treated medium was sufficient to reverse the growth defect (*Figure S2*), confirming the specificity of the iron-chelation phenotype. To determine whether Sef1 protein levels were affected in the *SEF1*OE strain, we utilized an epitope-tagged version of Sef1 in which 13 copies of the Myc epitope were fused in-frame at the C-terminus; this fusion protein is fully functional [6]. Surprisingly, the steady state level of Sef1-Myc was substantially reduced, particularly under iron-depleted conditions (*Figure S3*). The observations that overexpression of *SFU1* does not affect *SEF1* mRNA levels but strongly decreases Sef1 protein levels raised the possibility that Sfu1 may have a second function in the post-transcriptional regulation of Sef1.

To determine whether Sef1 localization is regulated, we used indirect immunofluorescence to visualize Sef1-Myc in wild-type cells exposed to varying concentrations of iron. Under iron-replete conditions, Sef1-Myc was localized primarily to the nucleus, with prominent areas of yellow overlap when the FFTC and DAPI channels were merged. Notably, examination of Sef1-Myc in an *sfu1*ΔΔ mutant revealed constitutive nuclear localization, even under iron-replete conditions (Figure 1b). Conversely, overexpression of *SFU1* resulted in substantial cytoplasmic localization of Sef1-Myc even under iron-depleted conditions in which it is usually nuclear (*SFU1*OE strain, *Figure 1b*). By comparison, an Sfu1-Myc fusion protein was found to be distributed between the nucleus and cytoplasm in wild-type cells propagated under iron-replete conditions and primarily cytoplasmic under iron-limiting conditions (*Figure S4b*).

These results established that Sef1 localization varies as a function of iron, that Sfu1 promotes Sef1 localization in the cytoplasm, and that the protein localizing activity of Sfu1—unlike its transcriptional repression activity (*Figure 1a*)—does not inherently require iron.

**Sn3 promotes Sef1 phosphorylation and nuclear localization under iron-depleted conditions**

Immunoblot analysis of Sef1-Myc recovered from wild-type cells grown under iron-replete vs. iron-depleted conditions demonstrated an inverse relationship between Sef1 protein abundance and iron levels (*Figure 2a*, lanes 1 and 2), which was expected based on the known, iron-dependent inhibitory activity of Sfu1 on *SEF1* gene expression. An unexpected finding was that the electrophoretic mobility of Sef1 also varied in an iron-dependent fashion. This subtle but reproducible decrease in Sef1 mobility under iron-depleted conditions was observed not only in wild-type cells, but also in an *sfu1*ΔΔ deletion mutant [lanes 3 and 4], arguing against a role for Sfu1 in this process.

We hypothesized that the lower mobility form of Sef1 might result from covalent phosphorylation. To test this hypothesis, we
Figure 1. Sfu1 regulates SEF1 gene expression and localization of Sef1 protein. Note that C. albicans gene deletion mutants are represented as ΔΔ because two alleles of target genes must be disrupted in this obligate diploid species. a) RT-qPCR results for SEF1 mRNA in wild-type (WT), sfu1ΔΔ, and SFU1-overexpression (SFU1OE) strains grown under iron-replete (H, high iron) or iron-depleted (L, low iron) conditions. Error
used a tandem affinity purification strategy to recover TAP-tagged Sef1 from C. albicans grown under iron-replete or iron-depleted conditions. Purified TAP-tagged Sef1 exhibited an iron-dependent mobility shift similar to that of Sef1-Myc, with protein from the iron-depleted cells running with slightly lower mobility (Figure 2b, compare lanes 1 and 3). Treatment of the purified proteins with lambda phosphatase, a broad specificity enzyme with activity on phospho-serine, phospho-threonine, and phosphotyrosine residues, resulted in conversion of the lower mobility form of Sef1-TAP to the higher mobility form (Figure 2b, compare lane 4 to lanes 1 and 2), in support of our hypothesis.

To identify the kinase responsible for low-iron-dependent phosphorylation of Sef1, we tested the 31 available homozygous knockout mutants affecting predicted kinases for sensitivity to BPS. Our reasoning was that, if phosphorylation of Sef1 is required for full induction of iron uptake genes, then a mutant lacking the relevant kinase might be hypersensitive to iron depletion, that is, phenotypically similar to ssn3Δ [6, 23]. Our screen identified the ssn3ΔΔ mutant as being hypersensitive to iron depletion (Figure S2). Further, an immunoblot of Sef1-Myc recovered from the ssn3ΔΔ strain revealed persistence of the higher mobility form under iron-depleted conditions (Figure 2c), consistent with a role for Ssn3 in phosphorylation of Sef1. The identical result was obtained when Sef1-Myc was examined in a strain encoding a predicted kinase-dead allele of Ssn3 (Ssn3D325A, Figure S5a).

Although C. albicans Ssn3 has not yet been characterized, its S. cerevisiae ortholog is a cyclin-dependent kinase with two known functions: first, it is a component of the Mediator complex with inhibitory activity on RNA polymerase II [24]; second, it phosphorylates a number of specific transcription factors to regulate their activity, nuclear-cytoplasmic localization, and/or stability [25, 26, 27]. To determine whether C. albicans Ssn3 influences the localization of Sef1, we performed indirect immunofluorescence on Myc-tagged Sef1 in the ssn3ΔΔ mutant. As shown in Figure 2d, deletion of SSN3 resulted in constitutive cytoplasmic localization of Sef1-Myc under both iron-replete and iron-depleted conditions; similar mislocalization was observed in a strain containing Ssn3D325A (Figure S5b). Unlike the case with Sfu1, however, overexpression of SSN3 via the TDH3 promoter (SSN3ΔΔ) had no obvious effect on Sef1-Myc localization (Figure S5b), perhaps indicating that the nuclear localization activity of Ssn3 is restricted to low iron conditions.

Sfu1 and Ssn3 each physically interact with Sef1 but play opposing roles

The preceding results were suggestive of a model in which Sfu1 and Ssn3 have opposite and competing roles in Sef1 localization, with Sfu1 promoting cytoplasmic localization and Ssn3 promoting nuclear localization. To test this model, we utilized the SFU1-overexpression strain that mislocalizes Sef1-Myc to the cytoplasm...
under iron-depleted conditions (Figure 1b). We predicted that, if Ssn3 competes with Sfu1 for localization of Sef1, then overexpression of Ssn3 might rescue this Sef1 mislocalization phenotype. Indeed, a strain in which both genes are driven by the strong TDH3 promoter exhibits substantial restoration of nuclear Sef1 expression, as observed in Figure 3a. These results indicate that Sfu1 and Ssn3 exert opposing roles on Sef1 localization, but only under iron-depleted conditions (when Sef1 is phosphorylated).

To determine whether Sef1 physically associates with Sfu1 and/or Ssn3, we created a series of double epitope-tagged strains, each containing a Myc-tagged version of one of the three potentially interacting proteins and a TAP-tagged version of another; the TAP epitope consists of a calmodulin binding domain fused to a TEV cleavage site and a Protein A domain (Figure S6a; [28,29]). Co-immunoprecipitation experiments were performed using whole cell extracts prepared from cells grown under iron-replete or iron-depleted conditions. Extracts were incubated with IgG-sepharose, which binds to the Protein A component of the TAP epitope, followed by extensive washing of the immunoprecipitated complexes and protein electrophoresis under denaturing conditions (SDS-PAGE; see Figure S6b for a schematic of the protocol). Finally, immunobots were probed with anti-Myc antibodies to determine the presence or absence of a Myc-tagged putative binding partner. Specificity of IgG-sepharose for the TAP tag was confirmed by performing experiments with strains containing Myc-tagged fusion proteins and an unfused TAP tag (Figure S6c), and specificity of the anti-Myc antibodies for the Myc epitope was confirmed using cells containing only the TAP-tagged fusion proteins (Figure 3b).

Shown in Figure 3b are the results with Sfu1-Myc and Sef1-TAP. Sfu1-Myc was efficiently co-immunoprecipitated with Sef1-TAP when cells were propagated in iron-replete medium (lane 3, IP), but not when iron-starved cells were used (lane 4, IP). On the other hand, when the epitope tags were reversed, co-immunoprecipitated Sef1-Myc was poorly visualized using extracts of iron-replete cells (Figure 3c, lane 3) but was easily seen using iron-starved cells (Figure 3c, lane 4). Together, these results suggest that Sef1 and Sfu1 interact physically in a manner that is independent of iron levels, whereas the sensitivity of our biochemical assay is a function of the relative abundance of the Myc-tagged protein in the extract.

Co-immunoprecipitation experiments combining either Sef1-Myc or Sfu1-Myc with Ssn3-TAP revealed a robust interaction between Ssn3 and Sef1, but no detectable interaction between Ssn3 and Sfu1 (Figure 3d). That is, Sef1-Myc was efficiently co-immunoprecipitated with Ssn3-TAP from an extract of iron-depleted cells (Figure 3d, lane 4, IP), which express relatively high amounts of Sef1-Myc protein (Figure 3d, lane 4, input), whereas Sfu1-Myc was not co-immunoprecipitated under any condition (Figure 3d, lanes 5 and 6, respectively). When the epitope tags were reversed, Ssn3-Myc was efficiently co-immunoprecipitated with Sef1-TAP using either iron-replete (Figure 3e, lane 3) or iron-depleted (Figure 3e, lane 4) cells; note that Ssn3-Myc is relatively abundant under both conditions. These results suggest that Sef1 physically associates with Ssn3 as well as Sfu1, but these appear to represent alternative complexes since Ssn3 and Sfu1 do not associate with each other.

Sef1 is likely destabilized in the cytoplasm

To learn whether the stability of Sef1 varies with its intracellular localization, we determined the half-life of Myc-tagged Sef1 in wild-type C. albicans and in mutants in which Sef1 is stably localized in either the nucleus or the cytoplasm. Under iron-replete conditions, Sef1 is predominantly cytoplasmic in wild-type C. albicans but is mislocalized to the nucleus in sfu1ΔΔ (Figure 1b). To obtain sufficient Sef1 protein for the analysis and to uncouple the role of Sfu1 in Sef1 localization from its effects on SEFI transcription, we replaced the endogenous SFU1 promoter with a constitutively active TDH3 promoter in both wild-type and sfu1ΔΔ strains; overexpressed Sef1-Myc exhibited the same pattern of iron-dependent nuclear vs. cytoplasmic localization as Sef1-Myc expressed from its endogenous promoter (Figure S7). The strains were propagated to mid log phase in iron-replete medium, followed by addition of cycloheximide to block further translation, and serial sampling for measurements Sef1-Myc abundance. Shown in Figure 4a is a quantitative immunoblot of Sef1-Myc and alpha tubulin, which was used as an internal control for protein loading. Under these iron-replete conditions, the calculated half-life of cytoplasmic Sef1-Myc was ~80 minutes (wild type, R² = 0.94) and that of nuclear Sef1-Myc was ~160 minutes (sfu1ΔΔ, R² = 0.92). Next, we examined Sef1-Myc stability under iron-depleted conditions, in which the protein is predominantly nuclear in wild-type cells (Figure 1b) but mislocalized to the cytoplasm in the ssn3ΔΔ mutant (Figure 2d). Wild-type and ssn3ΔΔ strains expressing SEFI-MYC from the endogenous SEFI promoter were propagated in iron-depleted medium to mid-log phase, then treated with cycloheximide and visualized as above (Figure 4b). Under these iron-depleted conditions, the calculated half-life of nuclear Sef1-Myc (~150 minutes in wild type; R² = 0.98) was once again more stable than that of cytoplasmic Sef1-Myc (~40 minutes in ssn3ΔΔ; R² = 0.96). The most parsimonious explanation for these results is that Sef1 is degraded more rapidly in the cytoplasm than in the nucleus; however, we cannot exclude the possibility that Ssn3 and Sfu1 exert independent effects on Sef1 degradation that are unrelated to its intracellular localization.

Our current model of Sef1 regulation, which integrates these results with previously published findings [6,10,22], is depicted in Figure 4c. According to the model, Sef1 is subject to two distinct forms of Sfu1-mediated repression when environmental iron is replete: 1) transcriptional repression of the SEFI gene, through direct binding and repression of transcriptional initiation; and 2) post-translational inhibition of Sef1 protein, through direct binding and retention in the cytoplasm, where Sef1 is more rapidly degraded. Alternatively, under iron-limiting conditions, when Sfu1 protein is depleted, Sef1 is bound by Ssn3, phosphorylated, and localized in the nucleus, where it activates expression of iron uptake genes. Our recent observation that Sef1-Myc is constitutively cytoplasmic in an sfu1ΔΔ/ssn3ΔΔ double mutant strain (Figure S8) suggests that Ssn3 may play actively promote the nuclear localization of Sef1, beyond merely extricating Sef1 from Sfu1.

Nuclear localization of Sef1 is associated with virulence

We previously demonstrated that SEFI gene expression is induced in the iron-limiting environment of the host bloodstream and that SEFI is required for virulence in a murine model of bloodstream candidiasis [6]. Conversely, we showed that SFU1 is not required for virulence but rather that the sfu1ΔΔ mutant exhibits increased competitive fitness relative to wild-type C. albicans, presumably because of an enhanced ability to take up extracellular iron [6]. Our current results suggest that the negative effect of Sfu1 on C. albicans virulence likely results from mislocalization of Sef1 to the cytoplasm rather than from repression of SEFI gene expression, since only the former activity is observed under conditions of iron depletion (compare Figure 1a with Figure 1b).
and Figure 1b). We tested this hypothesis by examining the virulence of mutants with moderate (**SFU1**OE, Figure 1b, low iron condition) to severe (**ssn3**DD, Figure 2d, low iron condition) defects in Sef1 nuclear localization. As shown in Figure 4d and 4e, both mutants were significantly attenuated in the murine bloodstream infection model, such that mice infected with either mutant survived longer than mice infected with wild type. Note also that the strength of the virulence defects paralleled the

![Figure 3. Sfu1 and Ssn3 each physically interact with Sef1 but play opposing roles in Sef1 localization.](image-url)
strength of the Sef1 mislocalization defects of the two mutants, with those of **ssn3**ΔΔ being worse, although contributions from additional misregulated targets of Ssn3 cannot be excluded.

**Discussion**

Human pathogenic microorganisms encounter a dearth of iron in the host bloodstream and internal organs [30,31,32], and specialized systems for iron acquisition have been demonstrated to be essential for the virulence of numerous bacterial, fungal, and parasitic pathogens, e.g. [33,34,35,36,37,38,39,40]. Meanwhile, commensal-pathogens such as *C. albicans* face the additional challenge of potential iron toxicity (from free radicals generated by the Fenton reaction [41]) in niches such as the gastrointestinal tract, where iron is relatively abundant [8,9]. We previously showed that the *C. albicans* transcription factors Sef1 and Sfu1 are key components of an iron homeostasis regulatory circuit that permits adaptation to these widely divergent host niches [6]. Sef1 protects against iron deficiency in the bloodstream through the induction of iron uptake genes, whereas Sfu1 protects against iron toxicity in the gut through repression of iron uptake genes as well as *SEF1*. Still unanswered are the questions of how the activities of Sef1 and Sfu1 are themselves tied to iron levels and whether additional regulatory inputs are involved. Here, we define a system for post-transcriptional, iron-dependent regulation of Sef1 protein that precisely controls the virulence of this obligate commensal-pathogen.

A novel post-transcriptional regulatory mechanism controls iron homeostasis in *C. albicans*

Sef1 plays a central role in *C. albicans* pathogenesis through promoting the expression of virulence factors as well as iron uptake genes, whereas Sfu1 is essential for commensalism [6]. Given its role in virulence and, perhaps, in the choice between commensal and virulent lifestyles, we hypothesized that Sef1 would be a prime target for regulation beyond transcriptional repression by Sfu1. Indeed, our analysis of Myc-tagged Sef1 in wild-type *C. albicans* has revealed multiple levels of iron-dependent regulation, including nuclear vs. cytoplasmic localization, phosphorylation, and differential protein stability. In wild-type cells, Sef1 protein is nuclear, phosphorylated, stable, and competent for transcriptional activa-
tion only under iron-depleted conditions such as those encountered in the bloodstream.

Our analysis of Sef1 in *C. albicans* mutants has shed further light on the mechanisms of Sef1 regulation. Surprisingly, in the *sfu1ΔA* mutant, Sef1-Myc is constitutively nuclear, whereas in an *SFU1*-overexpression strain it is predominantly cytoplasmic. These results clearly suggested a role for Suf1 in the cytoplasmic localization of Sef1. Our screen of *C. albicans* mutants affecting predicted kinases exposed a role for Ssn3 in promoting cellular resistance to iron depletion as well as phosphorylation of Sef1. Co-immunoprecipitation experiments indicating that Ssn3 forms a physical complex with Sef1 supported a direct role for Ssn3 in Sef1 phosphorylation. Our finding that Sef1-Myc is constitutively cytoplasmic in the *ssn3ΔA* mutant suggested that Ssn3 might oppose Sfu1 by promoting the nuclear localization of Sef1. This hypothesis was validated by the ability of overexpressed Ssn3 to overcome the cytoplasmic Sef1-mislocalization phenotype (under low iron conditions) of an *SFU1*-overexpression strain. Finally, our observations that Sfu1 and Ssn3 were both detectable in complexes with Sef1, but that neither could be found associated with the other, suggested that the functional antagonism between Sfu1 and Ssn3 occurs in part through competitive binding to Sef1 protein. Meanwhile, the observation that Sef1 is constitutively cytoplasmic in an *sfu1ΔA/ssn3ΔA* double mutant argues that Ssn3 plays at least one additional role in Sef1 nuclear localization.

These studies led to a revised model of Sef1 regulation (Figure 4c). According to the model, under iron-replete conditions, Sfu1 utilizes two distinct mechanisms to inhibit the function of Sef1: 1) Transcriptional repression, via direct binding to the *SEF1* promoter, and 2) Post-transcriptional repression, via binding to Sef1 protein and forced localization in the cytoplasm, where Sef1 is unstable and unable to participate in transcription. To our knowledge, this would be the first example of a regulatory factor that regulates its target by both transcriptional and post-transcriptional mechanisms. Under iron-limiting conditions, Sfu1 protein is depleted, and Sef1 associates with the predicted protein kinase, Ssn3. Ssn3 most likely phosphorylates Sef1 directly, and either the complex or free Sef1 is transported to the nucleus, where Sef1 functions as a transcriptional activator. A key goal of future studies will be to understand how iron regulates these newly described activities of Sfu1 and Ssn3.

Impact of Sef1 post-transcriptional regulation on virulence

The findings that Ssn3 and Sfu1 post-transcriptionally regulate Sef1, an important virulence factor, raised the question of whether these regulatory events impact *C. albicans* virulence. Previously, we observed that deletion of *SFU1* leads to hypervirulence in the murine bloodstream infection model, with the *sfu1ΔA* mutant significantly better at colonizing host kidneys than wild-type *C. albicans* [6]. We attributed this enhanced fitness to derepression of *SEF1* and iron uptake genes in the mutant, resulting in an increased capacity for iron acquisition. In light of our current results showing that Sfu1 requires iron for transcriptional repression activity, a more likely explanation for the fitness advantage of *sfu1ΔA* is that Sef1 is constitutively nuclear (and therefore transcriptionally active) in this strain, whereas in wild type some fraction of Sef1 is retained in the cytoplasm and degraded. Our current observations with *SFU1*OE and *ssn3ΔA* mutants dovetail with these findings by showing the converse, i.e. that mutants with incremental defects in the nuclear localization of Sef1 have proportional defects in virulence. Together, these results strongly support the hypothesis that *C. albicans* iron acquisition (and therefore virulence) can be modulated up or down, respectively, through the activities of Ssn3 or Sfu1 on Sef1 localization and stability. We hypothesize that the evolution of such fine-tuned regulation of a potent transcription factor is particularly advantageous to an obligate commensal-pathogen, such as *C. albicans*, which must continuously adapt to differing iron concentrations among the various microenvironments of its mammalian host, while avoiding excessive expression of pathogenicity genes during its usual role as a commensal.

Materials and Methods

Ethics statement

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco and were carried out according to the National Institutes of Health (NIH) guidelines for the ethical treatment of animals.

Media

*C. albicans* strains were routinely propagated in YPD, also referred to as “iron-replete” medium. “Iron-depleted” medium is YPD supplemented with one of the specific iron chelators, bathophenanthroline disulfonic acid (BPS) or 2,2′-dipyridyl (DIP), as previously described [6].

Plasmid and strain construction

All *C. albicans* strains used in this study are described in Table S2, primers are listed in Table S3, and plasmids are listed in Table S4. Construction of *C. albicans* knockout mutants, complemented (gene addback) strains, and strains containing Myc-tagged fusion proteins was performed as previously described [6,42,43,44].

For introduction of TAP epitopes at the C-terminus of Sef1, Sfu1, and Ssn3, a series of plasmids was constructed using PCR and homologous recombination in *S. cerevisiae* [45]. The vector was pRS316 [46], and the insert consisted of (5′ to 3′): a Pmcl restriction site; 350–450 bp of target ORF sequence up to, but not including, the stop codon; the TAP tag coding sequence [29]; a *SAT1* (dominant selectable marker)-flipper cassette [47]; 350–450 bp of sequence downstream of the target ORF; and a second Pmcl restriction site. Plasmids were called pSN150 (Sef1-TAP), pSN228 (Sfu1-TAP), and pSN219 (Ssn3-TAP). Pmcl-digested plasmids were transformed into wild-type *C. albicans* reference strain SN250 [42], and nourseothricin-resistant *C. albicans* transformants were screened by colony PCR to verify the correct orientation of the C-terminal TAP tag and *SAT1*-flipper cassette. Strains expressing both Myc- and TAP-tagged fusion proteins were constructed by transforming strains already expressing the Myc-tagged protein with the appropriate Pmcl-digested TAP-tag integration fragment, as described above.

Overexpression strains for *SEF1* and *SSN3* were created by replacing portions of the endogenous promoters with the highly active *TDH3* promoter. PCR and homologous recombination in *S. cerevisiae* [45] were used to create plasmids containing (5′ to 3′): a Pmcl restriction site; 350–450 bp of sequence homology ending ~500 bp upstream of the target ORF; the *SAT1* gene (dominant selectable marker); the *TDH3* promoter; 350–450 bp of sequence homology beginning with the start codon of the target ORF; and a second Pmcl site. The vector was pRS316 [46], the source of *NAT1-TDH3* promoter was pCN342 [48], and the resulting plasmids were named pSN147 (*SEF1*OE) and pSN229 (*SSN3*OE). Correct integration of the inserts in nourseothricin-resistant transformants was verified by colony PCR, and overexpression of *SEF1* and *SSN3* was confirmed by RT-PCR.
The SFU1 overexpression strain (SN742) was created using an analogous method. pSN141 was engineered to contain (5’ to 3’): a PmeI site; 350–450 bp of sequence upstream of the C. albicans LEU2 ORF; the C. dubliniensis ARG4 gene (selectable marker); the TDIH3 promoter; the SFU1 ORF; 350–450 bp sequence downstream of the LEU2 ORF; and a second PmeI restriction site. After digestion with PmeI, the plasmid was transformed into SN515 (sfu1ΔA). Correct integration of the insert in Arg+ transformants was verified by colony PCR, and overexpression of SFU1 was confirmed by RT-qPCR.

The Ssn3ΔD325A kinase-dead mutant (SN977) was created in a similar manner to that of the SFU1OE strain. First, PCR and primers SNO1394 through SNO1397 (Table S3) were used to create a D325A-encoding variant of the Ssn3 ORF. Next, plasmid pSN239 was engineered to contain (5’ to 3’): a PmeI site; 350–450 bp of sequence upstream of the C. albicans LEU2 ORF; the C. dubliniensis ARG4 gene (selectable marker); the TDIH3 promoter; the mutant Ssn3 ORF; 350–450 bp sequence downstream of the LEU2 ORF; and a second PmeI restriction site. A Myc-tagged version of Ssn3ΔD325A (SN987) was created using a plasmid (pSN273) that contains (5’ to 3’): a PmeI site; 350–450 bp of Ssn3 ORF sequence up to, but not including, the stop codon; sequence encoding 13×Myc; a SAT1-flipper cassette [47]; 272 bp of sequence downstream of the Ssn3 ORF; 350–450 bp of sequence downstream of the LEU2 ORF; and a second PmeI restriction site. PmeI-digested plasmid was transformed into SN977, and correct integration in nourseothricin-resistant transformants was verified by colony PCR. Sequences of all PCR products were verified by DNA sequencing.

Fluorescence microscopy

C. albicans was grown at 30°C for 5–6 hours in “iron-replete” (YPD) or “iron-depleted” medium (YPD supplemented with 500 μM BPS) to OD600 = 0.8–1.0. Cell fixation, cell wall digestion, and antibody hybridization were performed as previously described [19] except that the 9E10 anti-c-Myc antibody (Covance Research) was used at a 1:500 dilution of Cy2-conjugated secondary antibody (Jackson Immunoresearch) was used at a 1:300 dilution and detected with a 1:400 dilution of 500 μM BPS. Images were acquired under 100 oil × objective using a cooled CCD camera (Cooke Sensicam) mounted on an inverted microscope (Zeiss Axioskan 200 M; Carl Zeiss MicroImaging) or a Nikon Eclipse TE2000-E fluorescence microscope. All images were processed with ImageJ software (National Institutes of Health).

Protein extraction and immunoblotting

C. albicans protein extracts were prepared under denaturing condition using a protocol adapted from a previously described method [50]. Lysates corresponding to 1 OD600 of cells were analyzed by SDS-PAGE and immunoblotted with either anti-c-Myc (9E10, Covance Research) for Myc-tagged proteins or anti-peroxidase soluble complex antibody (Sigma, P2416) for TAP-tagged proteins. Immunoblots were also probed with anti-alpha tubulin antibody (Novus Biologicals, NB100-1639) as a loading control.

Quantification of Sef1 half-life

C. albicans strains were grown on YPD medium (“iron-replete”) or YPD medium supplemented with the specific iron chelator 2,2’-dipyridyl (DIP) at a final concentration of 0.5 mM (“iron-depleted”). A sample of 1–1.5 × 10^9 cells was obtained immediately (zero time point) before addition of cycloheximide to a final concentration of 2 mg/ml. At the indicated times, 1 OD value of cells was collected and harvested for protein preparations and immunoblotting. Semiquantitative detection of protein levels was performed using the LiCor Odyssey Infrared Imager (Lincoln, NE). Integrated fluorescence intensities of individual bands were measured and background subtracted using the Odyssey Application software. The signal from Sef1-Myc bands was normalized to that of alpha tubulin. Calculations of half-life were performed as previously described [51].

TAP pull-down analysis

Cells expressing TAP-tagged Sef1, Sfu1 or Ssn3 were grown in YPD medium to OD600 ~ 0.5–0.35 and centrifuged for 3 min at 5,000 rpm. Cell pellets were resuspended in “iron-replete” medium (pre-warmed YPD) or “low iron” medium (pre-warmed YPD supplemented with DIP), and grown for an additional 4 hours in the dark. Cells were collected by centrifugation, washed three times with ice-cold water, and resuspended in 1 ml of lysis buffer (20 mM Tris, pH 7.4, 100 mM KCl, 5 mM MgCl2, 20% glycerol) with protease and phosphatase inhibitors (Roche). Cells were lysed using a Bead Beater and one-third volume of glass beads. Cell lysates were centrifuged for 2 × 20 minutes at 14,000 rpm at 4°C. Protein concentration of the supernatants was measured by the Bradford assay. 3 mg of proteins was used for immunoprecipitation with 50 μl of immunoglobulin G-Sepharose resin (lgG Sepharose 6 Fast Flow, GE Healthcare). After 24 h of protein binding with rotation at 4°C, the resin was washed 4 times with lysis buffer and 2 times with tobacco etch virus (TEV) protease cleavage buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5 mM EDTA, 0.1% Tween-20). TEV protease (100 U) cleavage was performed in 1 ml buffer at 4°C overnight. The TEV chelate was collected and proteins were recovered by TCA (trichloroacetic acid) precipitation.

RNA extraction and RT-qPCR analyses

Total RNA was prepared using a hot-phenol method [52] and treated with DNaseI using the Turbo DNA-free kit (Ambion). Ten micrograms of RNA was used in standard RT reactions using oligo ([dT]20-N) primers. cDNAs were quantified by qPCR with the primers listed in Table S3 and normalized against ACT1.

Virulence analysis

As previously described [6], groups of 10 female (8- to 10-week-old) BALB/c mice (Charles Rivers) were injected by tail vein with 5 × 10^7 CFUs of wild type (SN425), SFU1OE (SN742), ssn3ΔA (SN982), or ssn3ΔA/Ssn3 (SN978). Mice were monitored twice daily and euthanized when morbidity criteria were met (weight loss >15%, hunched posture, inactivity).

Supporting Information

Figure S1 Fusion to TDIH3 promoter results in overexpression of SFU1 RNA and protein. Results are shown for strains propagated under iron-replete conditions. a) RT-qPCR analysis of SFU1 RNA in wild-type and SFU1OE strains. b) Immunoblot of Sfu1-Myc protein in wild-type and SFU1OE strains. (EPS)

Figure S2 BPS-sensitivity of C. albicans mutants. dilutions of equivalent numbers of wild-type and mutant cells were plated on iron-replete medium (YPD), iron-depleted medium (YPD +300 μM BPS), and iron-depleted medium with supplemental iron (YPD +300 μM BPS +100 μM FeCl3) and incubated at 30°C. Note that the BPS-related growth defects of SFU1OE, sfn1ΔA, and ssn3ΔA were complemented by FeCl3 supplementation.

Post-Transcriptional Mechanisms of Sef1 Regulation

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tion, indicating that the phenotypes resulted from iron limitation (vs. chelation of an alternative divalent cation).

(EPS)

Figure S3 Immunoblot of Sef1-Myc and alpha tubulin in wild type vs. $SEF1^{DD}$. Cells were propagated in iron-replete (H) or iron-depleted (L) liquid medium, and proteins from cell equivalents were visualized in lanes 1, 2, 4, and 5. To better visualize Sef1-Myc extracted from cells grown under iron-depleted conditions, proteins from 1/4 cell equivalents were visualized in lanes 3 and 6.

(EPS)

Figure S4 Specificity of anti-Myc antibody for indirect immunofluorescence and localization of Sfu1-Myc. a) Shown is indirect immunofluorescence of a C. albicans control strain that lacks a Myc epitope (SN250), showing minimal nonspecific staining with the anti-Myc antibody. b) Shown are indirect immunofluorescence images of an isogenic strain strain that lacks a Myc epitope (SN250), showing minimal nonspecific staining with the anti-Myc antibody. b) Shown are indirect immunofluorescence images of an isogenic strain

(C. albicans

mRNA levels in various strains.

(DOCX)

Table S1 Quantitation and statistical analysis of SEF1

mRNA levels in various strains.

(DOCX)

Table S2 Strains used in this study.

(DOCX)

Table S3 Primers used in this study.

(DOCX)

Table S4 Plasmids used in this study.

(DOCX)

Acknowledgments

The authors are grateful to Kalyan Pande for expert assistance with the virulence analysis of C. albicans mutants. We thank Guangshuo Ou, Cherilyn Elwell, and Jung Hwa (Jenny) Kim for help with microscopy and the Sil, Engel, and Vale laboratories for the generous use of their microscopes. We thank Sinem Beyhan and Nam Lee for technical assistance, Anita Sil for reagents, and Cliff Lowell for the use of his LiCor instrument. We are also grateful to Anita Sil and Hiten Madhani for useful discussions and to Hiten Madhani for helpful comments on the manuscript.

Author Contributions

Conceived and designed the experiments: CC SMN. Performed the experiments: CC CC. Analyzed the data: CC SMN. Contributed reagents/materials/analysis tools: CC SMN. Wrote the paper: CC SMN.

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