

The role of phase separation in a transcriptional repression mechanism in
Candida albicans

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Signature page

This dissertation by Maureen Alice Dowell is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Preface

The following is a thesis dissertation written by Maureen A. Dowell with input by Dr. Richard Bennett. The work presented in this thesis was performed at Brown University in the laboratory of Dr. Richard Bennett. Maureen A. Dowell performed all experiments and analyses reported herein with the following exceptions:

Chapter 2: Dr. Corey Frazer designed some plasmids related to Figures 1 and 4 and Extended Data Figures 1 and 3. Dr. Corey Frazer also purified some proteins related to Figure 4 and Extended Data Figure 3. Dr. Mae (Staples) Lux constructed a plasmid related to Figure 4 and Extended Data Figure 3, helped purify some proteins related to Figure 4, and fluorescently labelled some proteins related to Figure 4. Dr. Deepika Gunasekaran helped to edit a resource used for RNA sequencing analysis in Figure 5.

Chapter 3: Dr. Corey Frazer designed PrLD plasmids related to Figure 1 and the PrLD plasmids included in Figure 2. Dr. Corey Frazer also designed plasmids used for *E. coli* protein expression, purified proteins, and constructed several strains related to Figures 6 and 7. Dr. Mae Lux constructed the eYFP-Wor2-PrLD-LacI plasmid used in Figure 2, and one or more plasmids used for *E. coli* protein expression in Figures 6 and 7.

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Chapter 1: Introduction

1. Principles of biomolecular condensates

Phase separation occurs when a solution becomes supersaturated and demixes into a dense phase and a dilute phase [1, 2]. In this phenomenon, the molecules that make up the dense phase have a greater affinity for each other than for the surrounding solvent [3]. When this occurs in a biological context, the dense phase is referred to as a biomolecular condensate. Biomolecular condensates can exhibit a range of properties, from liquid-like, to gel-like, to solid-like [4]. These condensates are stable, but often remain highly dynamic [4-6].

The formation of biomolecular condensates has important implications for the cell (**Fig. 1**). Cellular processes are organized into compartments to facilitate efficient reactions. While cellular organelles are enclosed by lipid membranes to create a barrier between their components and the surrounding environment, many membraneless organelles are thought to be formed by phase separation, such as the nucleolus, stress granules, paraspeckles, and P granules [7-10]. Moreover, condensates have been implicated in human health concerns such as neurodegeneration, cancer, and developmental malformations [5, 11, 12].

Biomolecular condensates are complex structures often composed of proteins and/or nucleic acids, and their physical properties are determined by polymer interactions [1, 2]. In Flory-Huggins solution theory, three types of interactions are present: polymer-solvent, polymer-polymer, and solvent-solvent. According to this theory, phase separation occurs when polymer-solvent interactions are energetically unfavorable, causing formation of a polymer-rich dense phase and a solvent-rich dilute phase [2]. Electrostatic interactions have also been incorporated into polymer-solution theory, such that many condensates form as a way to neutralize opposing charges [2, 13]. More recently, percolation and entanglement theories have been discussed with respect to biomolecular condensate formation [3, 14].

The primary driver of biomolecular condensation is multivalency [15], the ability to interact with more than one other molecule, which increases the likelihood of polymer-polymer interactions. The repeating subunits of RNA, DNA, and many proteins give these polymer chains a high

valency [2, 5]. While repeats of ordered protein domains can give rise to multivalency, intrinsically disordered regions (IDRs) or low-complexity domains (LCDs) are commonly found in phase-separating proteins [10, 15]. IDRs lack a stable structure, and thus exhibit a high level of conformational heterogeneity [2-4, 16].

Prion-like domains (PrLDs) are a well-studied subset of IDRs that are rich in asparagine, glutamine, serine, and tyrosine, referred to as "prion-like" because of their similar amino acid composition to yeast prion proteins [15, 17]. Unlike classical prions, which are proteins that can form self-propagating aggregates [17], PrLDs do not necessarily form amyloid structures. A number of PrLD-containing proteins, including FUS, TDP-43, TAF15, and hnRNPA1, have been shown to undergo phase separation [17].

A stickers-and-spacers model was proposed to explain the contribution of valency to the phase separation behavior of PrLDs [18]. In this model, *stickers* are motifs that enable specific polymer-polymer interactions, while *spacers* engage in non-specific polymer-solvent interactions that affect the solubility of the molecule [3, 18]. The number of stickers thus determines the valency of the protein. While high sticker valency increases polymer-polymer interaction, these effects must be modulated by polymer-solvent interactions to maintain condensate dynamics. As shown by Martin et al., even spacing of aromatic stickers promotes liquid-like condensate formation, while clustering of stickers promotes aggregation [18]. Similarly, Das and Pappu had previously shown that alternating positive and negative charges promoted extended conformations (i.e. solvation), while clustering of like charges into blocks promoted collapsed conformations [19].

Numerous types of molecular interactions govern the phase separation behavior of biopolymers [15, 20]. In disordered proteins, pi-pi interactions frequently occur when amino acid side chains have delocalized electrons capable of creating regions of partial charge [21]. Aromatic residues (Y, F, W) are most commonly recognized as exhibiting this behavior [20], although Vernon et al. indicate that other residues such as arginine (R), glutamine (Q), asparagine (N), and

aspartic acid (D) are also capable of this behavior [21]. Pi electrons can also interact with positively charged cationic side chains such as arginine (R) or lysine (K) residues [15, 20]. Charge-charge interactions take place between oppositely charged residues (e.g. D and K) [15, 20], while hydrophobic interactions and hydrogen bonding can also contribute to phase separation [20]. Lastly, some PrLDs have been thought to form intermolecular cross-beta sheets that are similar to but less stable than those found in amyloid fibrils [15, 22, 23], although the occurrence of this type of secondary structure in phase-separated condensates is disputed [24, 25].

In the case of the disordered human protein FUS, several residue types contribute to condensation. FUS is an RNA-binding protein that is involved in many cellular processes, including transcription initiation, RNA splicing, DNA repair, and miRNA processing [26]. The saturation concentration of FUS is determined primarily by the tyrosine (Y) and arginine (R) content [27, 28]. Glycine (G) spacers are thought to maintain condensate liquidity, while increasing the proportion of glutamine (Q) or serine (S) spacer residues promotes FUS condensate hardening [28]. An opposing view disputes the stickers-and-spacers model, indicating that all of these residue types (Y, R, Q, S, and G) are involved in the intermolecular interactions that promote FUS phase separation [24, 29], suggesting that there are no true spacer residues. Regardless, the residues described are commonly enriched in phase-separating IDRs, in addition to phenylalanine (F), asparagine (N), lysine (K), aspartic acid (D), and glutamic acid (E) [2]. IDR length can also affect the ability of proteins to undergo phase separation and the material properties of condensates [30].

Alongside the identity of the amino acids, the addition of post-translational modifications (PTMs) to disordered proteins can also affect phase separation. The PTMs involved in phase separation are very diverse, including phosphorylation, arginine methylation, citrullination, acetylation, and poly(ADP-ribosylation) [31, 32]. The effect of a particular residue is system-dependent, highlighting the precarious nature and tunability of biomolecular condensates. For example, Lin et al. found that the phosphorylation of serines next to critical tyrosine residues in a

synthetic FUS model ablated condensate formation [27], while Wegmann et al. found that phosphorylation is necessary to promote phase separation of tau [33].

Even proteins that are not capable of phase separation still be incorporated into condensates [20, 34]. This finding led to the concepts of *scaffolds* and *clients* as proposed by Banani et al. [34] to describe systems where *scaffolds* nucleate phase separation and incorporate *client* proteins that may not be able to form condensates on their own. Along this line, RNA- and DNA-binding domains also present an opportunity for proteins to bind to a multivalent nucleic acid polymer scaffold [15], increasing the propensity for system phase separation beyond that of the protein alone. Some combinations of proteins form condensates more easily than do the individual proteins, as has been described for hnRNPA1 and FUS [35], emphasizing the importance of both homotypic and heterotypic polymer interactions.

Multiple other factors can affect condensate formation including pH, temperature, and salt [20]. Low pH is associated with stress granule formation in yeast [36]. Changing the pH can also have an effect on charged residues [20]. Temperature can affect phase separation in a variety of ways. Increasing temperature may promote phase separation by increasing the thermodynamic activity of component molecules, or by increasing active processes that result in phase separation [20]. Some systems also exhibit re-entrant phase behavior, where phase separation occurs at low and high temperatures, but not at an intermediate temperature range [20, 37]. Different salts have long been known to affect protein behavior [38]. Salts containing kosmotropic anions such as carbonate and sulfate tend to precipitate proteins, while salts containing chaotropic anions such as perchlorate or thiocyanate tend to promote solubility and denaturing of proteins [24, 38]. Ion valency can also have an effect, since multivalent ions can have a greater neutralizing effect on charged amino acid residues [20]. Recently, multivalent cations have been shown to specifically promote the phase separation of the negatively charged DDX4 helicase family, while having little effect on the related but less charged DDX3 family [39].

The properties of biomolecular condensates can also change over time [40, 41]. Alberti

and Hyman describe several ways in which a liquid condensate can undergo aging to a more solid-like state: 1) gelation, 2) glass formation, and 3) aggregation [40]. Gelation indicates the formation of a highly connected matrix that transitions suddenly from liquid to solid, while glass formation lacks this sudden transition, becoming more solid-like incrementally over time [40]. While gels and glasses are amorphous, aggregates are distinguished from these by low water content and reduction in molecular mobility [40]. These transitions in material properties can occur on timescales ranging from hours to days. Several factors can influence the solidification of biomolecular condensates, including environmental changes, temperature, mutations, high protein concentration, low water content, lack of physiological binding partners, accumulation of misfolded proteins within the condensate, and changes in intracellular chemistry associated with stress [40].

Lastly, recent advances have demonstrated that the chemical properties of condensates are distinct from those of the dilute phase. Dense phases formed by complex coacervation are depleted of ions compared to the dilute phase, since they are formed by charge neutralization of oppositely charged component polymers [42]. Condensates also modulate pH, with the pH of the dense phase moving away from that of the solution and towards that of the isoelectric point of the component polymers [43]. These ion and pH gradients could enable membraneless organelles formed by condensates to create molecular environments that enable specialized functions in a manner similar to lipid-enclosed organelles [42, 43]. Furthermore, the interfaces of condensates display properties that are distinct from both the dense phase and the dilute phase. While polymer chains on the interior or exterior of simulated hnRNPA1 condensates exhibited random configurations, chains located on the condensate interface are arranged perpendicularly to the interface [44]. Electrochemical potentials that exist at the interfaces of condensates have also been demonstrated to drive redox reactions [45]. Surface electrostatics could also provide a reason as to why all condensates in a system are not eventually observed to fuse into a single, large condensate [42].

2. Examples of biomolecular condensates

Phase separation is involved in a wide variety of cellular processes. Condensate formation plays a role in stress responses, cell signaling, transcriptional regulation, subcellular localization, and even occurs outside the cell in higher-order processes such as tight junction formation [46-51]. While new functions for biomolecular condensates are continually being uncovered, new techniques are also needed to address important questions about how phase separation occurs in a biological context, and how best to measure condensate properties in a native environment [4]. This section will review what is known the role of phase separation in a sampling of different cellular components, beginning with one of the best-studied [10]: the nucleolus.

2.1 The nucleolus

The nucleolus is the site of multiple important cellular processes, including the initiation of ribosome assembly, RNA polymerase I-mediated transcription, ribosomal RNA (rRNA) processing, and assembly of other ribonucleoproteins [52]. While the nucleolus is a large cellular structure, it is not bound by any lipid membrane [52]. It is composed of three layers: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC) [10, 52].

In 2011, Brangwynne et al. first published the liquid-like behavior of *Xenopus laevis* nucleoli, based on their spherical properties and ability to undergo fusion events [7]. Subsequently, disordered *Caenorhabditis elegans* nucleolar proteins FIB-1 and DAO-5 were shown to undergo condensation *in vitro*, with RNA promoting condensation at lower protein concentrations [53]. Another nucleolar protein, nucleophosmin (NPM1), was shown to form condensates when combined with proteins containing R-rich tracts [54], or on its own in the presence of the crowding agent Ficoll [55]. When FIB1 and NPM1 were combined with RNA, they were found to form immiscible liquid phases that formed a core-shell structure similar to the FC and DFC [56]. The formation of these subcompartments is dependent on the ability of the component proteins to bind rRNA, which affects the surface tension of the condensate layers and prevents mixing [56].

Later work has identified that nucleolar IDRs are enriched in acidic residues (D/E) and K-blocks, and that IDRs containing interspersed blocks of K and E drive the formation of the FC via complex coacervation [57]. RG-rich IDRs are depleted from the FC, but are more common in the DFC and GC [57]. Use of pH-sensitive dyes illuminated a pH gradient in reconstituted nucleoli, with the interior FC and DFC being more acidic than the outer edges of the GC [57]. The nucleolus as a whole is more acidic than the nucleoplasm or other nuclear structures such as Cajal bodies and nuclear speckles [57]. As rRNA is synthesized in the FC and DFC, the nucleolar pH gradient could represent a mechanism for egress of matured rRNAs into the GC where ribonucleoproteins are assembled, and then out into the nucleoplasm [52, 57]. Additionally, IDR mutations that produce R-rich sequences can cause aberrant localization to the nucleolus, resulting in altered nucleolar structure and defective rRNA production [12].

2.2 Stress granules

Stress granules (SGs) are cytoplasmic structures composed of protein and RNA that form when translation initiation is stalled [58]. This translation stalling can occur due to various stress conditions such as oxidative stress, glucose starvation, heat shock, or drug treatment [58, 59]. The exact protein-RNA composition of an SG is variable, although they typically have a dense, non-dynamic core surrounded by a less dense and more dynamic shell [60]. Overexpression of certain SG proteins such as TIA1 and TIAR can induce stress granule formation without the presence of stress conditions [61]. SGs are thought to store excess mRNAs when the capacity of translation or degradation machinery is exceeded [59], and they are disassembled when stress is removed [58].

In human cells, the proteins G3BP1 and G3BP2 (collectively referred to as G3BP) are necessary for SG formation [8]. G3BP1 can undergo phase separation in the presence of RNA [8, 62]. G3BP1 contains an R-rich region and an acidic tract: under non-stress conditions, these two regions of the protein self-interact, but during stress-induced translational inhibition, excess RNA

outcompetes G3BP1 self-interaction and scaffolds condensate formation [62].

Other SG component proteins such as hnRNPA1, FUS, TDP-43, Atx2, and TIA1 have been linked to neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia [58]. Disease-associated mutations in these proteins often increase their ability to self-assemble [58]. Defects in autophagy can lead to similar neurodegenerative pathologies, suggesting that the ability to clear SG components is necessary for neuronal survival [58]. Interestingly, the RNA-binding status of FUS and TDP-43 seems to regulate phase separation ability: RNA binding prevents phase separation, while RNA depletion causes aberrant condensate formation [63]. In neurons, RNA-bound TDP-43 localizes to SGs and does not impact cell health, while unbound TDP-43 forms separate condensates that cause neuronal death [64]. These studies point toward opportunities for new therapeutic development to modulate SG composition and protein condensation.

2.3 Paraspeckles

Paraspeckles are nuclear body found adjacent to nuclear speckles [65]. Although their exact function remains elusive, these membraneless organelles may have a role in regulating gene expression by sequestering RNAs in the nucleus [65]. While a number of proteins are required for paraspeckle formation (including NONO, SFPQ, HNRNPK, DAZAP1, FUS, RBM14, and HNRNPH3) [66, 67], the lncRNA NEAT1 forms the scaffold for this nuclear structure [68, 69].

Paraspeckles are enriched for PrLD-containing proteins, with FUS and RBM14 PrLDs required for paraspeckle formation [70]. NEAT1 contains multiple binding sites for disordered proteins, so it is likely that this RNA scaffold uses multivalency to seed condensate formation [71]. More recent work indicates that paraspeckles are a type of micelle that forms through microphase separation [9]. The 3' and 5' tail ends of NEAT1 have more solvent affinity so they localize at the surface, while the middle of NEAT1 localizes in the center of the paraspeckle [9]. Paraspeckles are also implicated in viral immunity, cancer, and disorders of the nervous system [67]. Notably,

disease-associated FUS mutations can cause the formation of paraspeckle protein aggregates in the cytoplasm [72].

2.4 Fungal biomolecular condensates

Several examples of phase separation have been uncovered in yeast and related fungal species. Phase separation is involved in diverse fungal processes such as stress response, autophagy, and cytoskeleton formation.

Yeast are an important model for SGs, which were introduced in section 2.2. Yeast SGs are thought to be less dynamic than those in higher eukaryotes [60], although they are not irreversibly aggregated into amyloid structures [73]. In yeast, different stresses have been found to cause the formation of SGs with different material properties: pH changes cause Pub1 SGs that are more gel-like, while heat stress causes more solid-like SGs that require Hsp104 for dissolution [36]. *In vitro*, Pub1 is capable of phase separation at low pH, while heat treatment reduces the dynamics of Pub1 condensates [36]. In the cell, these heat shock-induced SGs contain aggregated, amyloid-like structures [73]. Another yeast SG protein, Pab1, can also undergo phase separation, which was shown to be important for stress tolerance [46]. In both Pub1 and Pab1, the RNA recognition motifs are critical for phase separation [36, 46].

Classical prion proteins also play a role in yeast fitness and stress response [74, 75]. Some prion proteins are even able to form liquid-like states, not just amyloid structures [76]. For example, the translation termination factor Sup35 is a classical prion protein that regulates the response to starvation [47]. When cytosolic pH drops due to glucose starvation, Sup35 forms liquid-like condensates which dissolve when the stress is removed [47]. Sequestration of Sup35 into condensates stalls translation, which may be useful when resources are scarce [47]. Comparatively, yeast cells containing aggregated Sup35 prions [PSI⁺] had impaired recovery after removal of stress [47]. This finding indicates that the liquid-like behavior of Sup35 is important for fitness [47], although the formation of liquid-like condensates by prion proteins may

promote detrimental amyloid formation and prion conversion [77].

Phase-separating proteins also play a role in yeast autophagy [78]. The pre-autophagosomal structure (PAS) is the precursor to the autophagosome in yeast [79]. The ATG1 complex (made up of the proteins Atg1, Atg13, Atg17, Atg29, and Atg31) initiates formation of the PAS in response to nutrient starvation [79, 80]. Several of the Atg proteins contain IDRs [81], and the ATG1 complex forms condensates when reconstituted *in vitro* [80]. In cells, the entire PAS has been characterized as a liquid-like condensate based on observation of fusion events, fluorescence recovery, and treatment with 1,6-hexanediol [80], a chemical disruptor of weak hydrophobic interactions, which has also been reported to dissolve phase-separated condensates [82-84].

Autophagy is regulated by TORC1 signaling [81]. TORC1 inhibits autophagy by phosphorylating Atg13, reducing the affinity of Atg13 for other Atg1 complex proteins [81]. Evidence exists that during stress, TORC1 is partitioned into SGs [85], indicating a role for biomolecular condensates in TORC signaling. Condensation of the disordered Pbp1 protein has also been shown to inhibit TORC1 signaling, although the exact mechanism remains unclear [86, 87].

The cytoskeleton also makes use of condensation [88]. Condensates can act as reservoirs for monomer components at the ends of growing cytoskeletal polymers like actin and microtubules [88]. In *Saccharomyces cerevisiae*, the polarisome nucleates the formation of actin cables during cell division; this cytoplasmic structure is formed by a network of interacting proteins, including Bni1 and Spa2 [89]. Condensation of Spa2 with the disordered Aip5 (actin-interacting protein 5) has been shown to promote actin formation [90].

In the filamentous fungus *Ashbya gossypii*, phase separation of poly-Q proteins is responsible for patterning the cytosol [91, 92]. The poly-Q protein Whi3 is required for localizing *BNI1* mRNA to the hyphal tip, and for clustering *CLN3* mRNA [91, 93]. Whi3 undergoes phase separation when bound to RNA; this behavior is dependent upon the RNA-recognition motif and

not the poly-Q domain [92]. Interestingly, the identity of the RNAs bound by Whi3 affects the properties of the condensate. When Whi3 binds to *BNI1* mRNA, the condensate is more dynamic, whereas when Whi3 binds to *CLN3* mRNA, the condensates are less dynamic [92]. Subsequent work revealed that the different structures of the component RNAs drive the different physical properties of these condensates [94].

These examples represent a variety of different functions that condensation can play in fungal cells. As discussed, phase separation is involved in the stress response, autophagy, TORC1 regulation, cellular polarity, and cytosolic patterning. These mechanisms involve the formation of liquid-like condensates, as well as aggregates and true prions, and point to an important role that nucleic acid structure may play in specifying the activity and location of condensates.

3. Phase separation and transcriptional regulation

Phase separation has been proposed as a general mechanism of transcriptional regulation [48, 95, 96]. Condensate-forming proteins are involved in transcriptional regulation, chromatin remodeling, and heterochromatin formation [96]. This section is divided into three parts: 1) transcriptional activation, 2) heterochromatin formation and transcriptional repression, and 3) critiques of phase separation as a mechanism for transcriptional control.

3.1 The involvement of phase separation in transcriptional activation

Transcription is the production of RNA from a DNA template and is catalyzed RNA polymerases, that are often recruited by DNA-binding proteins called transcription factors (TFs). The transcriptional process involves three phases: 1) initiation, 2) elongation, and 3) termination [96].

In eukaryotic cells, the RNA polymerase II complex synthesizes all mRNAs [97]. The largest RNA pol II subunit, Rbp1, is responsible for the catalytic activity of the enzyme [97]. Rbp1 also contains a disordered C-terminal domain (CTD) composed of tandem YSPTSPS repeats [97,

98]. The CTD is involved in coordinating a wide variety of mRNA processes, including transcriptional initiation, elongation, and termination, and also capping, splicing, and 3' end processing of transcribed RNAs [97]. The number of CTD repeats varies by species; yeast CTDs have fewer YSPTSPS repeats, while humans have more repeats and thus a longer CTD [97-99]. The CTD has been shown to undergo phase separation [100]. Longer CTDs form more stable condensates than shorter CTDs; longer CTDs also produce more RNA pol II clustering in cells, and have an increased duration of chromatin association [100]. The increased phase separation propensity of long CTDs may be necessary to enable transcription in larger genomes (such as human vs. yeast), as the larger distances between regulatory regions and ORFs may make the recruitment of RNA pol II more difficult [99].

The RNA pol II CTD is also the target of multiple post-translational modifications [97, 98, 100]. During transcriptional elongation, the CTD accrues more phosphorylated residues, which are later dephosphorylated at the end of transcription [97]. Boehning et al. showed that phosphorylation dissolves CTD condensates [100]. This finding led to a model where the CTD forms condensates that promote the initiation of transcription, and then phosphorylation of the CTD allows the release of RNA pol II from the condensate, facilitating the transcriptional elongation step [100]. Subsequent dephosphorylation would then allow the CTD to be recruited into new condensates to repeat the cycle.

To initiate transcription, RNA pol II is first recruited to a gene locus by TFs. As a class, TF proteins are enriched for intrinsically disordered sequences [101]. This enrichment suggests that IDRs play an important role in the function of these proteins, which involves interaction with both DNA and the RNA polymerase complex to regulate which genes are transcribed. Boija et al. showed that the IDR-containing TF proteins OCT4, MYC, p53, NANOG, SOX2, RAR α , GATA-2, and ER concentrated into droplets with the IDR of the MED1 subunit of the Mediator Complex, a transcriptional coactivator that interacts with TFs and RNA pol II [48, 102]. They also demonstrated that the same residues required to promote phase separation of OCT4 were also

necessary to promote transcription [48]. TF IDRs expressed in mammalian cells can also form condensates when recruited to a synthetic landing pad; these IDR condensates recruit RNA pol II and drive transcription [84]. These TFs exhibited interaction specificity: while the FUS and TAF15 IDRs recruited each other into condensates, they could not recruit the Sp1 IDR, and Sp1 IDR condensates could not recruit the FUS or TAF15 IDRs [84]. TFs and coactivators are thought to form condensates together to concentrate transcriptional machinery into genomic compartments that are responsible for particular transcriptional programs [103, 104], consistent with previous data about the formation of DNA loops into gene neighborhoods that are transcriptionally correlated [105, 106]. Interaction specificity between IDRs could provide another mechanism for coordinating transcriptional programs.

Chromatin itself can also undergo phase separation, which is modulated by DNA linker length, acetylation, and histone H1 [107]. Acetylation by p300 dissolves these condensates, although acetylated chromatin will form condensates when combined with the TF BRD4 [107]. Unmodified chromatin droplets and BRD4-acetylated chromatin droplets adhere to each other but do not mix, indicating a mechanism for compartmentalization of the genome by phase separation [107]. RNA can cause compartmentalization of the genome as well; the accumulation of RNA at actively transcribed loci causes the recruitment of RNA-binding proteins that form microphases, which exclude inactive chromatin [108].

3.2 The involvement of phase separation in heterochromatin formation and transcriptional repression

Repressive chromatin also contains phase-separating proteins [109]. While transcriptionally active chromatin is bound by activating TFs, Mediator, and RNA pol II, transcriptionally repressed chromatin is bound by a different set of proteins [105, 109, 110]. Polycomb Repressive Complex (PRC) proteins and H3K27me3 modifications are typically associated with facultative heterochromatin, which is silenced in only certain cell types [109, 110]. HP1 and H3K9me3 are

commonly associated with constitutive heterochromatin, which is typically found in pericentromeric regions and is silenced in all cell types [109, 110].

The main complexes in the Polycomb group are PRC1 and PRC2, which deposit different histone modifications [109]. The disordered protein CBX2, a component of PRC1, forms condensates in cells and *in vitro*, which can incorporate DNA and nucleosomes [111]. CBX2 condensation is dependent on positively charged residues and phosphorylated serine-rich regions in the IDR [111, 112]. Mutation of these residues in CBX2 leads to a failure of PRC1 to compact chromatin in cells [112]. In the fungus *Fusarium graminearum*, the H3K27me3 reader protein BP1 has been shown to use phase separation to interact with PRC2 and mediate transcriptional repression [113].

The heterochromatin protein HP1 can also form condensates with DNA [114]. HP1-DNA condensates adhere to but do not mix with condensates formed by DNA only [114]. HP1 and H3K9me3 are commonly considered markers of constitutive heterochromatin [109, 110]. While H3K9me3 and unmodified chromatin will normally form condensates together, HP1 binds to and drives the segregation of H3K9me3 chromatin [115]. Paradoxically, active transcription can also be important for maintaining repressive chromatin. In actively transcribed regions, RNA-binding protein BAZ2A forms condensates with nascent RNA [116]. When BAZ2A RNA-binding is impaired, these condensates are lost, resulting in aberrant transcription of nearby H3K27me3 regions that are normally silent [116]. Thus transcriptionally active condensates may play an important role in sequestering transcriptional machinery away from silenced regions.

These studies indicate that phase separation likely has a role in segregating the genome into transcriptionally active and inactive regions.

3.3. Critiques of phase separation as a mechanism for transcriptional control

Questions have been raised regarding the phase separation behavior of proteins and its relevance inside the cell, calling for increased focus on polymers in their native cellular

environment [117, 118]. The formation of biomolecular condensates *in vitro* or in exogenous overexpression systems may be more artifactual than indicative of the true behavior of the native system.

Other studies have indicated that multivalency is more important for function than actual condensate formation. Trojanowski et al. found that multivalent interactions enhance transcription even without phase separation, with high multivalency increasing recruitment and residency time [119]. Similarly, Chong et al. found that while fusion oncoproteins that can form condensates can cause alternate patterns of transcription in cancer cells, increasing the phase-separation propensity of these TF proteins even further may actually repress transcription [120]. These studies indicate that transcription is a delicately balanced process requiring the correct level of dynamics and interaction—not too much, and not too little. Nonetheless, observations of condensate formation in exogenous systems may still provide a readout for valency, which is useful in its own right [118].

Mittag and Pappu [3] propose that even in the absence of distinct dense phases, pre-percolation dynamic clusters can form [121], as have been noted previously [84, 122]. These clusters refer to small assemblies of finite size that occur in a setting where the concentration of the solute is below both the saturation concentration and the threshold concentration for percolation [3]. They also propose that the active versus repressive functions of transcriptional condensates or pre-percolation clusters will be context-dependent [3], so the concentration as well as the presence of other components will impact transcription. The rise in studies on biomolecular microphase separation [9, 108, 123, 124] may represent an alternative possibility for *in vivo* presentation. Microphase separation is a distinct process where component polymers are made of blocks of sequences that repel each other, leading to the formation of small domains composed of compatible sequences; these domains are limited in size because of the attachment to other repulsive sequences in the same polymer [125]. Microphases can form spontaneously on a scale of 10-100 nm, which is relevant to a cellular environment, and their various

morphological properties are dependent on polymer composition [126].

In summary, studies on phase separation need to include an emphasis on the role of the cellular environment [117]. The formation of actual condensates may not be necessary for the function of many proteins that can undergo phase separation, as seems to be the case for some TFs. Other forms of dynamic multivalent interaction, such as clustering or microphase separation, may yet be physiologically relevant. However, observations about phase separation behavior can still be used as an indicator of valency and interaction ability [118]. These observations are likely to be useful for small systems such as fungal nuclei, where direct observation of condensation or clustering represents a particular technical challenge.

4. The white-opaque switch in *Candida albicans*

Candida albicans is an opportunistic human fungal pathogen that is found as a commensal in at least 70% of healthy adult humans [127, 128]. This fungus can cause systemic infection as well as oral, esophageal, vaginal, and nail infections [129]. The mortality rate for systemic candidiasis is approximately 30% [128, 130]. Immunosuppression, antibiotic treatment, and disruption of skin or mucosal barrier increase the risk of systemic candidiasis [128].

C. albicans can form multiple different morphologies, including both yeast and filaments [127]. Another cell type transition that *C. albicans* can undergo is the white-opaque switch, which was first identified in 1987 [131]. These cell types can be differentiated by their shape, size, and colony morphology [127, 131]. White cells are smaller and rounder, and form raised, shiny white colonies on standard laboratory medium. Opaque cells are larger and more elongated in shape, and form larger, flatter colonies with a darker color and less shine. These cell types also behave differently in a host environment. White cells are better at colonizing the gastrointestinal tract and are more successful in systemic infections, while opaque cells are specialized for mating and are better at colonizing the skin [127].

The transition between white and opaque cell states is epigenetically regulated by a

network of transcription factors (**Fig. 2**). Efg1 was the first TF found to regulate switching [132, 133], followed by the transcriptional repressor Tup1 [134]. In 2006, a novel TF, Wor1, was identified as the master regulator of opaque formation [135, 136]. This discovery was followed by the identification of other TFs that were critical for regulating the white-opaque switch: Czf1, and Wor2 [137, 138]. Subsequently, Wor3, Wor4, Ahr1, and Ssn6 were identified as regulators of white-opaque switching [139-142]. Efg1, Ahr1, Ssn6, and Tup1 promote the white cell state [28, 142, 143], while Wor1, Wor2, Wor3, Wor4, and Czf1 promote the opaque cell state [132, 135-138, 141, 144].

White-opaque TFs have been found to bind each other's promoter regions, forming feedback loops that maintain heritability of a single cell state [137, 140]. These TFs are often found at genomic locations despite a lack of consensus DNA binding sequences [140], pointing to protein-protein interactions as a critical factor for bringing TFs to regulatory regions.

5. Ssn6-Tup1 is a conserved transcriptional repression complex

Ssn6 has been characterized as a conserved transcriptional repressor in *S. cerevisiae*, where it is often referred to as Cyc8 [145-147]. Tup1 is a WD40 repeat-containing protein (**Fig. 3**) that is part of the Groucho/TLE family of proteins [146]. This protein family is involved in transcriptional repression and cell differentiation in many species, including humans, *Drosophila melanogaster*, and other yeast; TLE family proteins interact with other TPR-containing proteins like Ssn6 [146-149].

Together, Ssn6 and Tup1 form a complex that represses transcription [150]. The Ssn6-Tup1 complex is involved in regulating the expression of more than 7% of all genes in *S. cerevisiae* [151]. This complex is composed of one Ssn6 and four Tup1 molecules [152]. The alpha-helical N-termini of the Tup1 molecules form a dimer of dimers that is thought to be wrapped by the Ssn6 TPR (tetratricopeptide repeat, see **Fig. 3**) [153, 154]. Neither Ssn6 nor Tup1 contains a DNA-binding domain [147], so protein-protein interactions are critical for bringing these TFs to

DNA. Ssn6 is required for normal growth rates in both *S. cerevisiae* and *C. albicans* [142, 155], whereas deletion of Tup1 has not been noted to affect growth rate [153].

Initial assays found that Tup1 provides the repression function of the complex, while Ssn6 is thought to interact directly with DNA-binding proteins [156]. The Ssn6 TPR domain is required for interaction with Tup1 and other proteins [157-159]. Deleting different repeats from the TPR domain results in the de-repression of different genes [157]. Similarly, the WD40 repeats of Tup1 are required for repression of some genes and not others [156, 157]. Initial models posited that Ssn6 acts as a bridge to bring Tup1 to the site of repression. However, there is also evidence that Ssn6 and Tup1 can operate independently of one another, including in *C. albicans* [142, 160-162]. Additionally, Ssn6 may be able to act as an activator of transcription when Tup1 is not present [162].

In *S. cerevisiae*, Ssn6 can form a prion [*OCT*⁺] that causes de-repression of genes that are normally repressed by Ssn6-Tup1 and allows the use of lactate as the sole carbon source; overexpression of Hsp104 does not cure strains of [*OCT*⁺], and Hsp104 is required for inheritance of [*OCT*⁺] [151]. [*OCT*⁺] causes derepression of genes that are normally repressed by Ssn6-Tup1 [151]. In this case, prionization or aggregation can cause altered gene expression profiles in yeast cells and increased fitness in different environments.

In *C. albicans*, Ssn6 and Tup1 are both involved in regulating white-opaque switching [134, 142]. Ssn6 is essential for formation of the white cell type, as *C. albicans* Ssn6 knockout mutants are locked in the opaque state [142]. Tup1 deletion mutants are locked in a filamentous form [163], but overexpression of Tup1 causes opaque cells to convert to the white state [143]. Thus both Ssn6 and Tup1 are repressors of the opaque cell type, although it has been difficult to untangle the function of Tup1 from its role in hyphal regulation.

The human homolog of Ssn6, UTX, is frequently mutated in cancer cells [164]. UTX is also required for embryonic cell differentiation [165, 166]; UTX mutations are linked to Kabuki syndrome, a developmental disorder involving the neural crest [167]. UTX is also involved in

regulating the gene expression of CD8⁺ T cells, affecting the ability to clear cancer cells and viral infections [168, 169]. Recently, UTX has been shown to undergo phase separation [170]. Mutation of the disordered domain required for UTX phase separation decreases tumor suppression ability and prevents progression of embryoid body differentiation in mouse embryonic stem cells [170].

In summary, Ssn6 and its partner protein Tup1 play an important role in regulating gene expression in *C. albicans* and in other yeast. Ssn6 and Tup1 are prominently involved in cell fate determination in *C. albicans*. Homologs of these proteins are likewise involved in cell differentiation processes in higher eukaryotes, broadening the impact of studying the transcriptional control mechanism of Ssn6-Tup1.

6. *Candida albicans* transcription factors are capable of phase separation

Previously, our group showed that several of the regulatory TFs involved in white-opaque switching are capable of phase separation [171]. Efg1, Wor1, Wor2, Wor3, Wor4, Czf1, and Ssn6 all contain PrLDs, while Ahr1 does not. We showed that Efg1, Wor1, Wor4, and Czf1 can form phase-separated condensates alone or together *in vitro*. Deletion of the PrLDs from these TFs prevented the formation of liquid-like condensates *in vitro*, and also eliminated the ability of these TFs to promote the white-to-opaque transition. In a DNA curtains assay, which involves single DNA molecules stretched out in a flow cell, Efg1 was capable of forming condensates that could condense naked or nucleosomal DNA. When expressed in a mammalian nuclear model, PrLDs from Efg1, Wor1, Wor4, and Czf1 formed liquid-like condensates that could be dissolved by 1,6-hexanediol, a chemical often used to dissolve liquid condensates [82, 84]. Our group also showed that these PrLDs could colocalize with condensates formed by the Efg1 PrLDs. Acidic residues in the Wor1 PrLD were shown to be necessary for its interaction with Efg1 PrLDs. Interestingly, the Efg1 PrLDs could colocalize with the human FUS PrLD, but not with the Sp1 PrLD, indicating a degree of interaction specificity.

In other data from our lab, Ganser et al. showed that *C. albicans* TFs involved in biofilm

formation are capable of phase separation [172]. A biofilm is a structured community of microbes that adheres to a surface and is associated with extracellular matrix [173]. Biofilm formation is linked to increased resistance to antimicrobials and other damage-inducing agents [173]. A number of TFs are involved in the regulation of *C. albicans* biofilm formation, including Efg1, Flo8, Ndt80, Bcr1, Rfx2, Rob1, Brg1, Gal4, and Tec1 [172, 173]. Ganser et al. showed that the PrLDs of Efg1, Flo8, and Brg1 were capable of forming phase-separated condensates in a mammalian cell model, while the Bcr1 PrLD did not phase separate but could still co-localize with the PrLDs of Efg1. Deletion or mutation of PrLDs from these TFs reduced biofilm formation and filamentation, both *in vitro* and in the mouse gut. Interestingly, Efg1 was functional when its PrLDs were substituted by the human TAF15 PrLD, whereas Flo8 was not functional when its PrLD was replaced with that of TAF15. Aromatic residues in the Efg1 and Brg1 PrLDs were shown to be critical for the function of these domains in regulating gene expression in yeast and filamentation in the host. A subsequent NMR study [174] indicated that tyrosine (Y) residues play an important role in stabilizing transient alpha-helices that enable Efg1 phase separation.

These studies reveal that *C. albicans* TFs are capable of forming liquid-like condensates *in vitro* and in an exogenous nuclear model. The capacity to undergo phase separation seems to be critical for these TFs to regulate both gene expression and cell type formation in *C. albicans*.

7. Conclusions

Phase separation is now recognized as a common mechanism that organisms use to compartmentalize reactions in the cell [1]. Proteins and nucleic acids involved in the formation of nucleoli, stress granules, the cytoskeleton, and transcriptional hubs are capable of forming biomolecular condensates [7, 8, 48, 88]. Phase separation seems to be involved in both transcriptional activation and repression mechanisms [48, 103, 109, 113, 114]. The formation of biomolecular condensates may play a role in the compartmentalization of the genome into euchromatin and heterochromatin [107, 108, 111, 112, 114-116], which is critical for determining

gene expression and cell phenotype. One such model of cell differentiation is white-opaque switching in *C. albicans*. TFs involved in regulating white-opaque switching have already been shown to form phase-separated condensates [171, 172].

The topic of this thesis is the role of phase separation in the mechanism of the *C. albicans* transcriptional repressor Ssn6. In chapter 2, the ability of this TF to form condensates *in vitro* and in a nuclear model will be examined. The functions of the Ssn6 domains in regard to white-opaque switching and gene regulation will be dissected. Lastly, the way in which Ssn6 affects the properties of condensates formed by other TFs will be examined. In chapter 3, additional data related to the function of Ssn6 and other white-opaque TFs will be presented. The work contained here will contribute to the understanding of how transcriptional repressors can affect phase separation behavior and ultimately determine cell fate.

Figures

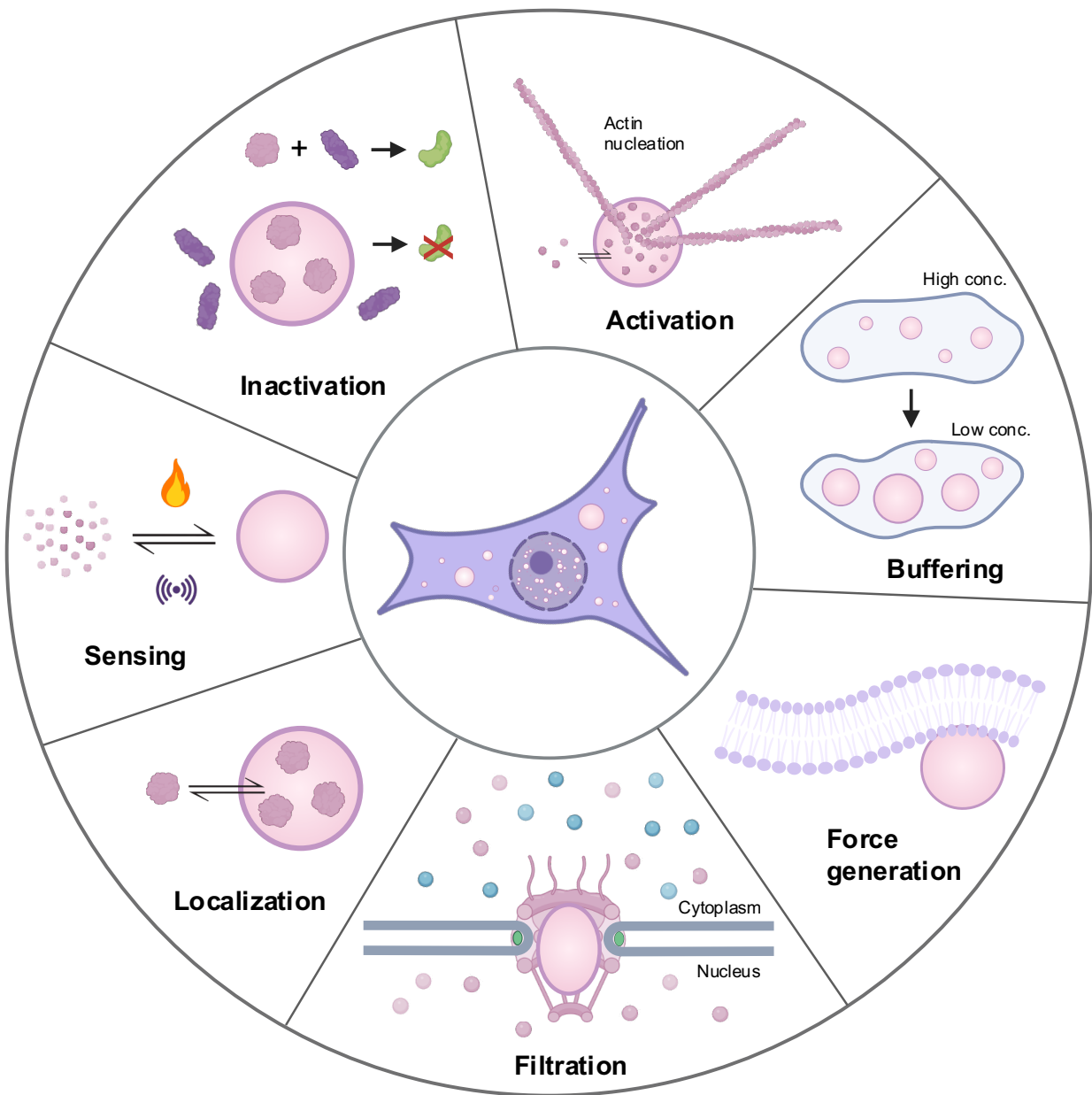


Figure 1: Biomolecular condensates play many roles in the cell. Biomolecular condensates are implicated in a diverse array of cellular functions. These functions include both activation and inactivation of reactions, localization and buffering of cellular components, generation of forces, and sensing of environmental stimuli. Figure adapted from Alberti et al. [4]. Made with biorender.com.

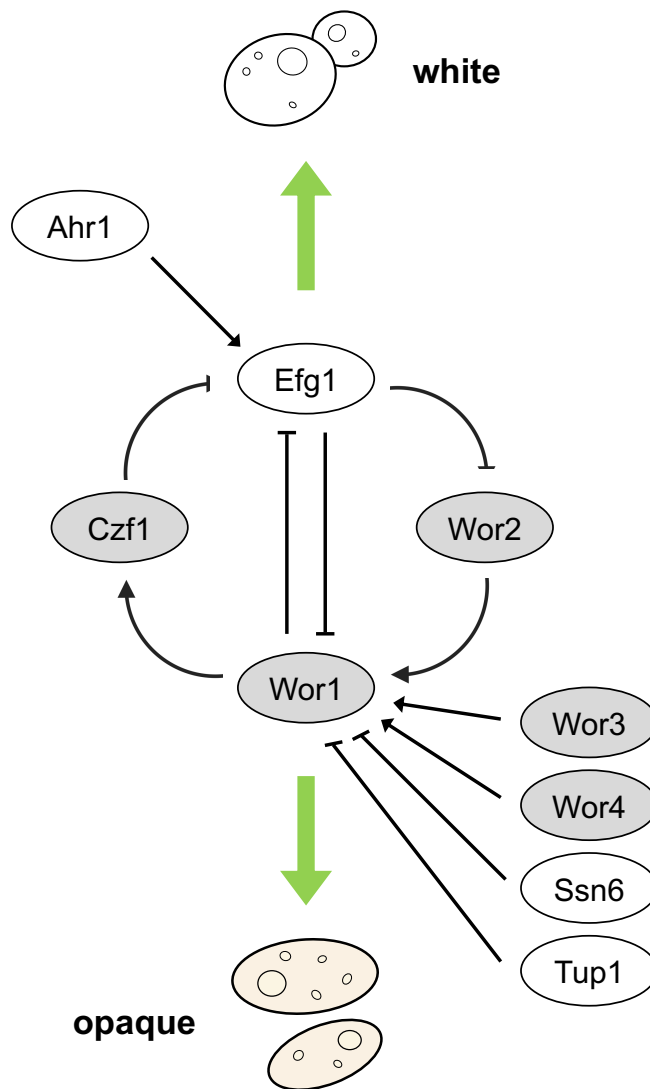


Figure 2: A network of TFs controls white-opaque cell type switching in *C. albicans*. TFs in white promote the white cell state, while TFs in grey promote the opaque cell state. Efg1 is the master regulator of the white state, while Wor1 is the master regulator of the opaque state. Adapted from Noble et al., 2017 [127]. Some elements from biorender.com.

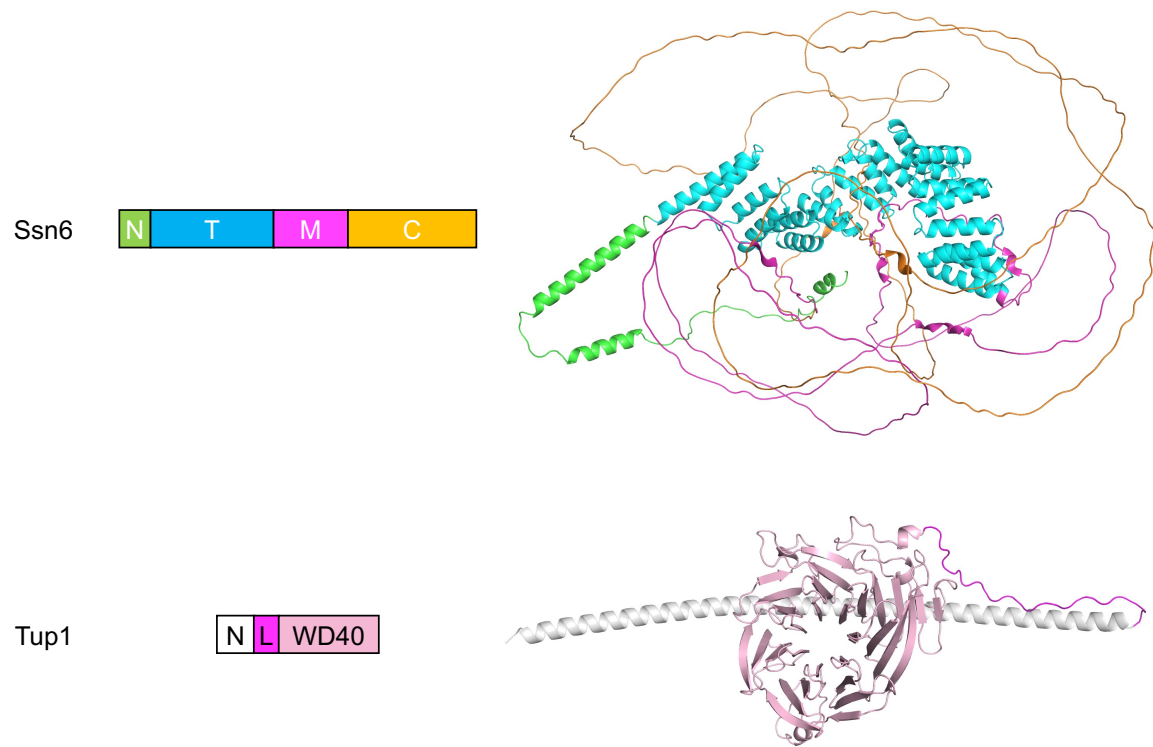


Figure 3: The structures of Ssn6 and Tup1. Ssn6 contains TPR repeats (T) [147], as well as two PrLDs (N and M) [171] and a C-terminus (C). Tup1 contains an alpha-helical N-terminus (N) [153], a linker region (L), and WD40 repeats at its C-terminus [175]. Predicted structures shown were downloaded from the AlphaFold Protein Structure Database [176-178] and edited with Pymol. Reference numbers are AF-Q5ADP3-F1-v4 (Ssn6) and AF-C4YFX2-F1-v4 (Tup1).

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**Chapter 2: The global transcriptional repressor Ssn6-Tup1 undergoes phase separation
to regulate gene expression**

**The global transcriptional repressor Ssn6-Tup1 undergoes phase separation
to regulate gene expression**

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Abstract

Ssn6-Tup1 is a transcriptional repression complex that is conserved from yeast to higher eukaryotes. Both of these proteins have been linked to phenotypic switching in *Candida albicans*, with the deletion of Ssn6 in particular completely abolishing the formation of the white cell state.

In this study, we examine the contribution of Ssn6 domains to both white-opaque switching as well as phase separation propensity. We find that the Ssn6 TPRs are critical for the function of the protein, in line with published data. Intriguingly, though, we also identify an important role for the intrinsically disordered C-terminal domain (CTD) of Ssn6, which has not been well-studied. When the CTD is deleted in *C. albicans*, the cells exhibit an overwhelming preference for the white cell state. We further show that Ssn6 is capable of nucleating phase-separated condensates in a mammalian cell model, and that deletion of the CTD reduces the size and dynamics of these condensates.

In vitro experiments using purified *C. albicans* TF proteins reveal that Ssn6 changes the material properties of condensates formed by other TFs, and that deleting the TPR or the CTD alters this behavior. This finding suggests that the CTD, and not just the TPR, may be critical for mediating protein-protein interactions necessary for Ssn6 to regulate gene expression. We further show the importance of the Ssn6 disordered regions in regulating gene expression by conducting RNA-seq on *C. albicans* cells. When expressed in *C. albicans*, Ssn6 mutants lacking either the TPR or the CTD exhibited altered gene expression profiles from control whites and opaques. We also show that the Ssn6 mutant lacking the TPR, which contains no canonical repression-mediating domain and consists solely of disordered regions, has a different gene expression profile than the Δ ssn6 strain. These observations indicate an important role for the disordered domains of Ssn6 in regulating gene expression and consequently, cell state.

Introduction

The opportunistic pathogen *Candida albicans* undergoes remarkable changes in cell morphology that contribute to the success of a fungal infection. One of these morphological transitions is the white-opaque switch, a heritable transition between the conventional white cell state and the alternative opaque cell state [1]. These two cell types show marked differences in a number of key phenotypes including differences in metabolism, tissue tropisms, virulence and mating competence [2].

The white-opaque switch is regulated by a network of transcription factors (TFs) [3-5], including the global transcriptional repressor Ssn6. Ssn6 function is essential for formation of the white cell state as the loss of this gene results in cells that are locked in the opaque state [4]. In the related yeast *Saccharomyces cerevisiae*, Ssn6 has been well-characterized as a conserved transcriptional co-repressor that forms a complex with its partner protein Tup1 [6, 7].

While this complex is capable of repressing the expression of up to 3% of the *S. cerevisiae* genome, neither Ssn6 nor Tup1 contains a DNA-binding domain, so the complex is likely recruited by the interaction of Ssn6 with other DNA-binding proteins [8]. Tup1 homologs include Gro/TLE family proteins that interact with tetratricopeptide repeat (TPR)-containing proteins similar to Ssn6 [8-11]. This family of proteins is critical for regulating cell differentiation in higher eukaryotes [10]. Indeed, *S. cerevisiae* Ssn6 interactions with human Tup1-like proteins can mediate the repression of genes in human cells, indicative of strong conservation of this mechanism [8, 9].

Previously, several TFs regulating the white-opaque switch were shown to form biomolecular condensates, either individually or in combination [12-14]. Condensates are formed when macromolecules phase separate by demixing into a denser phase that is surrounded by a more dilute phase [15, 16]. In a cellular environment, condensate formation can serve to recruit proteins and other molecules into compartments that facilitate cellular processes [17]. Many structures in the cell have now been shown to form via phase separation, including the nucleolus [18], stress granules [19], and P granules [20], and nuclear speckles [21]. Chromatin itself is

organized on a broad scale within the nucleus by phase separation of the DNA together with associated proteins [22]. Condensate formation is also implicated in the recruitment of RNA polymerase II to sites of active transcription via its repetitive C-terminal domain (CTD) [23], an intrinsically disordered subunit of the RNA polymerase II holoenzyme that is involved in transcription initiation. The ability of TFs to form condensates is generally associated with the ability to activate gene expression [24-26], although this remains a topic of debate [27-30].

Repressive chromatin proteins, such as the Polycomb Repressive Complex (PRC), have also been shown to undergo condensation [31, 32]. H3K27me3 reader protein BP1 uses phase separation to interact with PRC and mediate transcriptional repression in *Fusarium graminearum* [33]. The RNA-binding protein BAZ2A can form transcriptionally active condensates that sequester transcriptional machinery away from repressive H3K27me3 regions [34]. HP1 proteins facilitate the formation of phase-separated H3K9me2/3 chromatin condensates that demix from condensates composed of unmodified chromatin [35, 36]. These studies indicate that phase separation likely has a role in segregating the genome into transcriptionally active and inactive regions.

In this study, we show that the transcriptional repressor Ssn6 impacts the physical properties of *C. albicans* TF condensates *in vitro*. We also show that Ssn6 forms liquid-like condensates in the nuclei of mammalian cells with or without its partner Tup1. Deletion of the Ssn6 domains necessary for this behavior also alters white-opaque switching frequency and RNA expression in *C. albicans*. These findings contribute to the growing knowledge that transcriptional repressors can utilize phase separation in order to repress transcription and influence cell fate.

Results

The structured TPR domain and disordered C-terminal domain of Ssn6 affect white-opaque switching.

Prion-like amino acid composition (PLAAC) analysis was previously used to identify prion-like

domains (PrLDs) in several *C. albicans* TFs including Ssn6, which includes two regions with a prion-like sequence [12, 37]. Predictors of general disorder, such as VSL2 [38], identify most of the protein as being disordered, except for the structured TPRs located after the N-terminal PrLD. Based on these analyses, we describe the Ssn6 protein as having four domains: an N-terminal PrLD (N), a structured tetratricopeptide repeat domain (T), a medial PrLD (M), and a C-terminal domain (CTD or C) that is disordered but not prion-like (**Fig. 1a**).

Deletion of Ssn6 prevents *C. albicans* from forming white cells so that cells are locked in the opaque state [4]. Tup1, often considered a co-repressor with Ssn6, also acts to repress the opaque state in *C. albicans* [39]. To further investigate the role of Ssn6 in the regulation of white-opaque switching, strains expressing different Ssn6 variants were constructed in which variants were integrated into the endogenous Ssn6 locus of an *ssn6D/D* strain via homologous recombination [40]. A full-length Ssn6 re-integrand was used as the control. All Ssn6 variants were able to support the formation of the white cell state except for Ssn6 Δ T, indicating that the loss of TPRs blocks Ssn6 function (**Fig. 1b**). Expression levels and nuclear localization for each variant were analyzed by the addition of C-terminal fluorescent tag and microscopy. In general, expression levels were similar between Ssn6 proteins (although variations on the order of ~2-fold were observed) and all correctly localized to the nucleus (see **Extended Data Fig. 1**).

To quantify white-opaque switching in each strain, pure populations of white and opaque cells were plated for single colonies on SCD medium for 7 days to monitor cell type switching. These assays revealed that the N and M PrLDs were both found to have a modulatory role in switching. Loss of these domains resulted in higher rates of white-to-opaque switching than the control (**Fig. 1c**), consistent with a decrease in Ssn6 function. Interestingly, Ssn6 Δ C strains showed very low white-to-opaque switching under these assay conditions (**Fig. 1c**), although they could be coerced to form the opaque state by culturing on media containing N-acetyl glucosamine, a known chemical inducer of the opaque state [41]. Opaque Ssn6 Δ C cells also showed high frequencies of switching back to the white state under standard assay conditions (**Fig. 1d**).

Together, these experiments reveal (1) an essential role for the TPRs in Ssn6's ability to support the white state, (2) a modulatory role for Ssn6 PrLDs in white-opaque switching, and (3) that the unstructured Ssn6 CTD plays a surprisingly important role in supporting formation of the opaque state.

Ssn6 forms dynamic condensates when expressed in mammalian cells.

The PrLDs of several *C. albicans* TFs have been shown to promote the formation of phase-separated condensates when expressed in mammalian U2OS cells [12, 13]. We similarly evaluated the potential for Ssn6, or sub-domains of this protein, to form condensates using U2OS cells harboring ~50,000 copies of the LacO sequence [26, 42]. Full-length Ssn6 and Ssn6 variants were expressed as eYFP-Ssn6-LacI fusion proteins for recruitment to the LacO array. The full-length Ssn6 clearly formed large spherical foci at multiple positions in the nuclei (**Fig. 2a**). This behavior was unlike the LacI control which formed foci only at one or two positions in the nuclei, presumed to represent the LacO array. Ssn6 mutants lacking one or both PrLDs were also able to form large foci, whereas Ssn6 Δ T and Ssn6 Δ C variants produced visibly different puncta (**Fig 2a**). The Ssn6 Δ T variant was particularly striking as it formed large blobs that filled much of the nuclei and were therefore substantially larger than the foci formed by either the LacI control or the full-length Ssn6 (**Fig. 2c**). The Ssn6 Δ C variant produced smaller, more granular looking puncta than the full-length protein, suggesting that deletion of the C-terminal domain reduces dynamic interactions by Ssn6.

To test for liquid-like behavior, we treated the cells with 10% 1,6-hexanediol, a chemical that has been observed to dissolve phase-separated condensates [25, 26, 43, 44]. The full length Ssn6 puncta rapidly reduced in size and quantity within 30 seconds of 1,6-hexanediol addition (**Fig. 2d-e**). Puncta formed by Ssn6 Δ N and Ssn6 Δ NM also dissolved well, although Ssn6 Δ M seemed to only partially dissolve (**Fig. 2d-e**). The amorphous structures formed by Ssn6 Δ T also were reduced by hexanediol treatment (**Fig. 2d-e**). Notably, the Ssn6 Δ C puncta did not dissolve

with hexanediol treatment, indicating that the C-terminal domain may be important for dynamic interactions (**Fig. 2d-e**).

We also examined the phase separation properties of individual Ssn6 domains in the U2OS-LacO cell system (**Fig. 2f**). The TPR alone did not produce discrete puncta while the CTD produced large blobs similar to that formed by Ssn6 Δ T (**Fig. 2f**). The N and M PrLDs formed small, granular puncta that were significantly brighter than the LacI control (**Fig. 2f-g**), suggesting that these puncta are more densely packed. We also evaluated expressing the two halves of Ssn6 in U2OS cells. Expressing the N domain fused to the TPR domain (N-TPR) produced single puncta that resembled the LacI control in size (albeit somewhat weaker in intensity, **Fig. 2f-h**). Expressing the M domain fused to the CTD (M-C) again resulted in blob-like structures that were significantly larger than the LacI control (**Fig. 2f-h**).

Amino acid substitutions in the Ssn6 CTD alter condensate formation and white-opaque switching.

Given that deletion of the uncharacterized Ssn6 CTD had a major impact on both white-opaque switching and the size and dynamics of Ssn6 condensates, we constructed and examined four Ssn6 variants with specific amino acid substitutions in the CTD. The Ssn6 CTD is composed of approximately 20% acidic residues (**Extended Data Fig. 2a**), so a mutant Ssn6 was created where all D and E residues in the CTD were mutated to alanine. Several phosphorylation sites have been identified in the Ssn6 CTD [45], so these sites were mutated either to alanine to block phosphorylation, or to aspartic acid to mimic phosphorylation events. Finally, we created a mutant where all aromatic residues (Y/F) in the CTD were mutated to S or A, since aromatic residues have been frequently associated with the ability of IDRs to form phase-separated condensates [46, 47].

Each of the CTD variants were tested for their phase separation capacity when expressed in U2OS cells (**Extended Data Fig. 2b-d**), and for their role in white-opaque switching in *C.*

albicans (**Extended Data Fig. 2e-g**). Interestingly, the Ssn6 variant lacking acidic residues in the CTD was blocked from forming the white cell state, indicating that this variant is completely nonfunctional (**Extended Data Fig. 2e-g**). This mutant also formed altered puncta when expressed in U2OS cells, forming only very small, grainy puncta (**Extended Data Fig. 2b-d**). The aromatic and phosphorylation Ssn6 variants were able to form both white and opaque cells, although these were somewhat more likely to switch from opaque to white than the full-length Ssn6 control (**Extended Data Fig. 2e-g**). These variants also produced similar foci to the Ssn6 control when expressed in U2OS cells (**Extended Data Fig. 2b-d**). These experiments establish that removal of the CTD or modification of this domain by substitution of acidic residues can substantially alter the phase separation properties of Ssn6 and its role in white-opaque switching.

Tup1 co-localizes with Ssn6 and increases the size of Ssn6 condensates in U2OS cells.

Ssn6 function frequently requires association with its co-repressor Tup1 [6, 48, 49]; we therefore evaluated the effect of co-expression of Tup1 on Ssn6 in the U2OS cell model. Tup1 was expressed as a fusion protein with mCherry in U2OS-LacO cells that also expressed eYFP-Ssn6-LacI (**Fig. 3a**). Notably, Tup1 colocalized with the full length Ssn6 as well as all Ssn6 variants except for Ssn6 Δ T (**Fig. 3b**). This result is consistent with previous studies in *S. cerevisiae* that indicate that the TPR domain is required for Ssn6 to interact with Tup1 [49, 50]. Interestingly, the presence of Tup1 also resulted in expansion of the size of Ssn6 condensates, with Ssn6 foci being approximately 5 times larger when Tup1 was present versus its absence (**Fig. 3c**). We also co-expressed Tup1 with different individual Ssn6 domains, which showed that Tup1 co-localized with the Ssn6 TPR domain (T) but not with any other Ssn6 domain (**Fig. 3d-e**). Together, these experiments establish that *C. albicans* Ssn6 TPRs interact with Tup1, and this interaction increases the size of the condensates formed by Ssn6.

Purified Ssn6-Tup1 alters the properties of transcription factor condensates.

Previous studies have shown that several *C. albicans* white-opaque TFs can undergo phase separation when purified and examined *in vitro*, with some factors such as Efg1 even forming condensates in the absence of a molecular crowding reagent [12, 13]. We similarly purified Ssn6 from *Escherichia coli* as a fusion with maltose-binding protein (MBP, see **Extended Data Fig. 3a-b**). When cleaved from MBP, Ssn6 did not readily form condensates in the absence of a crowding reagent, but did form aggregated structures when polyethylene glycol (PEG) concentrations were 7.5% or above (see **Extended Data Fig. 3c**). To determine if Tup1 alters the propensity of Ssn6 to undergo phase separation *in vitro*, we co-expressed MBP-Ssn6 and MBP-Tup1 in the same bacterial cells and purified the Ssn6-Tup1 complex (gel **Extended Data Fig. 3b**). The Ssn6-Tup1 complex similarly did not form condensates in the absence of crowding reagents but again formed aggregates with PEG concentrations of 7.5% or above (**Extended Data Fig. 3d-e**). This aggregate-forming behavior of purified Ssn6 contrasts with the liquid-like characteristics of Ssn6 condensates in the U2OS cell model (**Fig. 2**).

Ssn6 is co-recruited to promoters via its interactions with sequence-specific DNA-binding TFs [4]. Therefore we examined the effect of Ssn6 (+/-Tup1) on the properties condensates formed by other TFs (gel **Extended Data Fig. 3b**). These included Efg1 and Flo8, two TFs with major effects on white-opaque switching frequencies [51-53]. We have previously showed that these purified TFs are capable of forming condensates [12, 13]; Efg1 readily forms condensates even in the absence of crowding reagents, whereas Flo8 forms liquid-like condensates only in the presence of 5% PEG. Efg1 condensates (10 μ M) were titrated with Ssn6-Tup1 (without crowding agent) and, remarkably, the addition of 2.5 μ M Ssn6/Tup1 essentially resulted in a complex dissolution of the Efg1 condensates. Lower concentrations of Ssn6/Tup1 resulted in gel-like or aggregate-like behavior in Efg1 condensates, even at ratios as low as 1:100 Ssn6:Efg1 (see 0.1 μ M Ssn6/Tup1 panel in **Fig. 4a**). These experiments were repeated in the presence of 7.5% PEG (**Fig. 4b**), where the inclusion of Ssn6/Tup1 caused Efg1 to form less liquid-like droplets and instead adopt chains of gel-like droplets or aggregates. Addition of Ssn6 alone (without Tup1)

similarly disrupted Efg1 condensates (**Fig 4c-e**) and also led to the disruption of droplets formed by Flo8 (**Fig. 4f-h**).

To determine if the Ssn6 TPR and CTD contribute to the observed effects on Efg1 and Flo8 condensates, we purified versions of Ssn6 lacking each of these domains (**Extended Data Fig. 3b**). We found that neither of these Ssn6 variants caused the same degree of disruption in TF condensate formation as the full-length Ssn6 (**Fig. 4c-d**). The addition of Ssn6 Δ T to Efg1 caused a slight reduction in the size of the condensates but no change in the roundness of the condensates (**Fig. 4c-e**), suggesting that these condensates retain their dynamic properties [18, 44]. Addition of Ssn6 Δ T to Flo8 condensates likewise did not affect either condensate area or roundness (**Fig. 4f-h**). In contrast, the addition of Ssn6 Δ C decreased both the size and the roundness of condensates formed by both Efg1 and Flo8, and condensates appeared more aggregated or gel-like in the presence of this Ssn6 variant (**Fig. 4c-h**). These results reveal how even small relative quantities of Ssn6 can drastically alter the properties of condensates formed by two key TF regulators of the white-opaque switch.

Ssn6 domains make distinct contributions to white and opaque gene expression profiles.

C. albicans white and opaque cells show marked differences in gene expression with >1000 genes differentially expressed between the two states [54]. To examine the role of the different Ssn6 domains in regulating gene expression, we performed RNA sequencing on strains expressing each Ssn6 variant in both white and opaque states. Principal component analysis (PCA) was performed using normalized expression values for genes that were differentially expressed between full-length Ssn6 white and opaque cells (**Fig. 5a**). We also plotted the top differentially expressed genes between full-length Ssn6 whites and opaques for all samples (**Fig. 5b**).

By PCA, white and opaque cells largely clustered as two separate groups. The Δ ssn6 strain, although generally considered to be locked in the opaque state [4], showed substantial

expression differences from control opaque cells and did not cluster with other opaque cell profiles (**Fig. 5a**). In particular, a substantial fraction of white-specific genes was expressed at a higher level in this mutant (**Fig. 5b**), consistent with Ssn6 acting to repress the expression of white-specific genes in opaque cells [4].

Ssn6 Δ T strains exhibited opaque-like cell and colony phenotypes that closely resembled those of the Δ ssn6 strain (**Fig. 1b**). Interestingly, however, the expression profile of Ssn6 Δ T opaque cells was distinct from that of both Δ ssn6 cells and wild type opaque cells (**Fig. 5a-c**). In fact, loss of the TPRs from Ssn6 resulted in an expression profile that more closely resembled that of control white cells rather than opaque cells (see clustering in **Fig. 5b**). This was largely the consequence of high expression of most white-specific genes in Ssn6 Δ T “opaque” cells as well as the repression of many opaque-specific genes (**Fig. 5b**).

Curiously, the expression profile of Ssn6 Δ C opaque cells also did not cluster with most other opaque expression profiles (**Fig. 5a**). These cells, like the Ssn6 Δ T cells, were defective in repressing a substantial number of white-specific genes, even more so than the Δ ssn6 strain. In fact, Ssn6 Δ C opaque cells appeared to show a general pattern of activation for both white- and opaque-specific genes (**Fig. 5b**). In contrast, the white cells formed by Ssn6 Δ C cells showed an expression profile that was similar to that of wildtype white cells (**Fig. 5a-b**), indicating that loss of the C-terminal domain has an outsized effect on the opaque state rather than the white state.

Analysis of the PrLD deletion strains (Δ N, Δ M and Δ NM) revealed that while PrLD-deletion white and opaque strains generally exhibited gene expression profiles that were similar to control white and opaque cells (**Fig. 5b**). This is also in line with the modulatory role of these domains on white-opaque switching (**Fig. 1**) and on phase separation properties (**Fig. 2**).

Taken together, these extensive expression profiling experiments provide an in depth look at the role of different Ssn6 domains on white-opaque gene expression and reveal surprising roles for the TPR and CTD in regulating subsets of white/opaque specific genes.

Discussion

In this study, we address how a conserved global repressor acts to regulate gene expression and cell fate in *C. albicans*. Ssn6, either independently or with its co-repressor Tup1, regulates a large fraction of the genome (~3% of *S. cerevisiae* and *C. albicans* genes) [8, 55]. Homologs of Ssn6 exist in humans, fruit flies, and other yeast species [8, 9, 56, 57]. In higher eukaryotes, homologs of Ssn6 have been linked to tissue-specific developmental patterning [58-60], highlighting that Ssn6 has a conserved role in defining cell fate from yeast to humans.

We dissected the role of the N, T, M and C domains of *C. albicans* Ssn6 to evaluate their roles in the regulation of the white-opaque switch. As was observed in *S. cerevisiae* [49], we found that the TPR repeats were critical for *C. albicans* Ssn6 function. The TPR domain mediates interactions with both Tup1 and with other proteins [49, 61, 62]. Deletion of the TPR resulted in *C. albicans* cells adopting an opaque-like phenotype similar to that of Δ ssn6 cells. Despite this opaque-like phenotype, RNA sequencing revealed that Ssn6 Δ T cells had an expression profile that more closely resembled control white cells rather than opaque cells. Surprisingly, the RNA profile of Ssn6 Δ T cells was also markedly different from that of Δ ssn6 cells, indicating that even in the absence of TPRs, Ssn6 had an impact on global gene expression.

The disordered CTD also plays an unexpectedly prominent role in Ssn6 function. Loss of this domain resulted in increased opaque-to-white switching, indicative of hyperactivity. In line with this, Ssn6 Δ C opaque cells showed an expression profile between that of control white and opaque cells, largely due to the repression of a subset of opaque-specific genes. Mutation of the acidic residues in the Ssn6 CTD had the opposite effect from deleting the CTD; this mutant showed a complete lack of opaque-to-white switching, further highlighting the importance of the CTD to Ssn6 function and the role of acidic residues in this domain. In contrast with the CTD, loss of the prion-like N and M domains generally did not have a major impact on Ssn6 function, either in white-opaque switching or on RNA expression profiles.

A major focus of this work was to examine the phase separation potential of Ssn6, both

individually and in combination with Tup1 or sequence-specific DNA binding TFs. We showed that purified Ssn6 showed little propensity toward liquid-like behavior by itself or when combined with Tup1. In contrast, Ssn6 showed a strong propensity to form liquid-like condensates when expressed in the human U2OS-LacO model [26, 42]. These condensates increased in size when Tup1 was co-expressed with Ssn6. The difference between *in vitro* and *in vivo* results could be due to several possibilities including: 1) differences in co-factors – Ssn6 could associate with other nuclear factors present in mammalian cells that enable it to form condensates, or 2) differences in post-translational modifications – these will differ between the *E. coli*-purified protein and that expressed in human cells. Interactions between Ssn6 and Tup1 in U2OS cells were strictly dependent on the presence of the Ssn6 TPRs, in line with previous studies in *S. cerevisiae* [49]. Individual domains of Ssn6 could also form condensates; the prion-like N and M domains formed small puncta while the disordered CTD formed large blobs. The Ssn6 TPR domain did not phase separate by itself and even inhibited puncta formation by the N domain when expressed as an N-TPR fusion in U2OS cells.

Purified *C. albicans* Ssn6 was able to form liquid condensates with Efg1 or Flo8, two TFs that regulate the *C. albicans* white-opaque switch [51-53, 63]. Ssn6 and Efg1, along with other white-opaque circuit TFs, colocalize to many of the same positions in the genome of opaque cells [3-5, 63-65], suggesting a possibility for co-condensation. Ratios higher than 1:4 Ssn6:TF essentially led to the complete disruption of Efg1 and Flo8 condensates, either in the presence or absence of Tup1. At a very low Ssn6:TF ratio (~1:100), the presence of Ssn6 led to the formation of less dynamic, gel-like condensates. Curiously, interactions between Ssn6 (+/-Tup1) and Efg1/Flo8 were not observed in the U2OS model (data not shown), suggesting that additional factors might act to bridge these TFs in *C. albicans* cells or that endogenous TFs could be blocking these interactions in U2OS cells. Deleting the TPR or CTD of Ssn6 altered the properties of both Efg1-Ssn6 and Flo8-Ssn6 condensates, consistent with the prominent role of these domains in the ability of Ssn6 to regulate gene expression. Thus Efg1 and Flo8 act as scaffolds for

condensate formation, while Ssn6 can be recruited into these condensates as a modulating client.

Recently, two human homologs of Ssn6, UTX and UTY, have been shown to form phase-separated condensates [66]. The phase separation properties of UTX were impacted by loss or mutation of a core IDR, and both the TPRs and IDR were found to play important roles in the tumor suppression activity of UTX [66]. Swapping the IDRs of UTX and UTY altered the properties of condensates formed by these proteins, with the UTY IDR having reduced dynamics compared to that of UTX [66]. Results from this study on Ssn6 suggest that reducing the dynamics of TF proteins may have consequences for the regulation of gene expression and for the plasticity of cell state. In *C. albicans*, cell fate is both heritable and plastic [3, 63]. Investigation into the regulation of this differentiation process could yield important insights into important areas such as regenerative capacity or cancer progression in higher eukaryotes.

We note that further work could be done to measure the mechanical properties of complex TF condensates, and to determine how these mechanical properties affect recruitment of RNA pol II and the transcriptional process. Especially interesting is the concept that transcriptional repressors can alter these complexes to make them more or less efficient at producing RNA. Understanding these properties will be critical for deciphering the complex molecular interactions that determine cell fate.

Materials and Methods

Plasmid construction. Specific details about all fragments, restrictions, and enzymes used can be found in Supplementary Table 1. Oligos used are described in Supplementary Table 2. Plasmid sequences were confirmed by sequencing the full plasmids with Plasmidsaurus.

To construct plasmids used in *C. albicans* strains, *C. albicans* Ssn6 sequences were amplified from SC5314 gDNA. Inserts for Ssn6 Δ N, Ssn6 Δ M, and Ssn6 Δ NM were assembled using fusion PCR. In this method, two non-contiguous sequences were amplified with an overlap region added to one product using oligos. In a subsequent PCR, these products are fused together using oligos at the extreme ends of the fusion product. Sequences were then inserted into pSFS2a [40] using restriction enzymes. A far 3' sequence was also inserted into the 3' end of all pSFS2a-Ssn6 cassettes to ensure good integration into the genome.

Ssn6 Δ T and Ssn6 Δ C inserts were designed for Golden Gate Assembly. Oligos for Golden Gate were designed using the New England Biolabs Golden Gate Assembly Tool. Ssn6 Δ T and Ssn6 Δ C inserts were assembled into a modified pSFS2a containing a BsaI landing pad (pSFS2a-BsaI). pSFS2a-BsaI was generated by digesting oligos 6048 and 6049 and ligating them into pSFS2a.

To construct plasmids for *E. coli* expression, synthetic codon-optimized sequences were used. Synthetic sequences were inserted initially into the MBP vector (RB523, pRP1B-MBP/THMT) via subcloning. Domain deletion variants of MBP-Ssn6 were produced using methods similar to that for *C. albicans* plasmids. Details are available in Supplementary Tables 1 and 2.

To construct plasmids for mammalian cell expression, synthetic codon-optimized sequences were also used. Inserts from this sequence were amplified and cloned into the eYFP-LacI vector [26].

To create CTD mutants, synthetic Ssn6 CTD sequences were ordered from Gene Universal. Sequences were either *C. albicans* sequences or codon-optimized sequences

depending on the desired application. These were fused to Ssn6 Δ C sequences using Golden Gate Assembly. *C. albicans* sequences were cloned directly into pSFS2a-BsaI, while sequences designed for the eYFP-LacI vector were assembled in pGGA select and subcloned later into eYFP-LacI.

Candida strain construction. Details about strain construction have been included in Supplementary Table 3. Oligos used for strain construction and integration checks are listed in Supplementary Table 2.

Wild type strains used were CAY6342 (white) and CAY6343 (opaque), which are equivalent to AHY135 and AHY136 that have been previously described [65]. The *ssn6/ssn6* strain used (TF121) has been previously described [67]. The *ssn6/Ssn6* strain used was CAY8083, which has been previously described as AHY337 [4]. These strains were gifted by the Johnson lab at UCSF.

Constructed pSFS2a-Ssn6 plasmids were digested with restriction enzymes, then transformed into TF121. Integration was checked by PCR in the forward and reverse directions. Nourseothricin-resistance cassettes were flipped out by incubating on SCM (Synthetic Complete Maltose) media, then checking for nourseothricin susceptibility.

Ssn6-Neon strains were constructed by amplifying an mNeon cassette from RB895 using long oligos. These long oligos contained 75 bp homology to the destination site and 25 bp homology to the mNeon cassette sequence. This PCR product was transformed into relevant strains, and then nourseothricin-resistance cassettes were flipped out on SCM.

White-opaque switching assays. Cells of a single phenotypic state were taken from YPD plates and resuspended in PBS. Cells per milliliter were estimated using optical density with $1 \text{ OD}_{600} = 2 \times 10^7$ cells/ml. Suspensions were serially diluted in phosphate-buffered saline (PBS) to 2×10^3 cells/ml. Approximately 100 cells were spread plated in triplicate on synthetic complete dextrose

(SCD) media. Plates were incubated at 22 °C for 7 days. Colonies were then counted and scored for opposite phenotype colonies or sectors.

Candida cell imaging. Cells were taken from plates and suspended in PBS, then imaged using a Zeiss Axio Observer Z1 inverted fluorescence microscope for fluorescence and DIC imaging equipped with Zen software (Zen v.3.0 blue edition). For Hoescht staining, cells were pre-incubated for 1 hour with 1 mg/ml of Hoechst 33258, then pelleted and resuspended in fresh PBS before imaging.

Mammalian cell culture, live-cell imaging and LacO array analysis. U2OS cells containing a LacO array (~50,000 LacO elements) were a gift from the Tjian Lab [26]. U2OS cells were cultured, transfected with plasmids, and imaged as described previously [12]. LacI focus area size and intensity was measured by masking the YFP signal and analyzing particles in FIJI. Background fluorescence intensity was corrected for by subtracting YFP signal outside the array spot in the cell nucleus. To quantify mCherry–PrLD enrichment at the LacO array bound by PrLD–LacI–EYFP constructs, we followed a method similar to that employed by Chong et al.⁸. The array spot was masked in the EYFP channel, then signal was measured in the mCherry channel. Two locations near the array in the mCherry channel were then measured and averaged to represent average background signal in the nucleus. The mCherry enrichment was then calculated by dividing the array mCherry signal by the background signal. Hexanediol treatment was conducted by adding 20% 1,6-hexanediol dissolved in pre-warmed DMEM to each well in a 1:1 ratio, for a final concentration of 10% 1,6-hexanediol. Timelapses were taken for 5 minutes with an interval of 10 seconds. Quantification was conducted by masking the YFP channel in FIJI and then analyzing the number of particles in each frame.

Protein purification. Proteins were purified as described previously [12]. Ssn6 protein variants

were induced at 16° C overnight.

Phase separation assays. Protein stocks were thawed at room temperature and diluted in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, then concentrated in centrifugal filter units (Amicon Ultra—0.5-ml centrifugal filter units) to 100 µl. Protein concentration was measured using a Nanodrop 2000c (Thermo Fisher Scientific). Proteins were then diluted in 10 mM Tris-HCl buffer with 150 mM NaCl to the desired concentrations. Protein reactions with TEV were set up in 10-µl total volumes (9.5 µl protein with 0.5 µl of 0.3 mg/ml TEV) and incubated for 30 min at room temperature [68]. Proteins were cleaved with TEV prior to addition of any PEG. Labelling of proteins with DyLight or AlexaFluor fluorescent dyes (Thermo Fisher Scientific) was carried out according to the manufacturer's instructions. Labelled proteins were added to assays at concentrations below .5 uM before TEV incubation. Proteins were imaged immediately after incubation on chamber slides (SPI Supplies, 10-chamber slides), with .8 µl solution per chamber, sealed with a glass coverslip. All images were acquired at ×63 initial magnification + x1.6 Optovar on a Zeiss Axio Observer Z1 inverted fluorescence microscope. Post-imaging processing was carried out in FIJI (ImageJ). Quantification of condensate size and shape properties was conducted by masking the fluorescent channel in FIJI, analyzing particles, and then measuring the relevant parameters.

RNA sequencing. *C. albicans* cells in one cell state were taken from YPD plates. These cells were inoculated into 15mL SCD and incubated for 18 hours in a rotator at 24° C. Cells per milliliter were estimated using optical density with $1 \text{ OD}_{600} = 2 \times 10^7$ cells/ml, and a volume equivalent to 10 OD_{600} of cells was harvested by pelleting, then snap-frozen with liquid nitrogen and stored at -80° C. RNA extraction was conducted using the RiboPure Yeast Kit (Invitrogen) according to the manufacturer's instructions. Library preparation was conducted using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) according to the manufacturer's instructions,

with unique i7 barcodes applied for each sample. Library concentrations were measured using a Qubit Fluorometer (Invitrogen) and then multiplexed. The final pooled library was sequenced on an Illumina NextSeq as SR85 and de-multiplexed by the UC Davis Genome Center.

Analysis of RNA sequencing. Fasta reads were trimmed with BBDuk, then aligned using STAR aligner. Assembly 21 chromosome FASTA files and GFF index file were downloaded from the Candida Genome Database. We noted that many reads were not captured because the annotated UTRs stopped before the mapped reads, so the GFF file was edited to extend the 3' sections by Deepika Gunasekaran. Reads were normalized in R using the edgeR package, and differential gene expression analysis was performed. Principal component analysis was performed in R using the factoextra package and visualized using ggplot2. Heatmap visualization was performed in R using pheatmap.

Figures

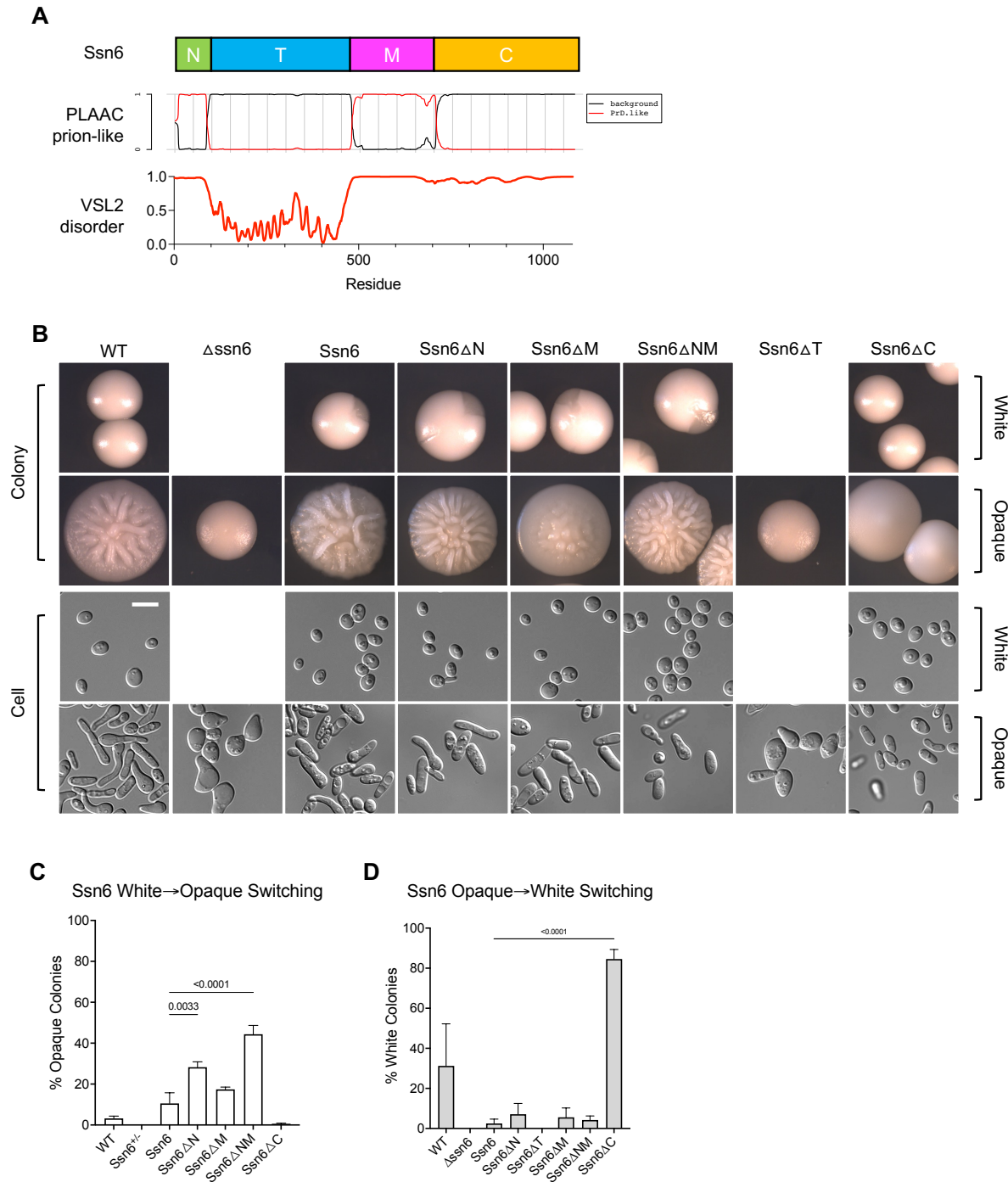


Figure 1: The Ssn6 TPR and C-terminal domains exhibit strong effects on phenotypic switching in *C. albicans*. **A.** Diagram of Ssn6 domains: N-terminal prion-like domain (N), tetratricopeptide repeat (T), medial prion-like domain (M), and disordered C-terminus (C). **B.** Images of *C. albicans* cells expressing Ssn6 variants. Top section shows colony morphology. Some white colonies shown display opaque sectors. Bottom row shows DIC images of cell morphology. **C.** Frequency of white Ssn6 variant cells switching to opaque. Student's t-test versus Ssn6 full-length control, $p \leq .05$. **D.** Frequency of opaque Ssn6 variant cells switching to white. Student's t-test versus Ssn6 full-length control, $p \leq .05$.

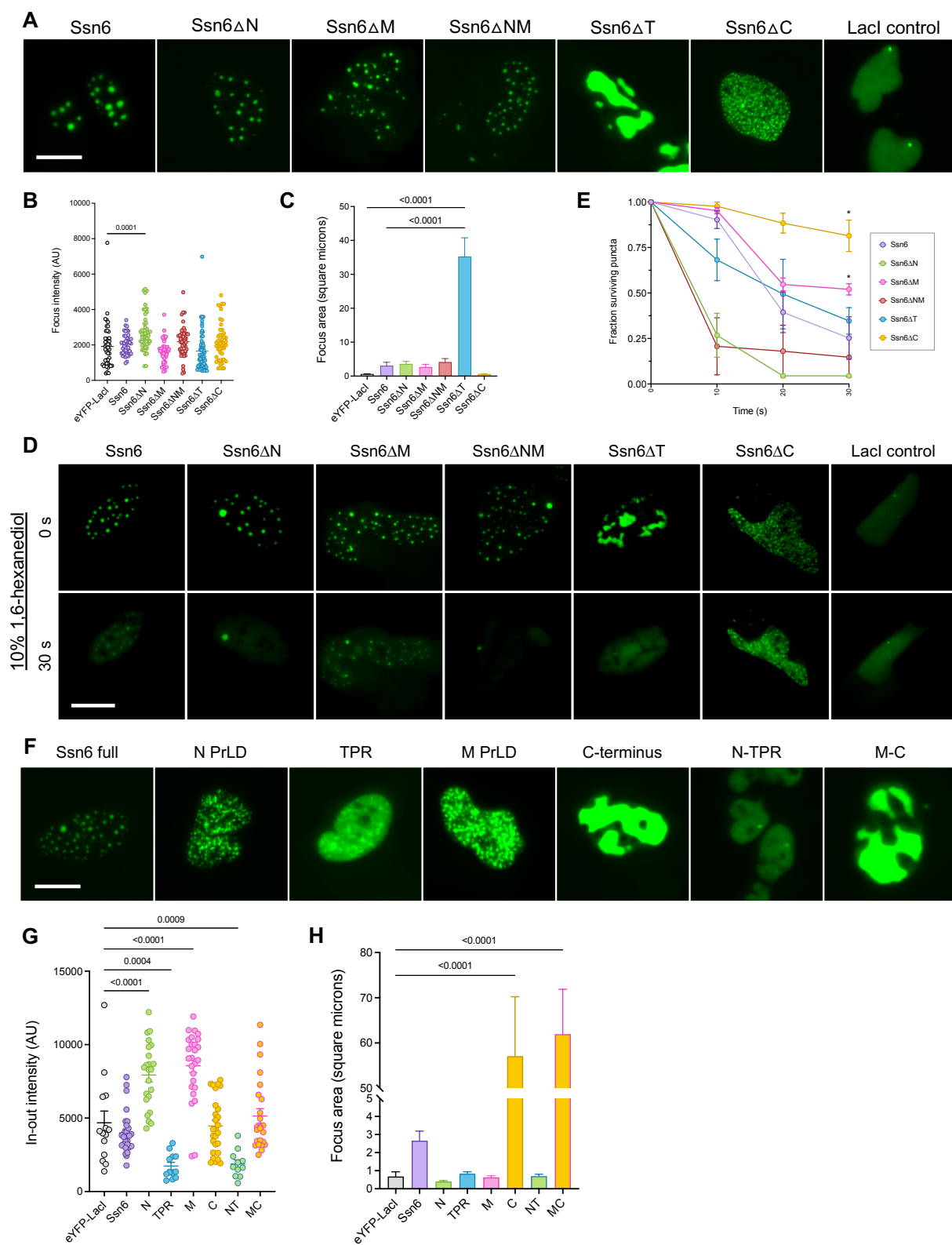


Figure 2: Ssn6 forms phase-separated condensates in U2OS cells. **A.** eYFP-Ssn6-LacI plasmid expressed in U2OS-LacO cells. Scale 10 μ m. **B.** Quantification of condensate area in eYFP-Ssn6-LacI

U2OS condensates. Statistics show only p-values of less than .05 in Student's t-test versus eYFP-LacI or Ssn6. **C.** Quantification of average fluorescence intensity of eYFP-Ssn6-LacI U2OS condensates compared to empty vector eYFP-LacI. Statistics show only p-values of less than .05 in Student's t-test versus eYFP-LacI or Ssn6. **D.** Images from timelapse of 10% 1,6-hexanediol treatment of U2OS-LacO cells expressing eYFP-Ssn6-LacI constructs. Scale 10um. **E.** Quantification of surviving puncta in D. Statistics represent Student's t-test vs. Ssn6 at 30s, $p \leq .05$. **F.** Expression of Ssn6 single domains in U2OS cells. Scale 10um. **G.** Quantification of condensate area in Ssn6 single domain U2OS condensates in F. Statistics show p-values of less than .05 in Student's t-test versus eYFP-LacI. **H.** Quantification of average fluorescence intensity of Ssn6 single domain U2OS condensates in F. Statistics show p-values of less than .05 in Student's t-test versus eYFP-LacI.

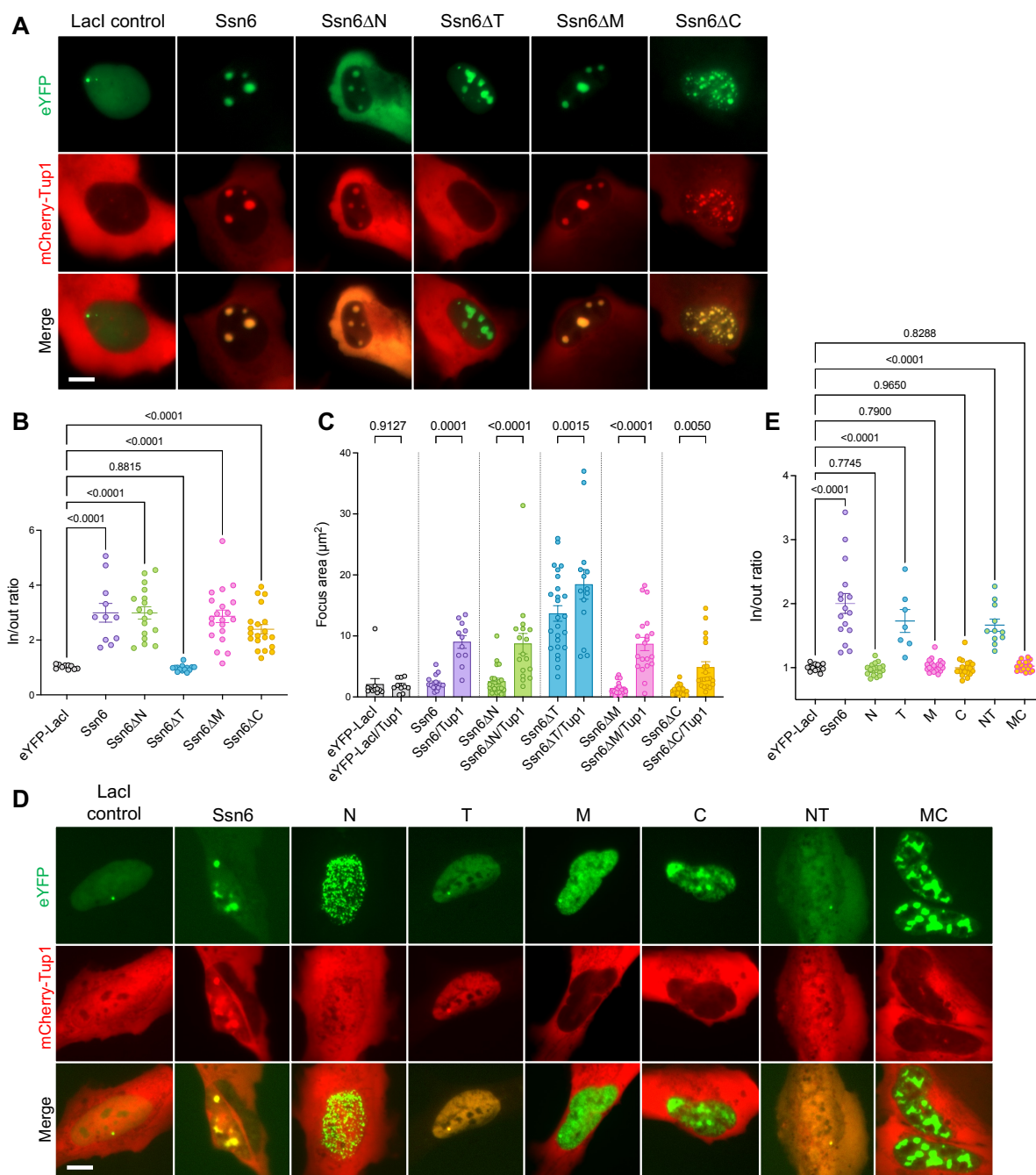


Figure 3: TPR domain is required for Ssn6 colocalization with Tup1 in U2OS cells. **A.** eYFP-Ssn6-LacI constructs co-transfected in U2OS-LacO cells with mCherry-Tup1. Scale 10μm. **B.** Quantification of eYFP and mCherry colocalization in A. Ssn6 puncta masked in eYFP channel, then intensity was measured in mCherry channel. mCherry signal is divided by background nuclear signal in mCherry channel. Statistics shown are all p-values for Student's t-test vs. eYFP-LacI control. **C.** Comparison of eYFP focus size when eYFP-Ssn6-LacI is expressed alone versus with mCherry-Tup1. **D.** Single Ssn6 domains in eYFP-LacI plasmid co-transfected with mCherry-Tup1. Scale 10μm. **E.** Quantification of eYFP and mCherry colocalization in D.

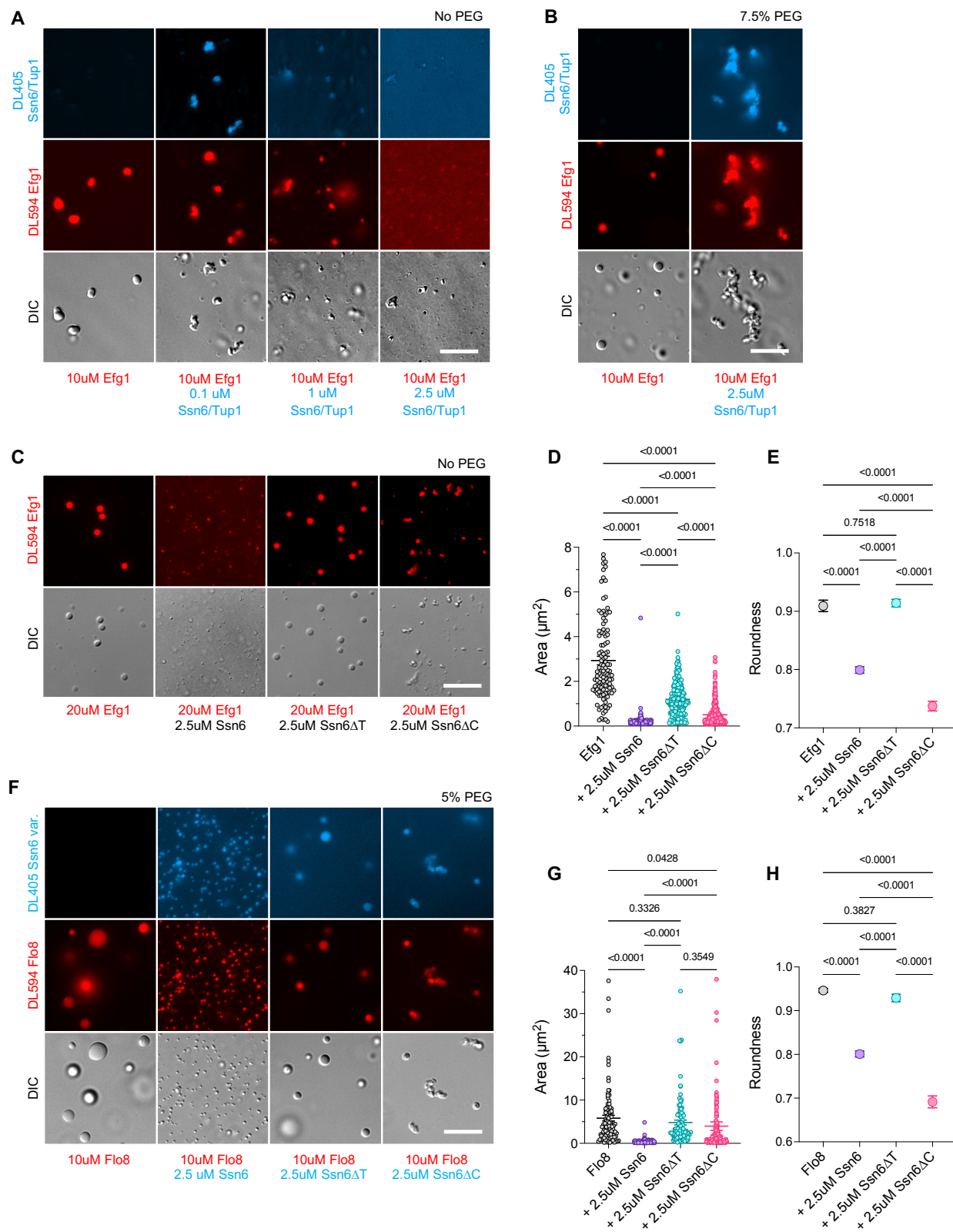


Figure 4: Ssn6 alters properties of other *C. albicans* transcription factor condensates *in vitro*. **A.** Titration of 10uM MBP-Efg1 with varying concentrations of MBP-Ssn6/MBP-Tup1. TEV-cleaved 20 minutes in 150mM NaCl 10mM Tris pH 7.4. Relevant samples spiked with <.5uM Dylight 594 MBP-Efg1 and/or Dylight 405 MBP-Ssn6/MBP-Tup1. Scale 10um. **B.** MBP-Efg1 +/- 2.5uM MBP-Ssn6/MBP-Tup1 in 7.5% PEG8000, TEV-cleaved 20 minutes in 150mM NaCl 10mM Tris pH 7.4. Relevant samples spiked with <.5uM Dylight 594 MBP-Efg1 and/or Dylight 405 MBP-Ssn6/MBP-Tup1. Scale 10um. **C.** 20uM MBP-Efg1 with 2.5uM MBP-Ssn6, MBP-Ssn6 Δ T, or MBP-Ssn6 Δ C. TEV-cleaved 20 minutes in 150mM NaCl 10mM Tris pH 7.4. Samples spiked with <.5uM Dylight 594 MBP-Efg1. Scale 10um. **D.** Quantification of Efg1 condensate area in C. Significance represents Student's t-test, all comparisons shown. **E.** Quantification of Efg1 condensate roundness in C. Significance represents Student's t-test, all comparisons shown. **F.** 10uM MBP-Flo8 with 2.5uM MBP-Ssn6, MBP-Ssn6 Δ T, or MBP-Ssn6 Δ C in 5% PEG. TEV-cleaved 20 minutes in 150mM NaCl 10mM Tris pH 7.4. Relevant samples spiked with <.5uM Dylight 594 MBP-Flo8 and/or Dylight 405 MBP-Ssn6/Ssn6 Δ T/Ssn6 Δ C. Scale 10um. **G.** Quantification of Flo8 condensate area in F. Significance represents Student's t-test, all comparisons shown. **H.** Quantification of Flo8 condensate roundness in F. Significance represents Student's t-test, all comparisons shown.

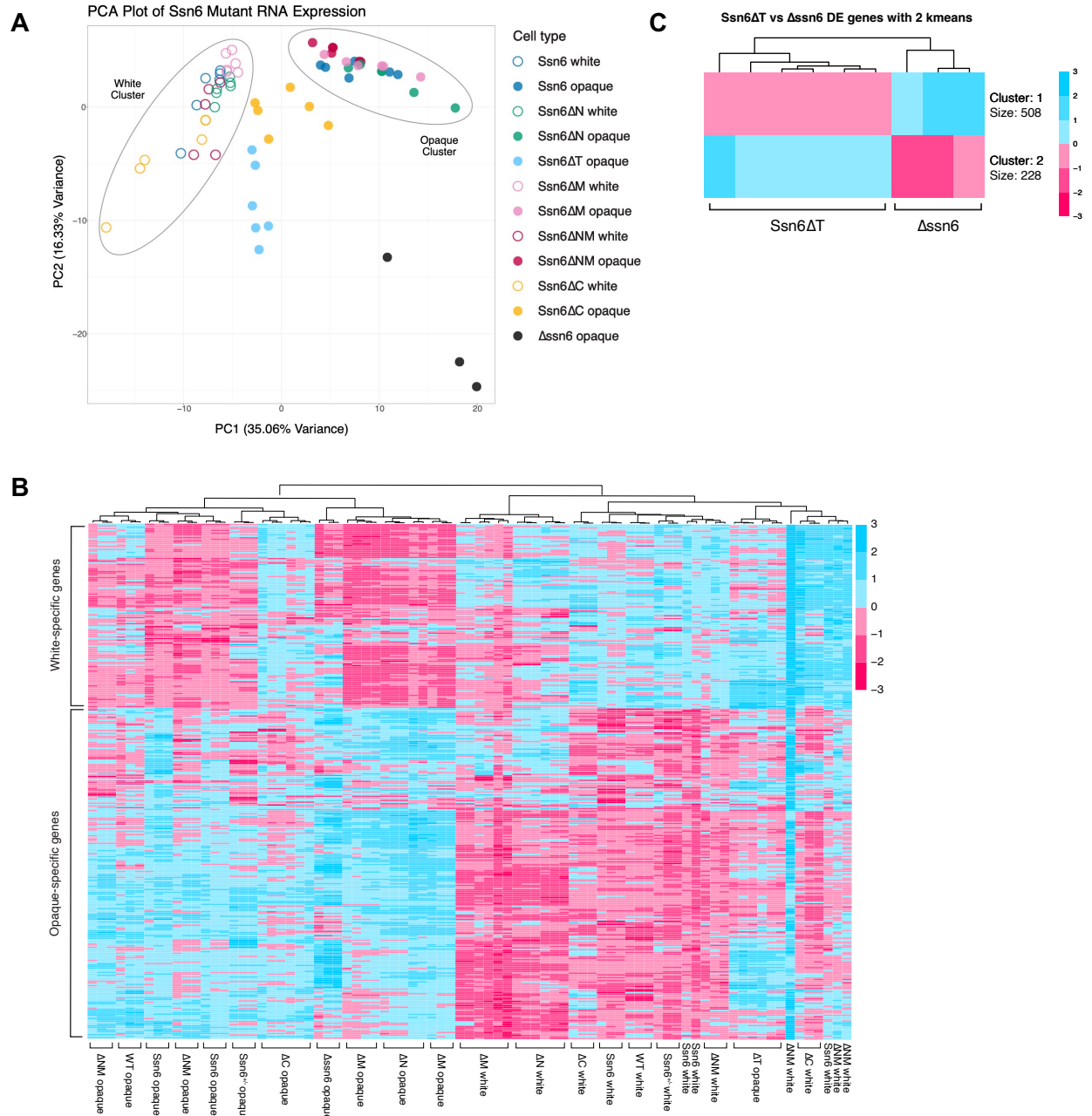
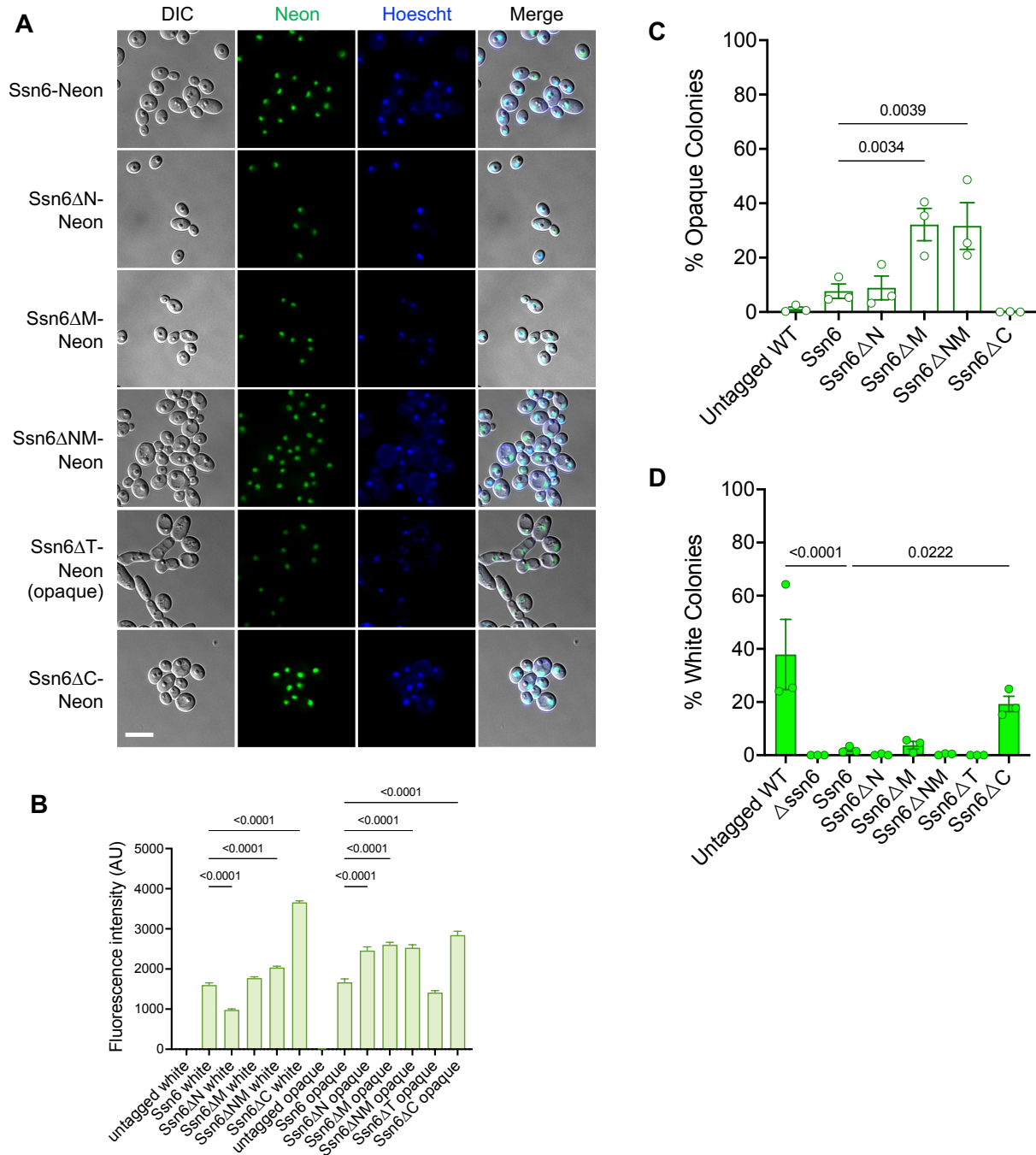
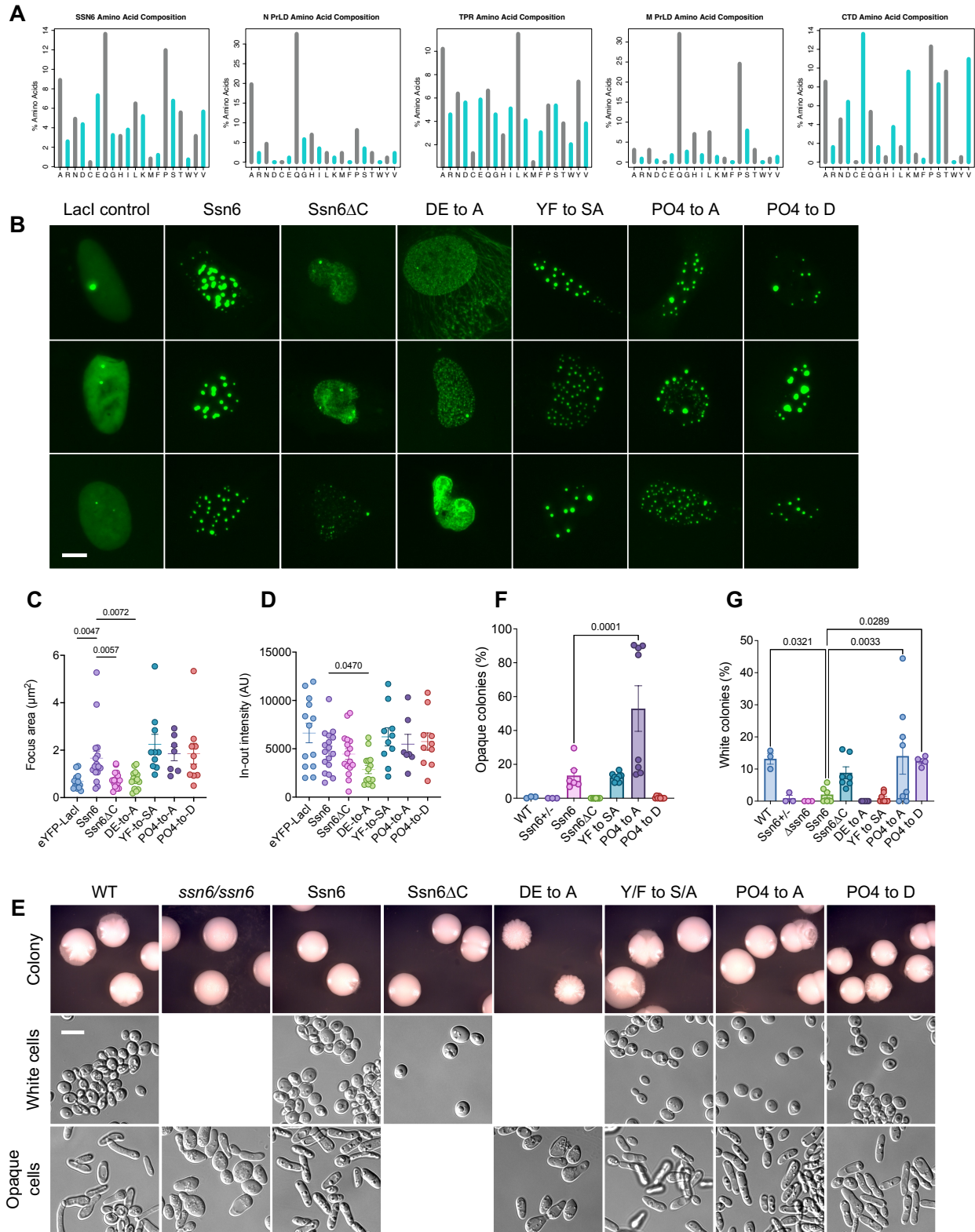


Figure 5: The Ssn6 TPR and C-terminal domains exhibit strong effects on RNA expression in *C. albicans*. **A.** Principal component analysis of Ssn6 mutant RNA-sequencing data. **B.** Heatmap showing differentially expressed genes between Ssn6 addback white and opaque strains. Scale represents z-score. **C.** Heatmap showing differentially expressed genes between Ssn6ΔT and Δssn6 strains with 2 kmeans. Scale represents z-score.

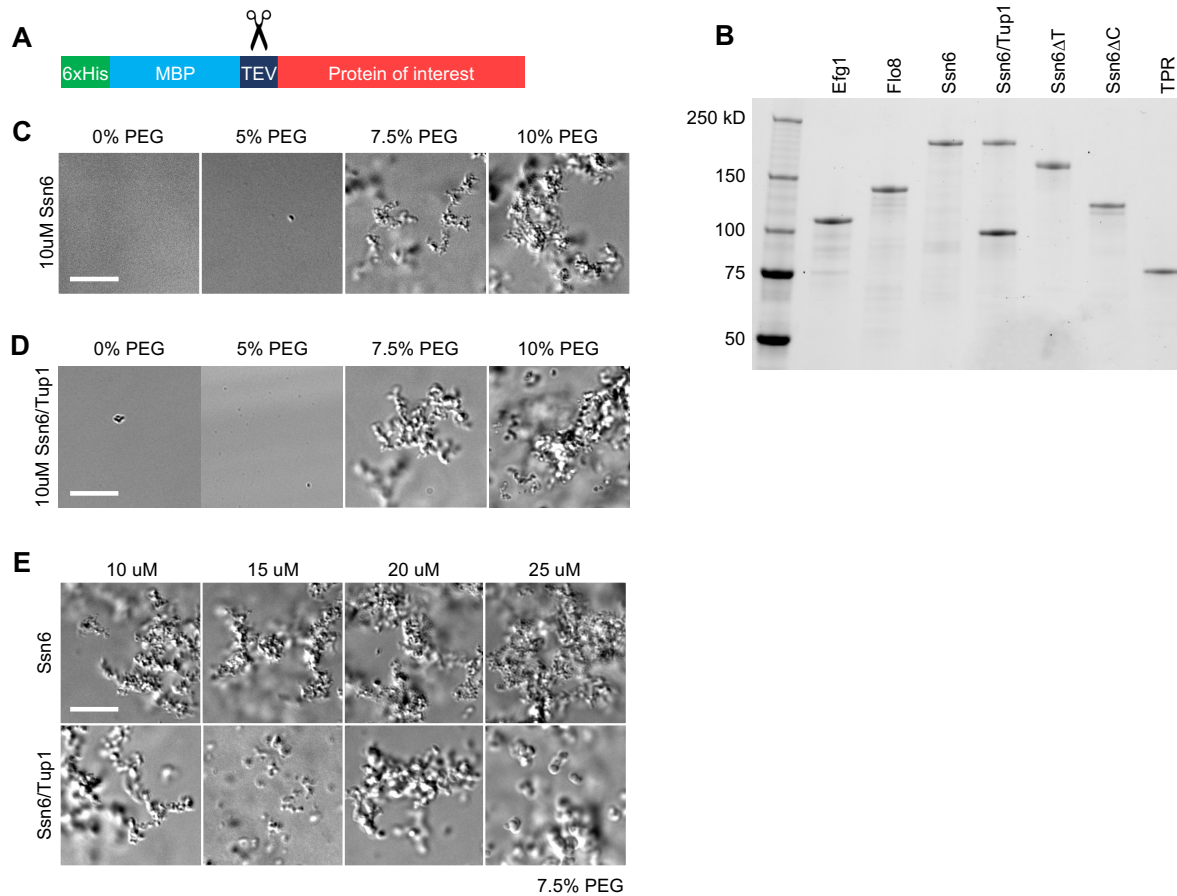
Supplementary Data



Extended Data 1: Quantification of Ssn6 mutant expression in *C. albicans*. **A.** Ssn6-Neon strains stained with Hoescht. Images shown are for white cells unless marked. Scale = 10μm. **B.** Quantification of A. Statistics shown are those significant versus the full-length Ssn6-Neon strain in the respective cell type, Student's t-test, $p \leq .05$. **C.** Ssn6-neon white-to-opaque switching assays. Statistics shown are those significant versus the full-length Ssn6-Neon strain in the respective cell type, Student's t-test, $p \leq .05$. **D.** Ssn6-neon opaque-to-white switching assays. Statistics shown are those significant versus the full-length Ssn6-Neon strain in the respective cell type, Student's t-test, $p \leq .05$.



Extended Data 2: Mutation of Ssn6 CTD acidic residues affect condensate properties in U2OS cells and switching properties in *C. albicans*. **A.** Amino acid composition of each Ssn6 domain. **B.** eYFP-Ssn6-LacI CTD amino acid mutation constructs in U2OS cells. Scale 10um. **C.** Size quantification of puncta in B. Statistics displayed are Student's t-test results that were significant versus eYFP-LacI, Ssn6, or Ssn6 Δ C, $p \leq .05$. **D.** Intensity quantification of puncta in B. Statistics displayed are all Student's t-test results that were significant, $p \leq .05$. **E.** Colony (top) and cell (middle and bottom) images of *C. albicans* Ssn6 CTD mutants. Scale 10um for DIC. **F.** *C. albicans* white-to-opaque switching data of Ssn6 CTD mutants. **G.** *C. albicans* opaque-to-white switching data of Ssn6 CTD mutants.



Extended Data 3: Ssn6 protein aggregates in vitro with or without partner protein Tup1. **A.** Diagram of MBP fusion proteins purified from *E. coli*. **B.** 10uM MBP-Ssn6 with various PEG8000 concentrations, TEV-cleaved 20 minutes in 150mM NaCl, 10mM Tris pH 7.4. Scale 10um. **C.** 10uM co-purified MBP-Ssn6/MBP-Tup1 with various PEG8000 concentrations, TEV-cleaved 20 minutes in 150mM NaCl, 10mM Tris pH 7.4. Scale 10um. **D.** MBP-Ssn6 alone or co-purified with MBP-Tup1 at various concentrations, TEV-cleaved 20 minutes in 7.5% PEG8000, 150mM NaCl, 10mM Tris pH 7.4. Scale 10um. **E.** Gel of MBP fusion proteins purified for Fig. 4 and Extended Data Fig. 3.

Tables

Supplementary Table 1: Plasmids used in this study

Plasmid	Name	Method	Starting Vector	Inserts	Template	Oligos	Restriction enzymes
pSFS2a	pSFS2a	Reuss et al. 2002					
RB523	MBP vector, pRP1B-MBP/THMT	Frazer et al., 2020, gift from Nick Fawzi					
RB1207	mCherry vector	Chong et al., 2018, gift from Tjian lab					
RB1208	eYFP-LacI vector	Chong et al., 2018, gift from Tjian lab					
RB1397	pSFS2a-Bsal	Oligo insertion	pSFS2a	Oligos 6048 & 6049			Apal, XhoI
RB895	pSFS2a-mNeon vector	Subcloning	pSFS2a	mNeon	RB880 (synthetic)	n/a	insert: XhoI; vector: XhoI, CIP
RB1277	pSFS2a-Ssn6	Cloning	pSFS2a	5'-Ssn6-3'	SC5314 gDNA	5607, 5608	Apal, XhoI
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1279	pSFS2a-Ssn6ΔN	Fusion PCR	pSFS2a	5'	SC5314 gDNA	5607, 5609	
				Ssn6ΔN-3'	SC5314 gDNA	5610, 5608	
				5'-Ssn6ΔN-3'	5' and Ssn6ΔN-3'	5607, 5608	Apal, XhoI
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1281	pSFS2a-Ssn6ΔM	Fusion PCR	pSFS2a	5'-Ssn6NT	SC5314 gDNA	5607, 5611	
				Ssn6C-3'	SC5314 gDNA	5612, 5608	
				5'-Ssn6ΔM-3'	5'-Ssn6NT and Ssn6C-3'	5607, 5608	Apal, XhoI
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1283	pSFS2a-Ssn6ΔNM	Fusion PCR	pSFS2a	5'	SC5314 gDNA	5607, 5609	
				Ssn6T	SC5314 gDNA	5610, 5611	
				Ssn6C-3'	SC5314 gDNA	5612, 5608	
				5'-	5' and	5607,	Apal, XhoI

				Ssn6 Δ N M-3'	Ssn6T and Ssn6C-3'	5608	
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1958	pSFS2a- Ssn6 Δ T	Golden Gate	pSFS2a- BsaI	5'- Ssn6N	RB1277	6905, 6906	BsaI-HF-v2
				Ssn6MC -3'	RB1277	6907, 6910	BsaI-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1960	pSFS2a- Ssn6 Δ C	Golden Gate	pSFS2a- BsaI	5'- Ssn6 Δ C	RB1277	6905, 6908	BsaI-HF-v2
				3'	RB1277	6909, 6910	BsaI-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1249		Cloning	RB523	Ssn6 Δ N	RB1170	5613, 5614	NdeI, XhoI
RB1251 *		Cloning	RB1170	Ssn6 Δ M	RB1254	n/a	BamHI, XhoI
RB1254		Fusion PCR	RB523	Ssn6T	RB1170	5613, 5616	
				Ssn6C	RB1170	5617, 5614	
				Ssn6 Δ N M	Ssn6T and Ssn6C	5613, 5614	NdeI, XhoI
RB1462	eYFP-Ssn6- LacI	Cloning	RB1208	Ssn6	RB1170	6038, 6107	insert: BsrGI- HF & XmaI; vector: BsrGI- HF & BspEI
RB1464	eYFP- Ssn6 Δ N-LacI	Cloning	RB1208	Ssn6 Δ N	RB1249	6039, 6107	insert: BsrGI- HF & XmaI; vector: BsrGI- HF & BspEI
RB1466	eYFP- Ssn6 Δ M-LacI	Cloning	RB1208	Ssn6 Δ M	RB1251	6038, 6107	insert: BsrGI- HF & XmaI; vector: BsrGI- HF & BspEI
RB1468	eYFP- Ssn6 Δ NM- LacI	Cloning	RB1208	Ssn6 Δ N M	RB1253	6039, 6107	insert: BsrGI- HF & XmaI; vector: BsrGI- HF & BspEI
RB1956	eYFP- Ssn6 Δ T-LacI	Cloning	RB1208	Ssn6 Δ T	RB1734 (Twist Bioscienc es)	6038, 6107	insert: BsrGI- HF & XmaI; vector: BsrGI- HF & BspEI
RB1957	eYFP- Ssn6 Δ C-LacI	Cloning	RB1208	Ssn6 Δ C	RB1170	6038, 6106	BsrGI-HF, BspEI
RB2024	eYFP-Ssn6- N-LacI	Cloning	RB1208	Ssn6N	RB1170	6038, 6153	insert: BsrGI- HF & XmaI; vector: BsrGI- HF & BspEI
RB2025	eYFP-Ssn6-	Cloning	RB1208	Ssn6T	RB1170	6039,	insert: BsrGI-

	T-LacI					6154	HF & XmaI; vector: BsrGI-HF & BspEI
RB2026	eYFP-Ssn6-M-LacI	Cloning	RB1208	Ssn6M	RB1170	6155, 6156	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB2027	eYFP-Ssn6-C-LacI	Cloning	RB1208	Ssn6C	RB1170	6157, 6107	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB2028	eYFP-Ssn6-NT-LacI	Cloning	RB1208	Ssn6NT	RB1170	6038, 6154	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB2029	eYFP-Ssn6MC-LacI	Cloning	RB1208	Ssn6MC	RB1170	6155, 6107	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB2182	eYFP-Ssn6-CTD-DE-to-A-LacI	Golden Gate, then subcloning	pGGAse lect, then RB1208	Ssn6ΔC	RB1462	8579, 8580	BsmBI-HFv2, then BsrGI-HF & BspEI
				CTD-DE-to-A	RB2168 (Gene Universal)	8581, 8582	BsmBI-HFv2, then BsrGI-HF & BspEI
RB2183	eYFP-Ssn6-CTD-YF-to-SA-LacI	Golden Gate, then subcloning	pGGAse lect, then RB1208	Ssn6ΔC	RB1462	8579, 8583	BsmBI-HFv2, then BsrGI-HF & BspEI
				CTD-YF-to-SA	RB2169 (Gene Universal)	8584, 8585	BsmBI-HFv2, then BsrGI-HF & BspEI
RB2184	eYFP-Ssn6-CTD-PO4-to-Ala-LacI	Golden Gate, then subcloning	pGGAse lect, then RB1208	Ssn6ΔC	RB1462	8579, 8586	BsmBI-HFv2, then BsrGI-HF & BspEI
				CTD-PO4-to-A	RB2170 (Gene Universal)	8587, 8588	BsmBI-HFv2, then BsrGI-HF & BspEI
RB2185	eYFP-Ssn6-CTD-PO4-to-Asp-LacI	Golden Gate, then subcloning	pGGAse lect, then RB1208	Ssn6ΔC	RB1462	8579, 8586	BsmBI-HFv2, then BsrGI-HF & BspEI
				CTD-PO4-to-D	RB2171 (Gene Universal)	8587, 8588	BsmBI-HFv2, then BsrGI-HF & BspEI
RB2178	pSFS2a-Ssn6-CTD-DE-to-A	Golden Gate	pSFS2a-BsaI	5'-Ssn6ΔC	RB1277	8567, 8568	BsaI-HF-v2
				CTD-DE-to-A	RB2164 (Gene Universal)	8569, 8570	BsaI-HF-v2
				3'	RB1277	8571, 8572	BsaI-HF-v2

		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB2179	pSFS2a- Ssn6-CTD- YF-to-SA	Golden Gate	pSFS2a- Bsal	5'- Ssn6 Δ C	RB1277	8567, 8573	Bsal-HF-v2
				CTD- YF-to- SA	RB2165 (Gene Universal)	8574, 8575	Bsal-HF-v2
				3'	RB1277	8576, 8572	Bsal-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB2180	pSFS2a- Ssn6-CTD- PO4-to-Ala	Golden Gate	pSFS2a- Bsal	5'- Ssn6 Δ C	RB1277	8567, 8573	Bsal-HF-v2
				CTD- PO4-to- A	RB2166 (Gene Universal)	8574, 8577	Bsal-HF-v2
				3'	RB1277	8578, 8572	Bsal-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB2181	pSFS2a- Ssn6-CTD- PO4-to-Asp	Golden Gate	pSFS2a- Bsal	5'- Ssn6 Δ C	RB1277	8567, 8573	Bsal-HF-v2
				CTD- PO4-to- D	RB2167 (Gene Universal)	8574, 8577	Bsal-HF-v2
				3'	RB1277	8578, 8572	Bsal-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1619	mCherry- Tup1	Cloning	pmCherry (RB1207)	Tup1	RB1172	6282, 6283	insert: BamHI- HF & XmaI; vector: BamHI- HF & BspEI
RB1170	MBP-Ssn6	Subcloning	RB523	Ssn6	synthetic	n/a	NdeI & XhoI
RB1172	MBP-Tup1	Subcloning	RB523	Tup1	synthetic	n/a	NdeI & XhoI
RB1227	MBP-Tup1- CmR	Subcloning	RB862 (MBP chloram phenicol vector)	Tup1	RB1172	n/a	NdeI & XhoI
RB514	MBP-Efg1	Cloning	RB523	Efg1	synthetic	n/a	NdeI & XhoI
RB971	MBP-Flo8	Subcloning	RB523	Flo8	RB959 (synthesized)	n/a	NdeI & XhoI
RB2258	MBP-Ssn6 Δ T	Golden Gate, then subcloning	pGGAse lect, then RB523	Ssn6N	RB1171	8782, 8783	BsmBI-HFv2, then NdeI & XhoI
				Ssn6MC	RB1171	8784, 8785	BsmBI-HFv2, then NdeI & XhoI
RB2257	MBP-Ssn6 Δ C	Cloning	RB523	Ssn6 Δ C	RB1171	5615,	NdeI & XhoI

						8781	
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*RB1251 construction used an existing BamHI site in TPR region to add rear portion of Ssn6 Δ NM into full Ssn6 sequence to create Ssn6 Δ M.

Supplementary Table 2: Oligonucleotides used in this study

Oligo #	Oligo name	Oligo sequence
4439	pSFS2 Right	GCGAAAAAGTGGGCACTAAG
5091	Neon rev	TTTATACAATTCATCCATACCCATAACATCA
5607	Ssn6 5 fwd Apal	GGACCGGGGGCCCCAGAAAGACAAAGAGAGAGCG
5608	Ssn6 3 rev XhoI	GGACCGCTCGAGCAACTATATTCCATCCAGAAAATATTTCTATC
5609	Ssn6 5 Rev	CATCTTTCCGGATCTTTGTTAATGA
5610	Ssn6 delta NPrLD Fwd	TCATTAACAAAGATCCGGAAAGATGTTACCTAGTTCAGCTGCTCTTAAT
5611	Ssn6 5 and N-ORF Rev	ATACTTTGTCAATTGTTCTAATCTTGC
5612	Ssn6 C-ORF Fwd overlaps N	GCAAGATTAGAACAATTGACAAAGTATATTGCACCTGTTAGA CAAGAAC
5613	MBP-Ssn6 dN-PrLD Fwd NdeI	GGACCGCATATGCTGCCGTCTAGCGCCG
5614	MBP-Ssn6 dN-PrLD Rev XhoI	GGACCGCTCGAGTTATTCGTCATCATAGTTTTTCGTCTTC
5615	MBP-Ssn6 Fwd NdeI	GGACCGCATATGTATGCTACCGCGCATACC
5616	MBP-Ssn6 N-ORF rev	GTATTTGGTCAGCTGTTCAAGAC
5617	MBP-Ssn6 C-ORF Fwd	GTCTTGAACAGCTGACCAAATACATTGCACCAGTGCGTCAG
5683	Ssn6-AB check left fwd	TCTTCTCTGCCCCATAAAATTCA
5684	Ssn6-AB check right rev	CACTAATACCATTACCAACAGTTTTCAA
5740	Ssn6 tail fwd (SacII)	GGACCGCCGCGGATTTGACTATCTTGTGAATTAAAGTTTCAA
5741	Ssn6 tail rev (SacI)	GGACCGGAGCTCTATTGCATGTTTTAAAAATGATTATTCACAAAC
5742	CaWor1 FL rev no stop XmaI	GGACCGCCCCGGGAGTACCGGTGTAATACGACC
5799	Ssn6 tail check rev	TTTCATTGGGAAAGAACAAGACAATAAAG
6038	EYFP Ssn6 fwd	ggaccgtgtacaaggggtgtagtggttatgctaccgcgcataccattaaac
6039	EYFP Ssn6 deltaN fwd	ggaccgtgtacaaggggtgtagtggtctgccgtctagcgccgcactg
6048	GGA Adapter for pSFS2A Fwd	CCAGTTGAGACCCATTAATGGTCTCTTACTC
6049	GGA Adapter for pSFS2A Rev	TCGAGAGTAAGAGACCATTAATGGGTCTCAACTGGGGCC
6106	mCherry ssn6 rev 2	ggaccgctcgagctataaccttctctctttttggatcttctgcatcatagtttctg
6107	LacI Ssn6 rev 2	ggaccgcccgggtctgcatcatagtttctgctctctc
6153	Ssn6 N PrLD LacI rev	GGACCGTCCGGAACGCTGTTGTTGCTGCTG
6154	Ssn6 TPR LacI rev	GGACCGTCCGGAGTATTTGGTCAGCTGTTCAAG
6155	Ssn6 M PrLD eYFP fwd	GGACCGTGTACAAGGGTGGTAGTGGTCAGCAGGAAGGCAA CACCC
6156	Ssn6 M PrLD LacI rev	GGACCGTCCGGAGTGTGGCTGCTGGTACTGCTG
6283	Tup1 BFP mCherry rev	ggaccgggatccctataaccttctctctttttggatctttttgtccatttccag
6342	Ssn6 N PrLD rev	tcgctgtgtgctgctgtgtgtgtgtgtggg
6350	Ssn6 upstream-C fusion fwd	caaagatccggaaagatgattgcacctgttagac
6455	Ssn6 addback T mid seq fwd	gtgaagtatggtatgatttgggg
6905	GG Ca Ssn6-5N fwd	GGCTACGGTCTCTCAGTCAGAAAGACAAAG AGAGAGCG
6906	GG Ca Ssn6-5N rev	GGCTACGGTCTCTCCTTCTTGTGTGCTGTTGTTGCTGCTG
6907	GG Ca Ssn6-MC3_fwd	GGCTACGGTCTCTAAGGTAATACTCACCCA C
6908	GG Ca Ssn6-deltaC_rev	GGCTACGGTCTCATCTCTAATGTGGTTGTT GATATTG
6909	GG Ca Ssn6-3near_fwd	GGCTACGGTCTCAGAGATAAAGAAAAGTGG TGAAATTG

6910	GG Ca Ssn6-3near rev	GGCTACGGTCTCCAGTACAACATATATTCCA TCCAGAAAATATTTTC
7113	Ssn6 neon tag long oligo fwd	gatgacgtcaaaaaagatgaaaatccagaacctccaatgagaaagattgaagaaga tgaaaattatgatgatgaaGGTGGTAGTGGTATGGTTTCTAAAG
7114	Ssn6 delta C neon tag long oligo fwd	ttacacaactctgtaatatcatatcagctccatcacaaagtaacctcaaccacaacaaca atatcaacaaccacatGGTGGTAGTGGTATGGTTTCTAAAG
7115	Ssn6 neon tag rev long oligo	ccccatccccctaccccgtaaatatataatattcaactatcatatacaatttcaccact ttctttatctggcgccgctctagaactagtggatc
8036	Ca Ssn6 N check rev	cttgcattgtagtccaccg
8567	GG Ca Ssn6 5 fwd	ggctacggtctcgagtcagaaagacaaagagagag
8568	GG Ca Ssn6 M PrLD DE to A rev	ggctacggtctcgctaacagggtgcaatatgtggtgtgatattgttg
8569	GG Ca Ssn6 CTD DE to A fwd	ggctacggtctcgtagacaagctcaagttaac
8570	GG Ca Ssn6 CTD DE to A rev	ggctacggtctccctttatcttaagcagcagcataattag
8571	GG Ca Ssn6 3 DE to A fwd	ggctacggtctccaaagaaaagtggtgaaattg
8572	GG Ca Ssn6 3 rev	ggctacggtctccagtacaactatattccatccagaaaatatttc
8573	GG Ca Ssn6 M PrLD rev	ggctacggtctcatgatattgtgtgtgtgtg
8574	GG Ca Ssn6 CTD fwd	ggctacggtctcaatcaacaaccacatattgcacctgtagacaag
8575	GG Ca Ssn6 CTD YF to SA rev	ggctacggtctcctctattcatcatctgaattttcatc
8576	GG Ca Ssn6 3 YF to SA fwd	ggctacggtctcctagagataaagaaaagtggtgaaattg
8577	GG Ca Ssn6 CTD PO4 rev	ggctacggtctcgctattcatcatcataattttcatcttc
8578	GG Ca Ssn6 3 PO4 fwd	ggctacggtctcgtagagataaagaaaagtggtgaaattg
8579	GG Ec Ssn6 BsrGI fwd	ggctaccgtctccggagtgtaaggggtgtagtg
8580	GG Ec Ssn6 M DE to A rev	ggctaccgtctcctggctgctggtactgctg
8581	GG Ec Ssn6 CTD DE to A fwd	ggctaccgtctccgccacacattgcaccagtgcgtcaggc
8582	GG Ec Ssn6 CTD DE to A rev	ggctaccgtctctatggtccggaggcgccgcgcata
8583	GG Ec Ssn6 M YF to SA rev	ggctaccgtctcatggctgctggtactgctg
8584	GG Ec Ssn6 CTD YF to SA fwd	ggctaccgtctcagccacacattgcaccagtgcgtcag
8585	GG Ec Ssn6 CTD YF to SA rev	ggctaccgtctctatggtccggattcgatcgctg
8586	GG Ec Ssn6 M PO4 rev	ggctaccgtctcggtgctggtactgctg
8587	GG Ec Ssn6 CTD PO4 fwd	ggctaccgtctcgccacacattgcaccagtgcgtcag
8588	GG Ec Ssn6 CTD PO4 rev	ggctaccgtctctatggtccggattcgatcatagttttc
8781	MBP-Ssn6deltaC Ec XhoI rev	ggaccgctcgagTTAGTGTGGCTGCTGGTACTGC
8782	GG MBP-Ssn6 Ec NdeI fwd	ggctaccgtctctggagcatatgtatgctaccgcgcATACC
8783	GG Ec Ssn6 N rev	ggctaccgtctcctgttgcttctgctgacgctgtgtgtgctgctg
8784	GG Ec Ssn6 M fwd	ggctaccgtctccaacacccatccgcccag
8785	GG MBP-Ssn6 Ec XhoI rev	ggctaccgtctcaatggctcgagttattcgatcatagttttcgtcttcTTC

Supplementary Table 3: *C. albicans* strains used in this study

Strain #	name	description
CAY6342	WT white	AHY135 from Lohse, et al. 2013, <i>PNAS</i> .
CAY6343	WT opaque	AHY136 from Lohse, et al. 2013, <i>PNAS</i> .
TF121	<i>ssn6/ssn6</i>	TF121 from Lohse, et al. 2016, <i>Genetics</i> .
CAY8083	<i>ssn6/SSN6</i>	AHY337 from Hernday, et al. 2016, <i>mBio</i> .
CAY11369	Ssn6 full with 3' homology satS	RB1277 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11371	Ssn6 full with 3' homology satS	RB1277 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11375	Ssn6 Δ N with 3' homology satS	RB1279 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11377	Ssn6 Δ N with 3' homology satS	RB1279 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11381	Ssn6 Δ M with 3' homology satS	RB1281 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11383	Ssn6 Δ M with 3' homology satS	RB1281 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11387	Ssn6 Δ NM with 3' homology satS	RB1283 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11389	Ssn6 Δ NM with 3' homology satS	RB1283 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683 & 5611. 5' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY13592	Ssn6 Δ T with 3' homology satS	RB1958 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY13593	Ssn6 Δ T with 3' homology satS	RB1958 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY13594	Ssn6 Δ C with 3' homology satS	RB1960 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY13595	Ssn6 Δ C with 3' homology satS	RB1960 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14850	Ssn6 CTD DE to A satS	RB2178 digested with PspOMI and SacI and transformed into TF121. Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14851	Ssn6 CTD DE	RB2178 digested with PspOMI and SacI and transformed into TF121.

	to A satS	Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14852	Ssn6 CTD YF to SA satS	RB2179 digested with PspOMI and SacI and transformed into TF121. Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14853	Ssn6 CTD YF to SA satS	RB2179 digested with PspOMI and SacI and transformed into TF121. Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14854	Ssn6 CTD PO4 Ala satS	RB2180 digested with PspOMI and SacI and transformed into TF121. Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14855	Ssn6 CTD PO4 Ala satS	RB2180 digested with PspOMI and SacI and transformed into TF121. Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14856	Ssn6 CTD PO4 Asp satS	RB2181 digested with PspOMI and SacI and transformed into TF121. Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14857	Ssn6 CTD PO4 Asp satS	RB2181 digested with PspOMI and SacI and transformed into TF121. Integration checked with oligos 5683/8036 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY13555	Ssn6 full Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11369. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13556	Ssn6 full Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11369. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13557	Ssn6 Δ N Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11375. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13558	Ssn6 Δ N Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11375. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13559	Ssn6 Δ M Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11381. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13560	Ssn6 Δ M Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11381. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13561	Ssn6 Δ NM Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11387. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.

CAY13562	Ssn6 Δ NM Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11387. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13563	Ssn6 Δ T Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY13592. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13564	Ssn6 Δ T Neon satS	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY13593. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13565	Ssn6 Δ C Neon satS	Long oligos 7114/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY13594. Integration checked with oligos 6455/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13566	Ssn6 Δ C Neon satS	Long oligos 7114/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY13595. Integration checked with oligos 6455/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.

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Chapter 3: Additional experiments and conclusions

Introduction

The following section contains preliminary data regarding the phase separation of Ssn6 and other white-opaque transcription factors (TFs). Data in this section examines colocalization of Ssn6 with itself and other TFs in a mammalian nuclear system, the behavior of full-length TFs vs. only prion-like domains (PrLDs) in this system, complementation of Ssn6 and its C-terminal domain (CTD), the role of the Ssn6 CTD in response to environmental cues, the endogenous level of TFs in *C. albicans*, and the effect of exogenous factors on Efg1 condensates.

Results

Ssn6 colocalization with Ssn6 and other TF PrLDs in U2OS cells.

In **Chapter 2** of this thesis, we examined the phase separation behavior of eYFP-Ssn6-LacI constructs expressed in the nuclei of a mammalian cell line (see **Chapter 2 Fig. 2-3**) [1, 2]. In this system, Ssn6 formed dissolvable condensates that interacted with the canonical partner protein of Ssn6, Tup1 (see **Chapter 2 Fig. 3**) [3-6]. In a similar manner, we co-transfected eYFP-Ssn6-LacI with an mCherry-Ssn6 plasmid in the U2OS cell model. Full-length Ssn6 colocalized with itself (**Fig. 1a**), as did PrLD deletion mutants of Ssn6 (**Fig. 1b**). eYFP-Ssn6 Δ T-LacI and eYFP-Ssn6 Δ C-LacI condensates showed no enrichment of mCherry-Ssn6 (**Fig. 1b-c**). This result suggests that both the TPR and the CTD of Ssn6 are necessary for the self-interaction that occurs inside the spherical and seemingly liquid condensates formed by eYFP-Ssn6-LacI, especially given that expression of only the TPR and CTD (Ssn6 Δ NM) is sufficient to produce spherical condensates in U2OS cells. Repetition to confirm this result may be useful.

Full-length Ssn6 did not colocalize with Efg1 or Czf1 PrLDs in U2OS cells (**Fig. 1d-g**). This observation contrasts with *in vitro* data (see **Chapter 2 Fig. 4**) where Efg1 and Ssn6 are capable of forming condensates together. Since only Efg1 PrLDs were expressed in this system, Ssn6 may instead interact with the structured DNA-binding domain of Efg1. Alternatively, Ssn6 may be

interacting with other proteins that are natively present in the mammalian cell nucleus, which prevent it from interacting with the exogenously expressed *C. albicans* TFs. Proteins expressed in eukaryotic systems may also contain post-translational modifications (PTMs), whereas those expressed in *E. coli* typically do not. The presence of PTMs could affect interactions with other proteins, leading to different interaction patterns being observed in mammalian cells vs. *in vitro*.

Additionally, we performed some preliminary experiments examining whether mCherry-Flo8-PrLD and mCherry-Wor4-PrLD could interact with other TF PrLDs in U2OS cells. The Flo8 PrLD curiously did not co-localize with itself, the *Candida maltosa* Wor1 (CmWor1) PrLD, or the Efg1 PrLDs in U2OS cells (**Fig. 1h**). The eYFP-Flo8-PrLD-LacI construct forms distinct round condensates (see top row **Fig. 1h**), so a lack of self-interaction seems odd. Validation of the full mCherry-Flo8-PrLD plasmid sequence may be necessary. On the other hand, the Wor4 PrLD does seem to interact with the CmWor1 PrLD and perhaps also with the Efg1 PrLDs, although this interaction is less distinct than with CmWor1 (**Fig. 1i**). Replication is needed to validate these results, along with quantitative analysis.

Future experiments could examine the colocalization of full-length *C. albicans* TFs in U2OS cells. It will be interesting to see if there are any TFs that interact with more partners when the structured domains are present. Differences in interaction from our published *in vitro* experiments [7, 8] would also be interesting, and could lead to further investigation of other factors such as PTMs. Imaging on the new Evident fluorescence microscope recently acquired by the Bennett lab could provide better resolution for colocalization experiments.

Expression of full-length *C. albicans* TFs in U2OS cells.

Our previously published studies only included expression of TF PrLDs in the U2OS system [7, 8]. Since the expression of full-length Ssn6 in the eYFP-LacI vector in this system yielded spherical condensates (**Chapter 2 Fig. 2**), we decided to examine other full-length TFs in this system as well. Plasmids were created encoding eYFP-TF-LacI variants, and also eYFP-TF-NLS

variants, which have only a nuclear localization signal (NLS) instead of the LacI module. eYFP-TF-NLS constructs will thus only be recruited to DNA by the innate DNA- or protein-interaction ability of the TF.

The Czf1 PrLD is able to form numerous small puncta when expressed as an eYFP-LacI fusion (**Fig. 2a**), in contrast to the singular punctum and diffuse nuclear signal typically formed by eYFP-LacI alone (**Fig. 2b**) [7]. Full-length Czf1 fused to LacI was able to form many small, grainy puncta as well (**Fig. 2a**). Curiously, when full-length Czf1 was expressed with only an NLS, it localized to large structures within the nucleus that are likely the nucleoli. A nucleolar stain could confirm the identity of these structures.

Expression of eYFP-Wor4-PrLD-LacI has previously led to the formation of numerous small puncta [7], although expression in this experiment was poor, so another trial was conducted (**Fig. 2c**). Full-length Wor4-LacI appeared to be able to form small grainy puncta, as did full-length Wor4 fused to only an NLS (**Fig. 2c**). Wor4 contains a putative zinc finger domain [9], so it likely has intrinsic DNA-binding capability. Expression of Wor2 variants was also tested (**Fig. 2d**). The eYFP-Wor2-PrLD-LacI variant expressed very poorly in two separate trials, so its ability to form condensates cannot be confirmed (**Fig. 2d**). Existing images of cells expressing full-length Wor2-LacI appear similar to the LacI control (**Fig. 2b**). Full-length Wor2-NLS cells have large, bright puncta, although these cells do not appear particularly healthy (see DIC in **Fig. 2d**).

Expression of full-length Efg1 without LacI (**Fig. 2e**) formed distinct granular puncta that do not appear quite as spherical as previously published data of eYFP-Efg1-LacI [7]. Full-length Flo8 formed spherical puncta when fused to LacI, but did not form any discrete puncta without LacI (**Fig. 2f**). The full-length Flo8-LacI puncta appear smaller than previously published data of the Flo8 PrLD [8], although quantification is needed. We also expressed a full-length CaCmWor1, where the DNA-binding domain is from the *C. albicans* sequence, while the PrLD is from the *C. maltosa* sequence. CaCmWor1 is able to complement for *C. albicans* Wor1 [7]. This full-length TF was able to form puncta when fused to LacI (**Fig. 2g**). CaCmWor1 without LacI formed some

nuclear structures that are not quite puncta, but also do not appear diffuse like the eYFP-LacI control (**Fig. 2b**). Expression of full-length Ssn6 without LacI (**Fig. 2h**) produced a diffuse signal similar to that of Flo8. This result indicates that the ability of both Ssn6 and Flo8 to form condensates is dependent on recruitment to DNA, although neither of these TFs has an inherent DNA-binding domain.

Future directions include repetition of experiments, particularly for constructs that expressed poorly, such as Wor2. Quantification of full-length TF droplet properties, including size and shape, would also be interesting, especially in comparison to expression of only the PrLDs. Treatment with 1,6-hexanediol to disrupt the condensates [2, 10, 11] may also reveal information about the stability of the structures produced by full-length TFs.

Expression of full-length TFs with mCherry-Ssn6 in U2OS cells.

To test whether Ssn6 could colocalize with full-length TFs in a nuclear system, we co-expressed eYFP-TF-NLS plasmids with mCherry-Ssn6 in U2OS cells. Ssn6 does not appear to colocalize with Efg1 (**Fig. 3a**), Wor2 (**Fig. 3b**), Czf1 (**Fig. 3c**), Wor4 (**Fig. 3d**), or CaCmWor1 (**Fig. 3e**) in this system, although no quantification has been performed. Ssn6 has been found to colocalize with these other TFs by ChIP data in *C. albicans* [12], so this result was unexpected. Most of these TFs form small grainy structures when not fused to LacI, so performing the experiment with a LacI fusion construct may yield different results. Additionally, other factors such as mammalian protein binding partners or PTMs may affect TF interactions in the U2OS cells. Another possibility is that Ssn6 may be recruited to the same genomic locations as these other TFs via a separate mechanism that does not involve direct interaction with the TFs tested in this experiment.

Complementation of Ssn6 with *Saccharomyces cerevisiae* Ssn6.

Ssn6 has homologs in various eukaryotic species, including *S. cerevisiae* [13, 14]. *C. albicans* and *S. cerevisiae* have 38% amino acid sequence identity, although the TPR domains of the

proteins have 70% amino acid sequence identity [14]. Predictions of general disorder [15] and prion-like character (PLAAC) [16] were used to characterize the amino acid sequences of *S. cerevisiae* Ssn6 (Sc Ssn6) and *C. albicans* Ssn6 (Ca Ssn6). Sc Ssn6 and Ca Ssn6 exhibited similar general profiles (**Fig. 4a**), with both including a short N-terminal PrLD, followed by structured TPR regions, a larger medial PrLD, and a CTD that is disordered but not prion-like. Since these general domains seemed similar, we created *C. albicans* strains where the endogenous Ssn6 genes were removed, and replaced with Sc Ssn6.

Initially, Sc Ssn6 strains kept in ambient conditions did not seem capable of forming white cells (**Fig. 4b**). Incubation at 37 °C promotes white cell formation [17], so these strains were incubated at 37 °C for 24 hours, then returned to ambient conditions for 6 days. Sc Ssn6 plates grown under these conditions showed colonies that were fully white or contained white sectors (**Fig. 4c**). Although Sc Ssn6 colony morphology is similar to that of Δ ssn6 (**Fig. 4c**), Sc Ssn6 cell morphology appears to show a partial rescue of the Δ ssn6 cell integrity defect (**Fig. 4b**).

Pure populations of Sc Ssn6 white and opaque cells were isolated, plated for single colonies, and grown in ambient conditions to quantify switching frequency (**Fig. 4d-e**). Sc Ssn6 white cells reverted to opaque at a frequency of ~60%, which was significantly higher than that of the Ca Ssn6 control (**Fig. 4d**). Sc Ssn6 opaque cells never spontaneously formed white colonies or sectors under ambient conditions (**Fig. 4e**), although this experiment is lacking a Ca Ssn6 control.

Since Sc Ssn6 strains had been observed to form white cells when exposed to high temperature (**Fig. 4b-c**), this effect was quantified by performing another switching assay where opaque cells were plated and incubated at 37 °C for 24 hours, then returned to ambient conditions for 6 days. Sc Ssn6 formed white colonies or sectors at a frequency of over 70% in this condition, although some diverging behavior between the two Sc Ssn6 strains used is apparent (**Fig. 4f**). WT and Ca Ssn6 control strains exhibited 100% white colonies, while the control Δ ssn6 strain never formed white cells (**Fig. 4f**).

This data indicates that Sc Ssn6 partially complements Ca Ssn6 in terms of white-opaque switching. Growth curves would also be useful to further describe the extent of Sc Ssn6 complementation in *C. albicans*. Further assays could characterize cell wall and cell membrane integrity rescue in more detail, as well as exposing cells to a variety of osmotic conditions to pinpoint the exact cause of Δ ssn6 cell distension and lysis. Future directions could also include analysis of the amino acid content of Sc Ssn6 domains, mutation of residue types of interest, and domain-swapping experiments.

Ssn6 Δ C fusions with Fus, TAF15, CmWor1, and Sp1 IDRs in U2OS and *C. albicans*.

The Ssn6 CTD is a disordered domain that has a strong effect on white-opaque switching in *C. albicans* (**Chapter 2 Fig. 1**). When the CTD is deleted, *C. albicans* strains are hyperactive in promoting the white cell state (**Chapter 2 Fig. 1b-c**). Work from our lab has shown that the human TAF15 IDR is able to complement the Efg1 PrLD in biofilm formation in *C. albicans*, but does not complement the Flo8 PrLD [8]. To determine if exogenous IDRs could complement for *C. albicans* Ssn6 CTD, Ssn6 variants were constructed where the CTD was replaced with IDR sequences from FUS, TAF15, CmWor1, and Sp1.

When these Ssn6 Δ C-TF fusions were expressed in the U2OS cell model [2, 7], they appeared similar to Ssn6 Δ C (**Fig. 5a**). Image quantification could be used to test for statistical significance in punctum size differences. In *C. albicans*, Ssn6 Δ C-TF fusions were able to form both white and opaque cells (**Fig. 5b-c**), although these opaque cells are not as long as the WT opaque cells from this assay. Ssn6 Δ C-CmWor1 opaques appear particularly short and mixed with white cells (**Fig. 5c**), so these may be similar to an intermediate or grey cell state, as previously described [18, 19].

Quantitative switching assays were performed on Ssn6 Δ C-TF fusion strains (**Fig. 5d-e**). Ssn6 Δ C-FUS and Ssn6 Δ C-CmWor1 white cells formed zero opaque colonies, similar to Ssn6 Δ C strains. Ssn6 Δ C-TAF15 white cells formed a few opaque colonies, so these were significantly

different from Ssn6 Δ C, although none of the strains formed opaques at the frequency of the Ssn6 control (**Fig. 5d**). Ssn6 Δ C-FUS and Ssn6 Δ C-CmWor1 opaques both formed white colonies at a higher frequency than the WT strain, although Ssn6 and Ssn6 Δ C strains are needed as controls in this experiment (**Fig. 5e**).

Future directions could include quantification of cell length differences between Ssn6 Δ C-TF fusion strains and WT or Ssn6 control opaque cells. None of the Ssn6 Δ C-TF fusion strains exhibited the crenulated opaque colony morphology common to the strain background (see WT and Ssn6 in **Fig. 5b**), suggesting that these strains may also be defective in filamentation.

The Ssn6 CTD plays a role in environmental sensing.

As discussed in Chapter 2, the Ssn6 Δ C mutant is hyperactive in promoting the white cell state (see **Chapter 2 Fig. 1**). Environmental factors such as high CO₂ and the carbohydrate N-acetyl glucosamine (NAG) can promote the opaque cell state [20, 21].

To test the response of Ssn6 Δ C strains to CO₂, white cells were plated and incubated with 20% CO₂ at 25 °C for 7 days. Surprisingly, none of the Ssn6 Δ C colonies exhibited any opaque morphology at the end of the experiment, in contrast to the WT and Ssn6 controls, which all showed opaque sectoring (**Fig. 6a-b**). This data indicates that the Ssn6 CTD is involved in the pathway that regulates CO₂-mediated opaque formation.

To test the response to NAG, white cells were plated on Lee's NAG media and incubated at 25 °C for 7 days. WT and Ssn6 controls exhibited opaque colonies with "cloud-like" morphology, alongside white colonies with invasive filamentous borders (**Fig. 6c**). The Δ ssn6 strain formed few, very tiny colonies, indicating particularly slow growth on Lee's NAG media (**Fig. 6c**). Ssn6 Δ C colonies all exhibited "cloud-like" opaque colony morphology, with the cells appearing either like typical oblong opaque or distended with granular interiors under the microscope (**Fig. 6c**). Ssn6 Δ C opaque formation frequency was 100% on Lee's NAG media, significantly higher than that of the controls (**Fig. 6d**). Further experiments could validate the opaqueness of Ssn6 Δ C cells

grown on Lee's NAG, such as qPCR for expression of OP4 or assessment of mating competency. This experiment needs to be repeated with an additional Ssn6 Δ C strain, since one did not grow on the Lee's NAG media, likely due to auxotrophy.

A preliminary RNA-sequencing experiment was also performed on single Ssn6 and Ssn6 Δ C strains exposed to 20% CO₂ or Lee's NAG media (**Fig. 6e-f**). Cells were spread-plated, then incubated in the relevant conditions described above (**Fig. 6a-d**). Samples were collected at 6, 24, and 72 hours. Only single samples of each timepoint were prepared for RNA-seq, so to perform differential gene expression analysis, the 6, 24 and 72 hour samples were combined into an "exposure" group, and the zero timepoint sample was used as the comparison. In the 20% CO₂ condition, the Ssn6 Δ C strain showed more differentially expressed genes (DEGs) than the Ssn6 strain (**Fig. 6e**), with Ssn6 having 416 DEGs, while Ssn6 Δ C had 598. In the Lee's NAG condition, the opposite was observed, with Ssn6 now having 612 DEGs, while Ssn6 Δ C had 426 (**Fig. 6f**). The majority of DEGs in either condition do not overlap. This experiment could be validated by performing it again with strain duplicates and more timepoint replicates.

In other experiments, Ssn6 Δ C opaque colonies were observed to lack the crenulated morphology of other opaque colonies in this strain background (see **Chapter 2 Fig. 1b**). This observation suggested that Ssn6 Δ C cells may have a filamentation defect, so a filamentation assay was performed by incubating cells in liquid culture with or without filamentation-inducing cues (**Fig. 6g**). Ssn6 Δ C cells were observed to have a filamentation defect in the strong inducing condition (YPD + serum, 37 °C). Δ ssn6 cells have been previously reported to exhibit a moderate defect in filamentation, being only able to form stubby pseudohyphae, and not true hyphae [14]. This data suggests that the Ssn6 CTD is involved in the filamentation pathway in *C. albicans* as well.

Protein levels of white-opaque TFs in *C. albicans* and *in vitro* droplet reconstitution.

The white-opaque switching in *C. albicans* is regulated by a number of different TFs. We have

previously proposed that these TFs form condensates together to regulate cell identity [7], but the exact protein levels of these TFs are not known. To determine the protein expression levels of these, TFs were C-terminally tagged with mNeon in *C. albicans* cells. White and opaque colonies from each strain were isolated, then cells were stained with Hoescht, and mNeon fluorescence was observed under the microscope (**Fig. 7a**). Nuclear mNeon fluorescence was measured for both white and opaque cells and normalized against an untagged strain (**Fig. 7b-c**). Efg1 is particularly abundant in white cells, followed by Ssn6 and Tup1 (**Fig. 7b**). In opaque cells, Tup1 is the most abundant TF examined, followed by Ssn6, Wor3, Wor4 and Efg1 (**Fig. 7c**).

Condensates composed of endogenous levels of TFs were reconstituted by setting the most abundant TF concentration at 20uM, and then adding other factors at levels relative to this value. Due to the number of proteins in the opaque sample, the 20uM concentration for Tup1 could not be maintained, but the relative protein ratios are similar to that measured in Fig. 6c. The white condensates were small and round, while the opaque condensates appeared more textured and gel-like (**Fig. 7d**), although these effects have not been quantified.

Although this data quantifies the protein levels of each of these TFs in the nucleus, these relative levels may not be present at regulatory regions in the genome. Properties of potential nuclear condensates formed by white-opaque TFs may also be affected by PTMs or the presence of other molecules including RNA or as-yet unidentified TFs.

The effect of time, temperature, and other TFs on Efg1 condensates.

Other studies have shown that condensates often become more viscous or aggregated over time [22-24]. To test this effect, Efg1 condensates were allowed to form, and then rested for 24 hours either at room temperature or at 37 °C. Other samples included 10% 1,6-hexanediol (a chemical that disrupts weak hydrophobic interactions) [2, 10, 11], 7.5% PEG (a molecular crowding agent), or both.

At the initial timepoint, Efg1 formed small round condensates (see top row **Fig. 8a**).

Inclusion of 1,6-hexanediol prevented condensate formation, while PEG caused the formation of larger spherical condensates. Inclusion of both 1,6-hexanediol and PEG interestingly caused the formation of small aggregates. After resting for 24 hours at room temperature, the same samples were imaged again (see middle row **Fig. 8a**). The control and 1,6-hexanediol conditions appeared similar to the initial timepoint, while the PEG sample now contained small aggregates. The PEG and 1,6-hexanediol sample contained clumpy chains of gel-like condensates and aggregates. The samples rested at 37 °C were also imaged after 24 hours (see bottom row **Fig. 8a**). All samples now contained large chunky structures. The control sample appeared as a chain of rounded condensates. Interestingly, the 1,6-hexanediol condition had now formed large aggregates with a tight structure after incubation with raised temperature.

The effect of Wor1 protein on Efg1 condensates was also tested. 10 μ M Efg1 was titrated with Wor1, ranging from concentrations of .1 μ M to 10 μ M (**Fig. 8b**). Although the Efg1 only sample in this experiment appeared to form gel-like condensates on its own, the addition of Wor1 seemed to cause the formation of larger chains of condensates. Notably, the addition of Wor1 does not have the effect of "dissolving" Efg1 condensates in the same way that Ssn6 does (**Chapter 2 Fig. 4**).

Other experiments were performed to test a method of measuring the dynamics of Efg1 condensates by microscopy. In this method, Efg1 condensates are formed with or without the addition of another TF. These condensates are applied to a welled slide and allowed to adhere to the bottom. Labelled Efg1 is then spiked into the well, and a timelapse records the incorporation of labelled protein into the adhered condensates. Differences were observed between the ability of Efg1 condensates and Efg1-Wor1 condensates to incorporate labelled protein (**Fig. 8c**), with Efg1-Wor1 incorporating label at a lower rate, which may indicate slower dynamics. Aging these condensates for 24 hours had little effect on the result. The addition of Ssn6-Tup1 also appeared to reduce the dynamics of Efg1 condensates by this method (**Fig. 8d**). Aging for 24 hours further slowed the apparent dynamics of the Efg1-Ssn6-Tup1 condensates (**Fig. 8d**), although little effect

was observed on aged condensates composed of Efg1 alone, similar to the observations for **Fig. 8c**. The results of this experiment indicate that both Wor1 and Ssn6-Tup1 are capable of altering the properties of Efg1 condensates.

Data summary

The experiments included in this chapter further investigated the phase separation of TFs involved in white-opaque switching in *C. albicans*, building upon our previous studies [7, 8]. We examined the behavior of full-length TFs in an exogenous nuclear model system, and found that full-length TFs often behaved differently than the PrLDs alone. Several TFs were dependent on the addition of a DNA-binding module to form condensates in this model. We also examined colocalization of Ssn6 with several TFs in the U2OS system, and found that Ssn6 does not seem to colocalize strongly with any of the TFs tested aside from Tup1 (**Chapter 2 Fig. 3**).

In *C. albicans*, we assayed complementation of Ssn6 with *S. cerevisiae* Ssn6, which seemed to partially complement Ssn6, but was not able to replace *C. albicans* Ssn6 as a regulator of white-opaque switching. Replacement of the Ssn6 CTD with IDRs from other TFs (FUS, TAF15, and CmWor1) was not able to restore the behavior of full-length Ssn6 in either the U2OS system or in *C. albicans*. Future studies may examine differences between IDRs in more detail. The Ssn6 CTD was also found to play a role in environmental sensing, affecting CO₂ sensing, response to N-acetyl glucosamine, and filamentation. The endogenous levels of white-opaque TFs in both white and opaque cell nuclei were determined. A variety of factors, including temperature, crowding agents, and the addition of other TFs such as Wor1 and Ssn6, were found to promote aging of Efg1 condensates *in vitro*.

While much of this data is preliminary, it may inform future experiments on white-opaque switching, the phase separation behavior of *C. albicans* TFs, and functional differences between TFs encoded in the amino acid sequences of their IDRs.

Discussion

The white-opaque network is fine-tuned to regulation of cell state in *Candida albicans*. While we have shown that several of these TFs are capable of phase separation [7], the interactions of the disordered domains of these proteins are specific [7, 8], and may be modulated by factors such as post-translational modifications or the stoichiometric balance of different TFs in the genome.

How does the transcriptional regulation of white-opaque switching actually work? It may be the case that spatial rearrangement of the genome takes place during cell type switching. If this were true, the white-activated genes may be physically rearranged into an active chromatin space in white cells, while the opaque-activated genes are sequestered into a repressive chromatin space. In opaque cells, opaque-specific genes would be moved into the active space, while white-specific genes would be sequestered into the inactive chromatin space. In mammalian cells, chromatin is reorganized during cell differentiation [25], and heterochromatin formation provides a mechanism for turning off genes during the differentiation process [26, 27]. The demixing of modified and unmodified chromatin into distinct condensates has been described [28], as well as compartmentalization of RNA-rich active genomic locations [29]. Heterochromatin formation has also been linked to phase separation [29-34]. These studies give credence to the idea that phase separation is involved in genomic organization, which may be the case in *C. albicans* as well.

Our *in vitro* data indicates that some of our key white-opaque TFs (Wor1 in **Fig. 8b-c**, Ssn6 in **Chapter 2 Figs. 4 and S3**) lack the dynamic behavior associated with transcriptionally active TFs in other systems [35, 36]. For this reason, it may be the case that some white-opaque TFs are actually involved in creating a condensate with reduced dynamics to sequester particular genes away from the transcriptional machinery and turn off their expression ('repressive sequestration'). Activity of a particular gene would then depend on the composition of TFs at that location [37].

Other studies have indicated that while phase separation may activate transcription in

some conditions [2, 35, 36], too much phase separation may actually reduce transcriptional activity [38]. If this were the case for white-opaque TFs, it may explain why many of these TFs display characteristics of both transcriptional activators and repressors [39]. Phase separation in this case may act as a negative self-regulator where up to a certain threshold, transcription is enabled, while beyond the threshold, transcription becomes unfavorable due to size or reduced dynamics of the condensate. This proposed negative self-regulation may be beneficial for the cell, as an uncontrolled positive feedback loop that produces a PrLD-containing TF such as Wor1 may lead to impaired cellular function as the nucleus becomes filled with this protein.

Another model has been proposed where nascent RNA stimulates transcriptional condensate formation, while the accumulation of larger amounts of RNA during transcriptional elongation leads to the dissolution of the condensate [40]. In this model, positively charged TFs interact with negatively charged RNA to form a complex coacervate, but as RNA concentration increases, the charge balance no longer supports coacervation. RNA could play a similar role in regulating transcriptional condensate in *C. albicans*. Likewise, the Ssn6 CTD also contains a large proportion of acidic residues (**Chapter 2. Fig. S2a**), which may be responsible for interaction with other white-opaque TFs that have a high proportion of basic residues. A high proportion of these acidic residues may exhibit a similar function to RNA, where above a certain threshold condensation is no longer supported. This hypothesis could potentially explain the observations in **Chapter 2 Fig. 4**, where Ssn6 seems to shrink Efg1 and Flo8 condensates *in vitro*.

Hernday et al. indicates that Ssn6 is involved in repression of the opaque transcriptional program in white cells [12]. If the idea of repressive sequestration is correct, Ssn6 may be recruiting these loci to transcriptionally inactive gel-like condensates. Further reduction of condensate dynamics, such as is the case for deletion of the Ssn6 CTD (**Chapter 2 Figs. 2 and 4**), would then lead to an inability for these opaque loci to escape negative regulation, and a predisposition to the white cell type, as is also seen in Ssn6 Δ C strains (**Chapter 2 Fig. 1**).

Furthermore, in the related yeast *S. cerevisiae*, Ssn6 interacts with histone modifying

complexes. Ssn6-Tup1 and the Swi-Snf complex have been shown to act antagonistically in *S. cerevisiae*, with Ssn6-Tup1 promoting repressive chromatin structures, while Swi-Snf promotes active chromatin structures [41]. Ssn6 interacts directly with the histone deacetylases Rpd3, Hos1, and Hos2 [42, 43], while Tup1 interacts with deacetylated H3 and H4 histone tails [42]. Ssn6 and Tup1 both promote repressive nucleosome positioning [44].

Interestingly, Hos2 is part of the Set3 complex in yeast [45, 46]. The SET/MLL family of histone methyltransferases is involved in hematopoietic cell differentiation and cancer [47, 48]. The human homolog of Ssn6, UTX, interacts directly with MLL4 via the UTX TPR domain [49]. In embryonic stem cells, UTX has been shown to recruit both MLL4 and p300 to facilitate conversion of inactive enhancers to active ones via co-occurrent deposition of H3K4me1 and H3K27ac modifications [50]. Additionally, the Set3-Hos2 histone deacetylase complex acts as a negative regulator of filamentation in *C. albicans* [46]. Interaction of Ssn6 with this complex may represent a mechanism whereby Ssn6 contributes to the regulation of filamentation (**Chapter 2 Fig. 1b** and **Chapter 3 Fig. 6g**). These studies suggest that Ssn6 and Tup1 are involved in epigenetic modification, and present another mechanism where recruitment of interacting proteins may change the local genomic landscape from repressive to transcriptionally active.

The work in this thesis contributes to the understanding of phase separation in regulating cell type, which is relevant to the study of development as well as oncogenesis. Understanding how different proteins impact the properties of phase-separated condensates is important for developing treatments that can target these properties to affect the function of the condensate, potentially promoting healthy development or preventing cancer cell proliferation. The development of peptides or small molecules to alter the properties of these condensates represents a future avenue of therapeutic research, with a wide range of potential applications from antimicrobials to treatments for cancer and neurodegeneration.

Materials and Methods

Plasmid construction. Specific details about all fragments and restriction enzymes used can be found in Supplementary Table 1. Sequences for oligos used in this study are listed in Supplementary Table 2. Plasmid sequences were confirmed by sequencing the full plasmids with Plasmidsaurus.

To construct plasmids for mammalian cell expression, synthetic codon-optimized sequences were used. Inserts from this sequence were amplified and cloned into the eYFP-LacI vector [2]. FUS, TAF15, and Sp1 coding sequences were a gift from the Tjian lab [2].

To construct plasmids used in *C. albicans* strains, *C. albicans* Ssn6 sequences were amplified from SC5314 genomic DNA. *S. cerevisiae* Ssn6 was amplified from *S. cerevisiae* genomic DNA. Inserts were assembled either by fusion PCR or by Golden Gate cloning. Fusion PCR involved the joining of two non-contiguous sequences. This is carried out by amplifying fragments with an overlap region added to one product using oligos. In a subsequent PCR, these products are fused together using oligos at the extreme ends of the fusion product. Sequences were then inserted into pSFS2a [51] using restriction enzymes. A far 3' sequence was also inserted into the 3' end of all pSFS2a-Ssn6 cassettes. Some inserts were designed for Golden Gate Assembly. Oligos for Golden Gate were designed using the New England Biolabs Golden Gate Assembly Tool. These inserts were assembled into a modified pSFS2a containing a BsaI landing pad (pSFS2a-BsaI). pSFS2a-BsaI was generated by digesting oligos 6048 and 6049 and ligating them into pSFS2a.

To construct plasmids for *E. coli* expression, synthetic codon-optimized sequences were used. Synthetic sequences were inserted initially into the MBP vector (RB523, pRP1B-MBP/THMT) via subcloning.

Mammalian cell culture, live-cell imaging and LacO array analysis. U2OS cells containing a LacO array (~50,000 LacO elements) were a gift from the Tjian Lab [2]. U2OS cells were cultured,

transfected with plasmids, and imaged as described previously [7]. LacI focus area size and intensity was measured by masking the YFP signal and analyzing particles in FIJI. Background fluorescence intensity was corrected for by subtracting YFP signal outside the array spot in the cell nucleus. To quantify mCherry enrichment at the LacO array bound by eYFP-LacI constructs, we followed a method similar to that employed by Chong et al [2]. The array spot was masked in the eYFP channel, then signal was measured in the mCherry channel. Two locations near the array in the mCherry channel were then measured and averaged to represent average background signal in the nucleus. The mCherry enrichment was calculated by dividing the array mCherry signal by the background signal.

Candida strain construction. Details about strain construction have been included in Supplementary Table 3. Oligos used for strain construction and integration checks are listed in Supplementary Table 2.

Wild type strains used were CAY6342 (white) and CAY6343 (opaque), which are equivalent to AHY135 and AHY136 that have been previously described [52]. The *ssn6/ssn6* strain used (TF121) has been previously described [53]. The *ssn6/Ssn6* strain used was CAY8083, which has been previously described as AHY337 [12]. These strains were gifted by the Johnson lab at UCSF.

Constructed pSFS2a-Ssn6 plasmids were digested with restriction enzymes, then transformed into TF121. Integration was checked by PCR in the forward and reverse directions. Nourseothricin-resistance (NAT) cassettes were flipped out by incubating on SCM media, then checking for NAT susceptibility.

TF-Neon strains were constructed by amplifying an mNeon cassette from RB895 using long oligos. These long oligos contained 75 bp homology to the destination site and 25 bp homology to the mNeon cassette sequence. The PCR product was transformed into relevant strains, then NAT cassettes were flipped out on SCM.

White-opaque switching assays. Cells of a single phenotypic state were taken from YPD plates and resuspended in PBS. Cells per milliliter were estimated using optical density with $1 \text{ OD}_{600} = 2 \times 10^7$ cells/ml. Suspensions were serially diluted in phosphate-buffered saline (PBS) to 2×10^3 cells/ml. Approximately 100 cells were spread plated in triplicate on synthetic complete dextrose (SCD) media. Plates were incubated at 22 °C for 7 days. Colonies were then counted and scored for opposite phenotype colonies or sectors.

Candida cell imaging. Cells were taken from plates and suspended in PBS, then imaged using a Zeiss Axio Observer Z1 inverted fluorescence microscope for fluorescence and DIC imaging equipped with Zen software (Zen v.3.0 blue edition). For Hoescht staining, cells were pre-incubated for 1 hour with 1 mg/ml of Hoechst 33258, then pelleted and resuspended in fresh PBS before imaging.

RNA sequencing and analysis. *C. albicans* cells in the white cell state were taken from YPD plates. These cells were inoculated into 15mL SCD and incubated for 18 hours in a rotator at 24 °C. The initial timepoint was harvested immediately, and cells were spread-plated onto 4-8 plates of either SCD or Lee's NAG media. SCD plates were incubated at 25 °C with 20% CO₂, while Lee's NAG plates were incubated at 25 °C. Samples were collected by scraping at 6 hours, 24 hours, and 72 hours. Cells per milliliter were estimated using optical density with $1 \text{ OD}_{600} = 2 \times 10^7$ cells/ml. A volume equivalent to 10 OD_{600} of cells was harvested by pelleting, then snap-frozen with liquid nitrogen and stored at -80 °C. RNA extraction was conducted using the RiboPure Yeast Kit (Invitrogen) according to the manufacturer's instructions. Library preparation was conducted using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) according to the manufacturer's instructions, with unique i7 barcodes applied for each sample. Library concentrations were measured using a Qubit Fluorometer (Invitrogen) and then multiplexed. The final pooled library was sequenced on an Illumina NextSeq as SR85 and de-

multiplexed by the UC Davis Genome Center.

Fasta reads were trimmed with BBDuk, then aligned using STAR aligner. Assembly 21 chromosome FASTA files and GFF index file were downloaded from the Candida Genome Database. We noted that many reads were not captured because the annotated UTRs stopped before the mapped reads, so the GFF file was edited to extend the 3' sections by Deepika Gunasekaran. Reads were normalized in R using the edgeR package, and differential gene expression analysis was performed.

Protein purification. Proteins were purified as described previously [7].

Phase separation assays. Protein stocks were thawed at room temperature and diluted in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, then concentrated in centrifugal filter units (Amicon Ultra—0.5-ml centrifugal filter units) to 100 μ l. Protein concentration was measured using a Nanodrop 2000c (Thermo Fisher Scientific). Proteins were then diluted in 10 mM Tris-HCl buffer with 150 mM NaCl to the desired concentrations. Protein reactions with TEV were set up in 10- μ l total volumes (9.5 μ l protein with 0.5 μ l of 0.3 mg/ml TEV) and incubated for 30 min at room temperature. Proteins were cleaved with TEV prior to addition of PEG. Labelling of proteins with DyLight or AlexaFluor fluorescent dyes (Thermo Fisher Scientific) was carried out according to the manufacturer's instructions. Labelled proteins were added to assays at concentrations below .5 μ M before TEV incubation. Proteins were imaged immediately after incubation on chamber slides (SPI Supplies, 10-chamber slides), with .8 μ l solution per chamber, sealed with a glass coverslip. All images were acquired at $\times 63$ initial magnification + $\times 1.6$ Optovar on a Zeiss Axio Observer Z1 inverted fluorescence microscope. Post-imaging processing was carried out in FIJI (ImageJ).

Figures

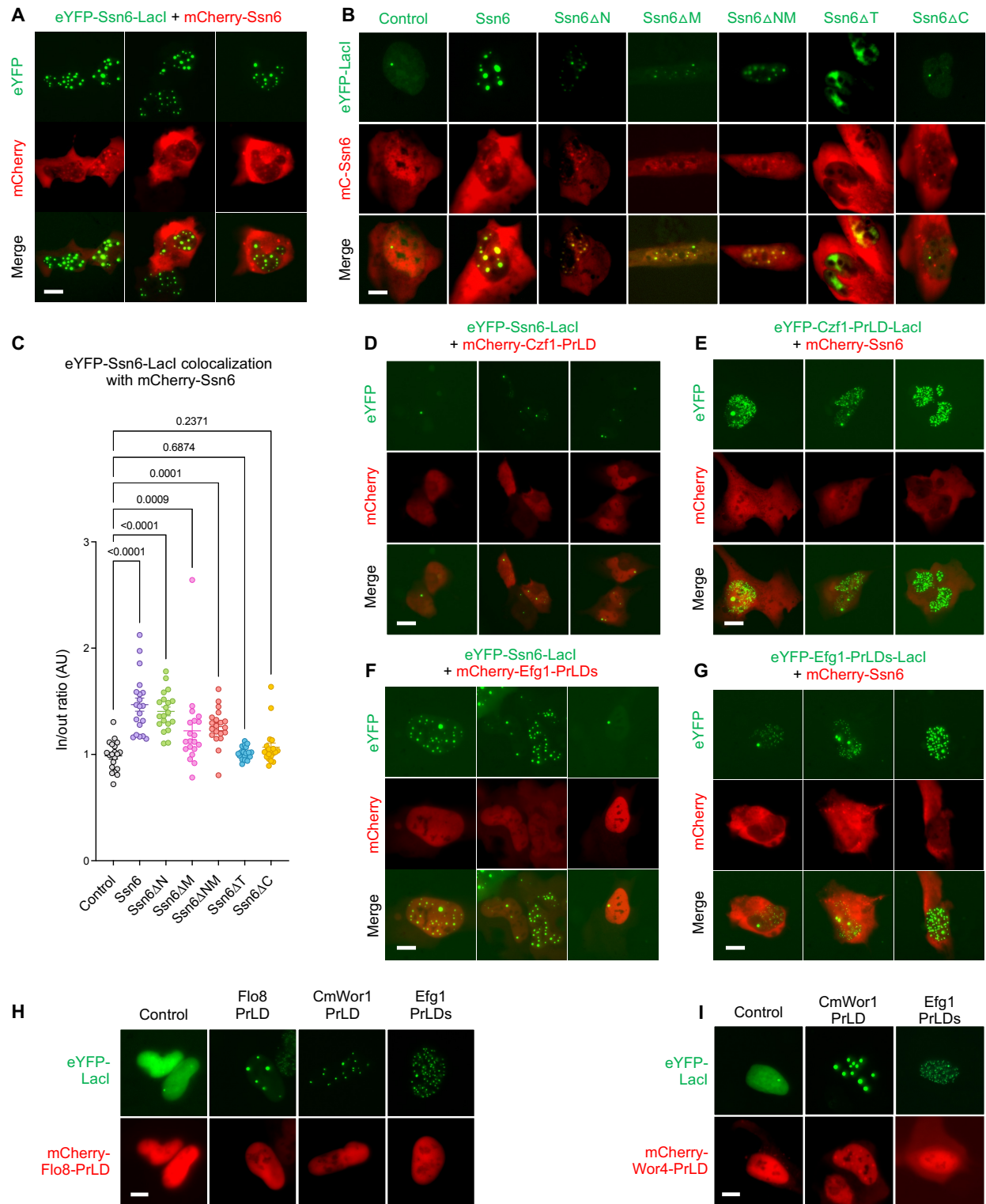


Figure 1: Colocalization of Ssn6 with itself and the PrLDs of other TFs in U2OS cells. **A.** eYFP-Ssn6-LacI co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **B.** eYFP-Ssn6-LacI domain deletion constructs co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **C.** Quantification of eYFP and mCherry colocalization in B. Ssn6 puncta masked in eYFP channel, then intensity was measured in mCherry channel. mCherry signal is divided by background nuclear signal in mCherry channel. Statistics shown are all p-values for Student's t-test vs. eYFP-LacI control. **D.** eYFP-Ssn6-LacI co-transfected in U2OS-LacO cells with mCherry-Czf1-PrLD. Scale 10µm. **E.** eYFP-Czf1-PrLD-LacI co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **F.** eYFP-Ssn6-LacI co-transfected in U2OS-LacO cells with mCherry-Efg1-PrLDs. Scale 10µm. **G.** eYFP-Efg1-PrLDs-LacI co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **H.** mCherry-Flo8-PrLD co-transfected in U2OS-LacO cells with various eYFP-LacI constructs. Scale 10µm. **I.** mCherry-Wor4-PrLD co-transfected in U2OS-LacO cells with various eYFP-LacI constructs. Scale 10µm.

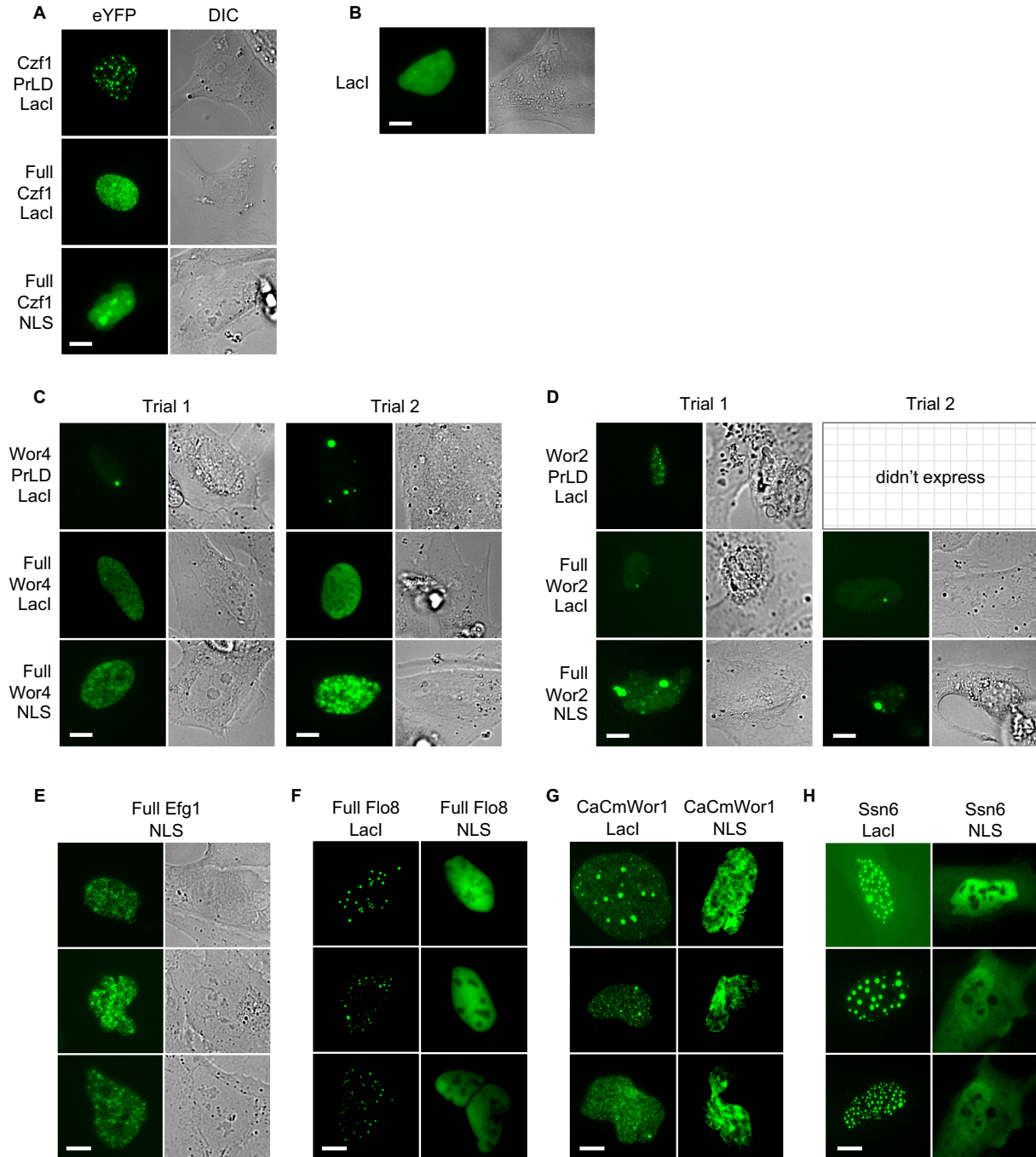


Figure 2: Expression of full-length TFs in U2OS cells. **A.** Expression of eYFP-Czf1-PrLD-LacI, eYFP-Czf1-LacI, and eYFP-Czf1-NLS in U2OS-LacO cells. Scale 10μm. **B.** Expression of the eYFP-LacI empty vector control in U2OS-LacO cells. Scale 10μm. **C.** Expression of eYFP-Wor4-PrLD-LacI, eYFP-Wor4-LacI, and eYFP-Wor4-NLS in U2OS-LacO cells. Scale 10μm. **D.** Expression of eYFP-Wor2-PrLD-LacI, eYFP-Wor2-LacI, and eYFP-Wor2-NLS in U2OS-LacO cells. Scale 10μm. **E.** Expression of eYFP-Efg1-NLS in U2OS-LacO cells. Scale 10μm. **F.** Expression of eYFP-Flo8-LacI and eYFP-Flo8-NLS in U2OS-LacO cells. Scale 10μm. **G.** Expression of eYFP-CaCmWor1-LacI and eYFP-CaCmWor1-NLS in U2OS-LacO cells. CaCmWor1 is a fusion TF composed of the DNA-binding domain of *C. albicans* Wor1 and the PrLD of *C. maltosa* Wor1. Scale 10μm. **H.** Expression of eYFP-Ssn6-LacI and eYFP-Ssn6-NLS in U2OS-LacO cells. Scale 10μm.

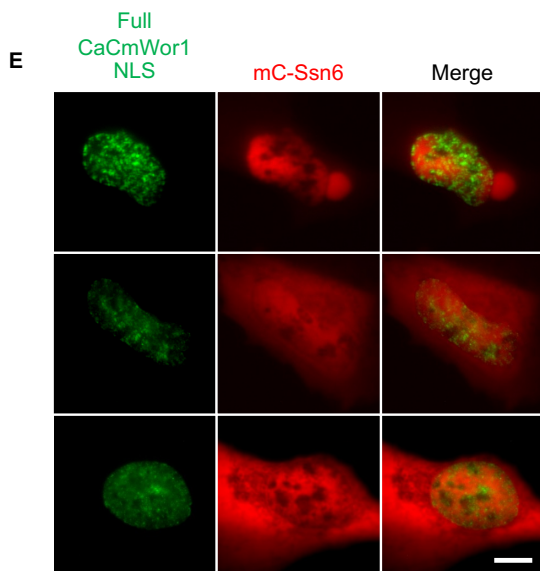
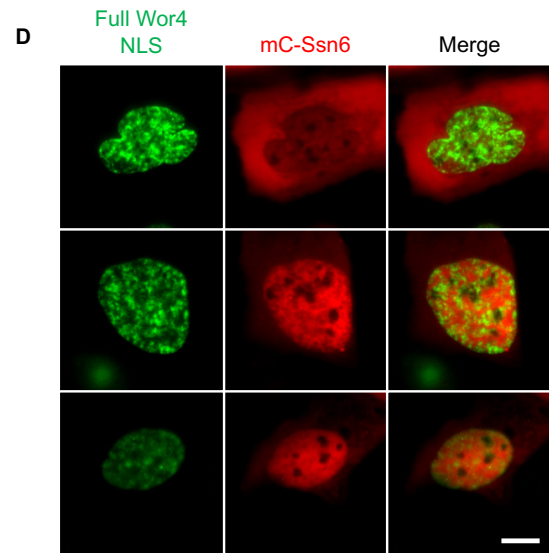
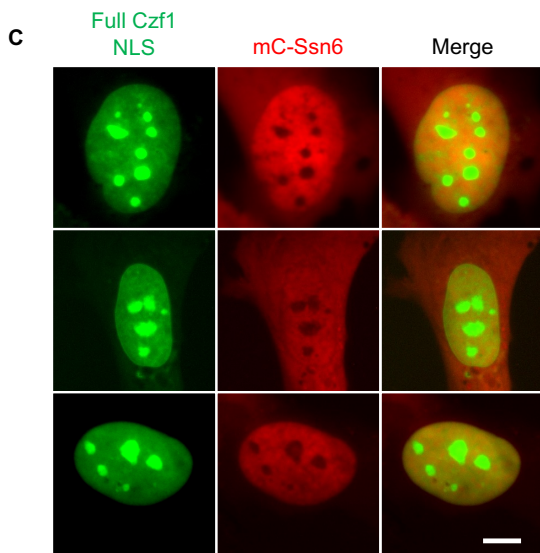
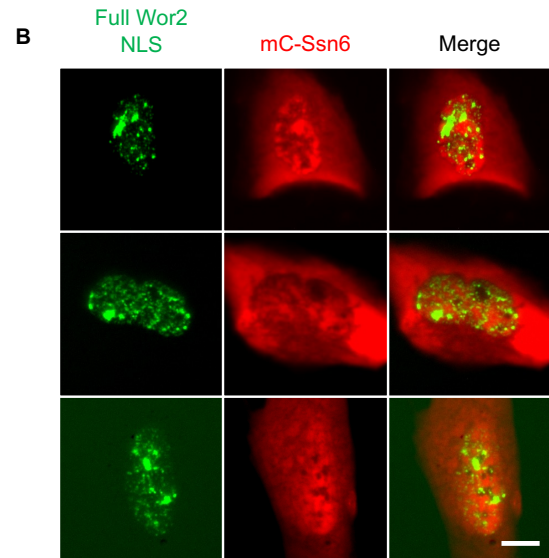
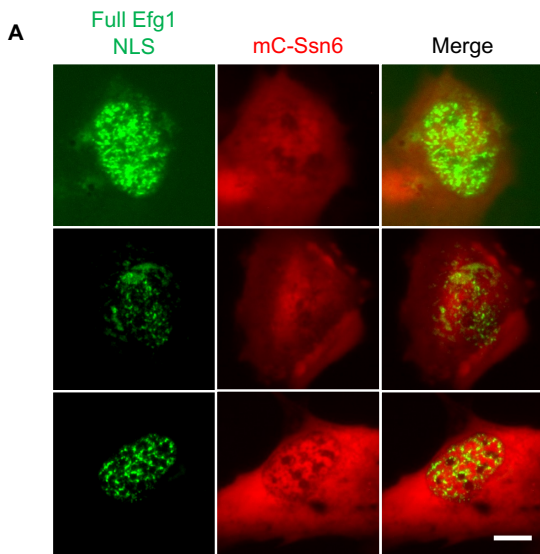


Figure 3: Ssn6 colocalization with full-length TFs in U2OS-LacO cells. **A.** eYFP-Efg1-NLS co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **B.** eYFP-Wor2-NLS co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **C.** eYFP-Czf1-NLS co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **D.** eYFP-Wor4-NLS co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm. **E.** eYFP-CaCmWor1-NLS co-transfected in U2OS-LacO cells with mCherry-Ssn6. Scale 10µm.

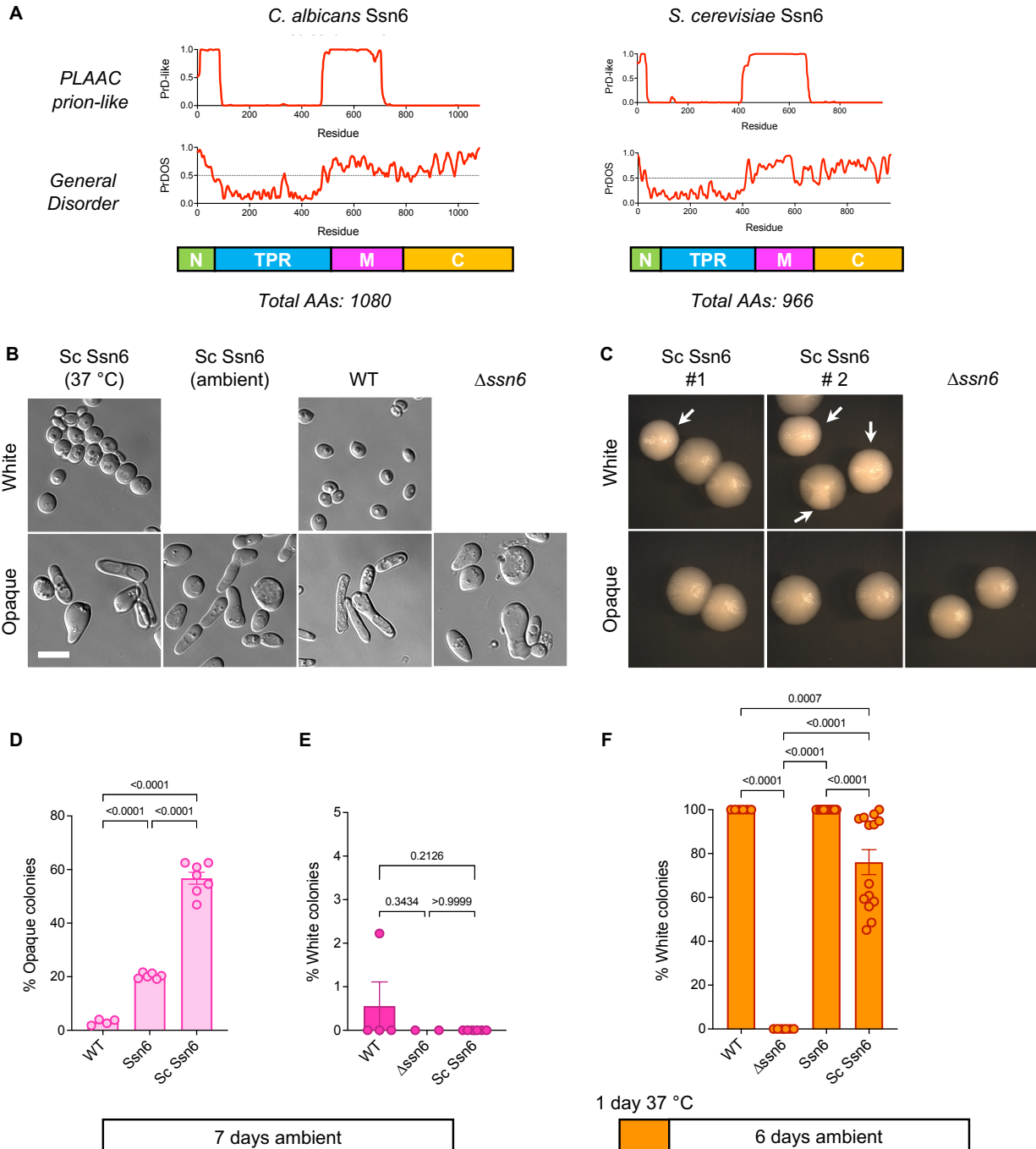


Figure 4: Ssn6 complementation with *S. cerevisiae* Ssn6. **A.** Plots of prion-like character and general disorder for *C. albicans* Ssn6 and *S. cerevisiae* Ssn6. Bottom row shows approximate alignment to Ssn6 domains: N-terminal prion-like domain (N), tetratricopeptide repeat (T), medial prion-like domain (M), and disordered C-terminus (C). **B.** DIC images of *C. albicans* cells. Scale 10 μ m. **C.** Images of *C. albicans* colonies expressing ScSsn6. Comparison to Δ ssn6 colonies is shown. **D.** Frequency of white ScSsn6 cells switching to opaque under ambient conditions. Statistics shown are p-values from Student's t-test for all comparisons. **E.** Frequency of opaque ScSsn6 cells switching to white under ambient conditions. Statistics shown are p-values from Student's t-test for all comparisons. **F.** Frequency of white ScSsn6 cells switching to opaque after 1 day at 37 °C followed by 6 days of ambient conditions. Statistics shown are p-values from Student's t-test for all comparisons.

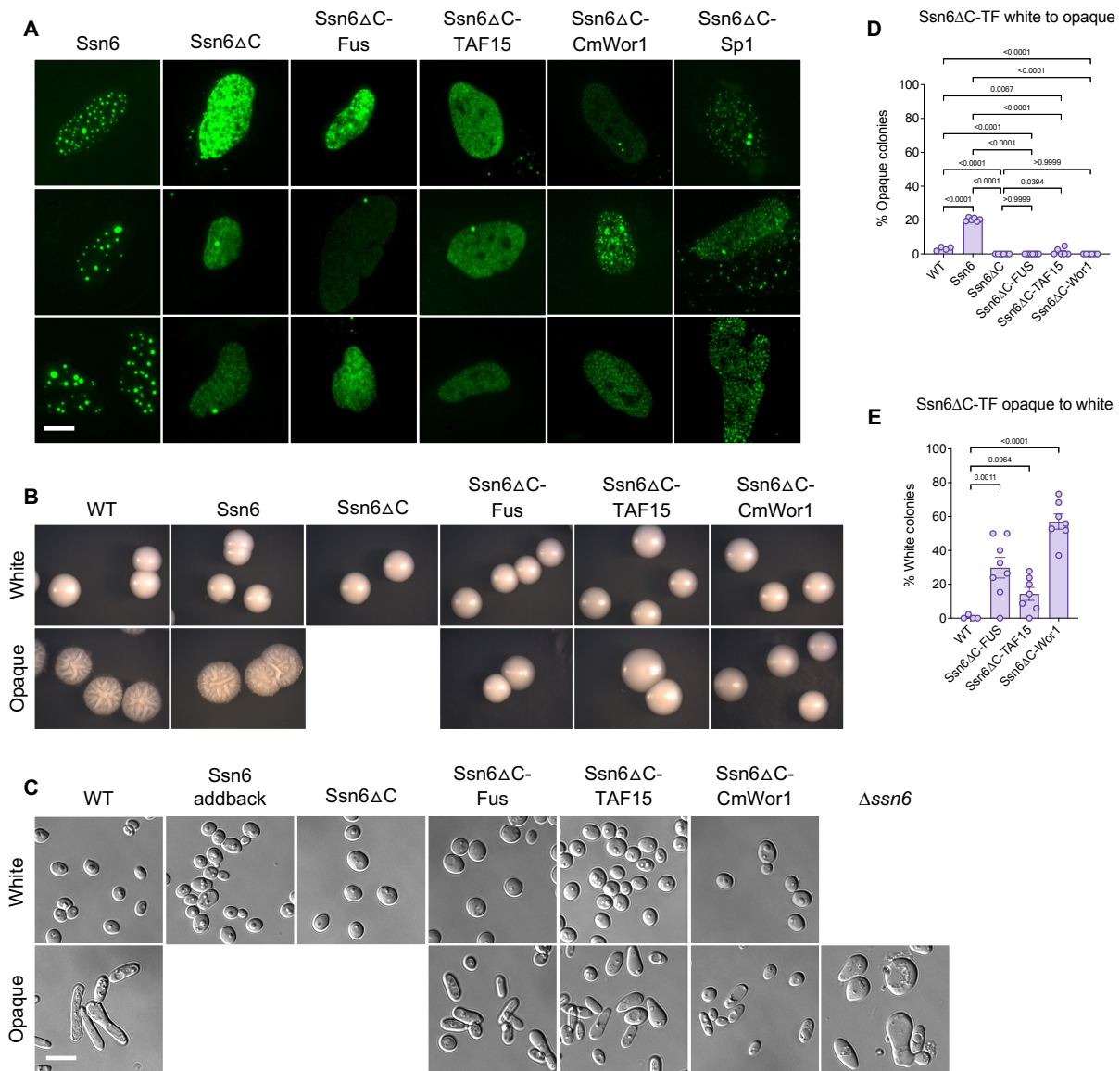
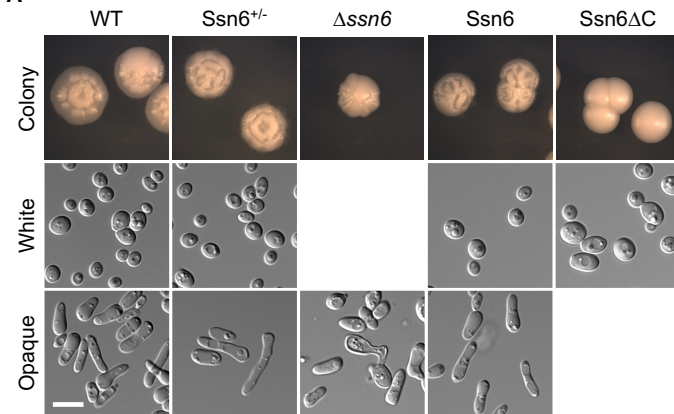
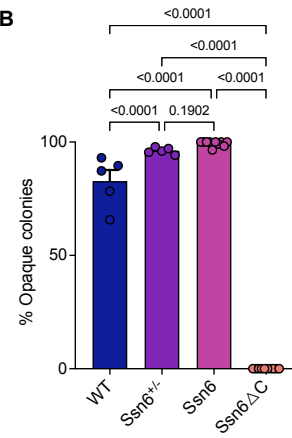


Figure 5: Ssn6 Δ C fusions with Fus, TAF15, CmWor1, and Sp1 IDRs in U2OS-LacO cells and *C. albicans*. **A.** Expression of eYFP-Ssn6-LacI constructs in U2OS-LacO cells. Scale 10 μ m. **B.** Colony morphology of *C. albicans* cells expressing Ssn6 Δ C-TF fusions. Top row shows white colonies; bottom row shows opaque colonies. **C.** DIC images of *C. albicans* cells expressing Ssn6 Δ C-TF fusions. Top row shows white cells; bottom row shows opaque cells. Scale 10 μ m. **D.** Frequency of white cells switching to opaque. Statistics shown are p-values from Student's t-test for comparisons against WT, Ssn6, and Ssn6 Δ C. **E.** Frequency of opaque cells switching to white. Statistics shown are p-values from Student's t-test for comparisons against WT.

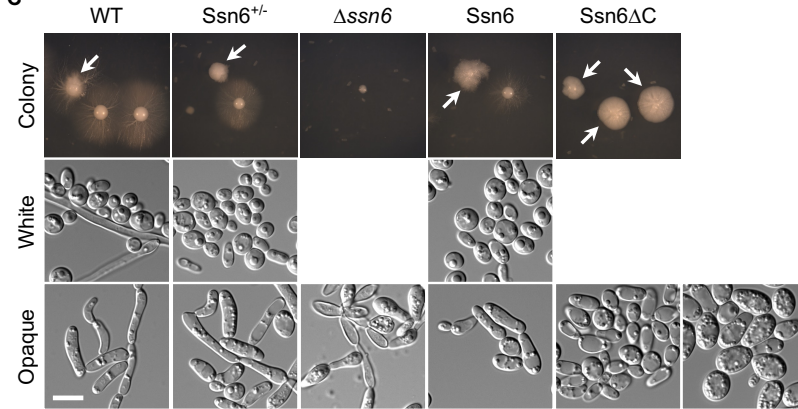
A 7 days 25 °C SCD 20% CO₂



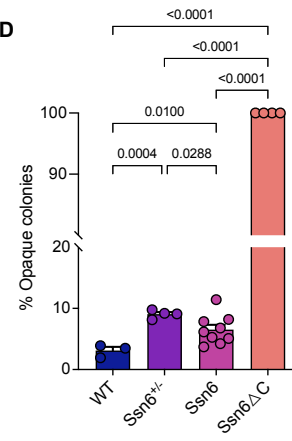
B



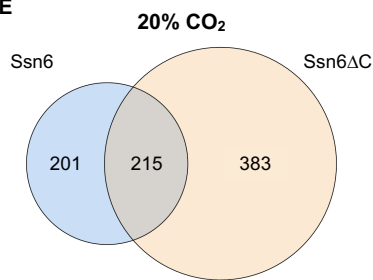
C 7 days 25 °C Lee's NAG



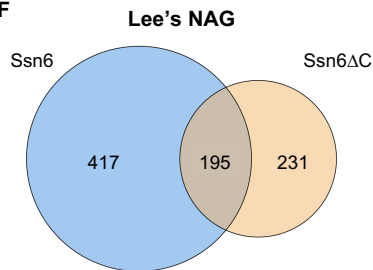
D



E



F



G

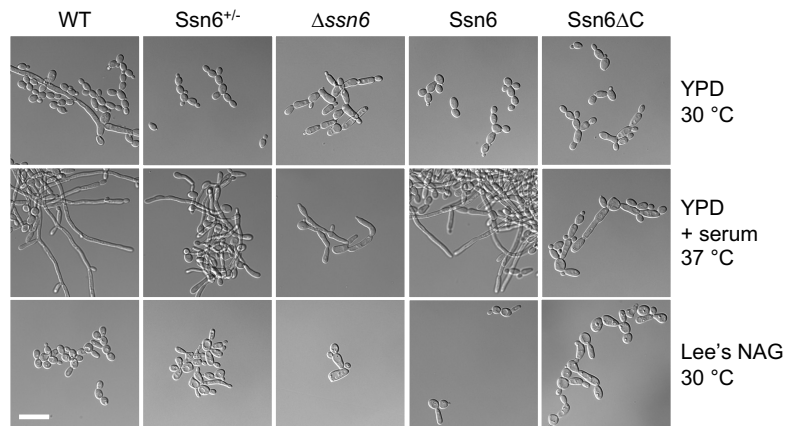


Figure 6: Ssn6 CTD affects *C. albicans* response to environmental stimuli. **A.** Images of colonies and cells after white-to-opaque switching assay on SCD with 20% CO₂ at 25 °C. Scale for cell images = 10µm. **B.** Switching assay data for A. Statistics are p-values for Student's t-test, all comparisons shown. **C.** Images of colonies and cells after white-to-opaque switching assay on Lee's NAG at 25 °C. Scale for cell images = 10µm. Arrows indicate "cloud-like" opaque colony morphology. **D.** Switching assay data for C. Statistics are p-values for Student's t-test, all comparisons shown. **E.** Preliminary RNA-seq data showing overlap in differentially expressed genes between Ssn6 and Ssn6ΔC on SCD plates with 20% CO₂ at 25 °C (differential expression performed versus the same strain at the time of inoculation). **F.** Preliminary RNA-seq data showing overlap in differentially expressed genes between Ssn6 and Ssn6ΔC on Lee's NAG plates at 25 °C (differential expression performed versus the same strain at the time of inoculation). **G.** DIC images from filamentation assay, incubated for 4 hours in liquid media in rotator. Scale = 20µm.

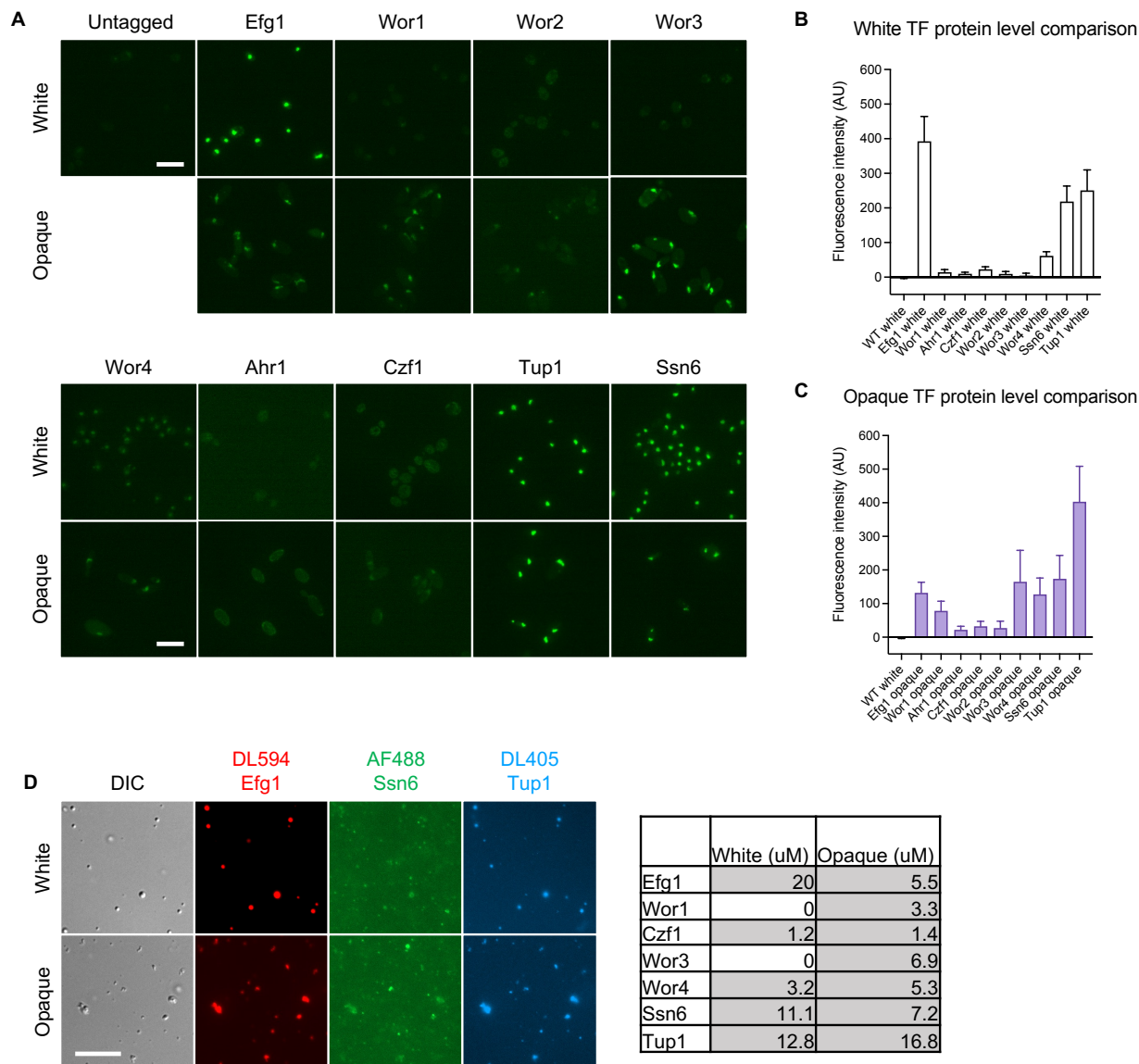


Figure 7: Levels of white-opaque TFs in *C. albicans* cells and *in vitro* droplet reconstitution. A. Images of *C. albicans* cells expressing TFs C-terminally tagged with mNeon. Scale 10μm. **B.** Average fluorescence intensity of mNeon-tagged TFs in white cells, normalized to untagged cells. **C.** Average fluorescence intensity of mNeon-tagged TFs in opaque cells, normalized to untagged cells. **D.** *In vitro* condensates composed of relative endogenous nuclear ratios of TFs as determined in B and C. Final TF concentrations used are shown in the table. 150mM NaCl 10mM Tris pH 7.5. Scale 10μm.

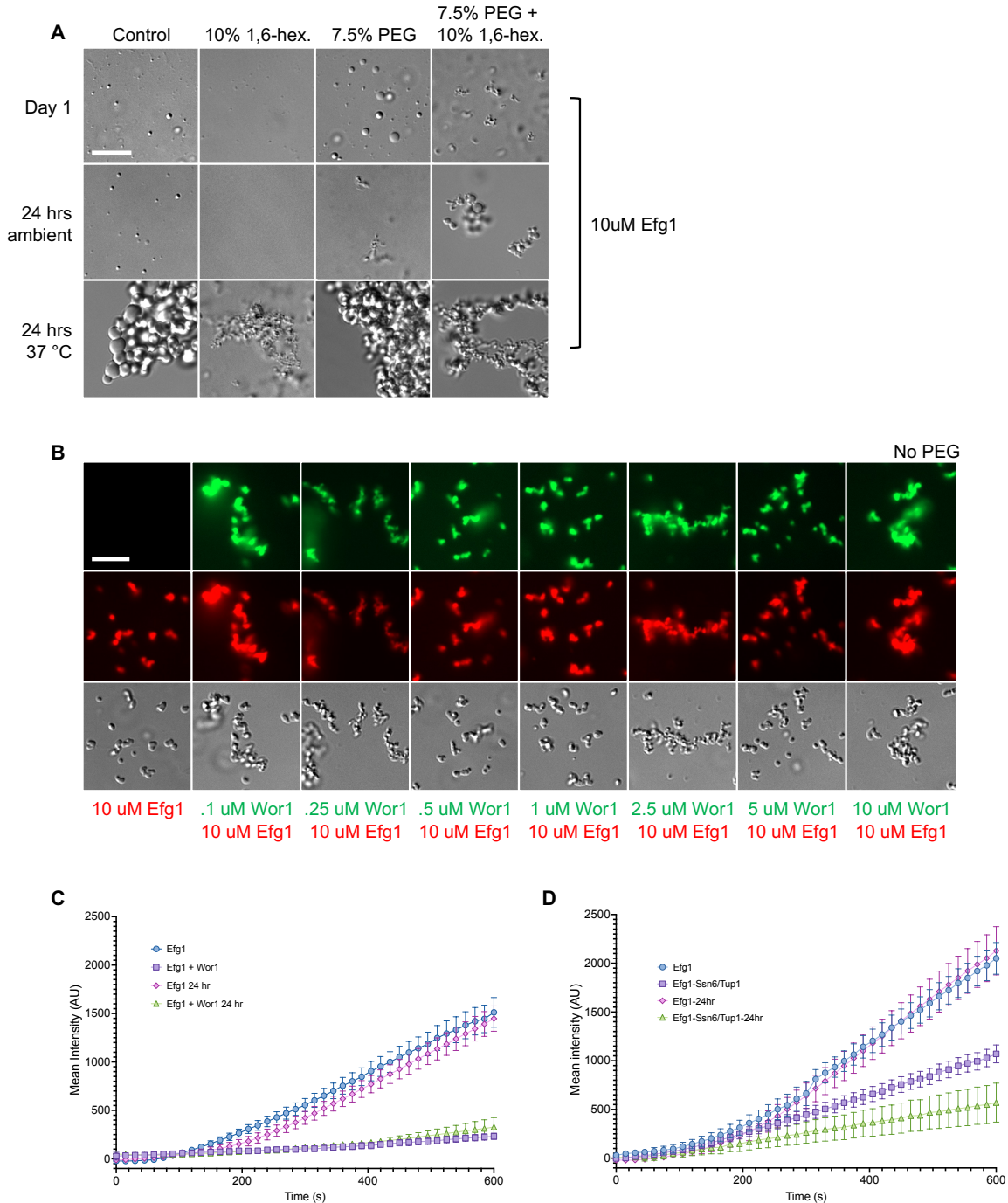


Figure 8: Efg1 droplet dynamics are affected by time, temperature, and addition of other TFs. A. 10 μ M MBP-Efg1 TEV cleaved +/- 7.5% PEG +/- 10% 1,6-hexanediol. TEV cleaved at 20 μ M, then diluted 1:1 with relevant buffer. 150mM NaCl 10mM Tris pH 7.4. Scale = 10 μ m. **B.** 10 μ M Efg1 with 0-10 μ M CaCmWor1. 150mM NaCl 10mM Tris pH 7.4, no PEG. Scale = 10 μ m. **C.** Incorporation of .25 μ M AF488 Efg1 into 15uM Efg1 +/- .25 μ M CaCmWor1 at time zero or 24 hours. n = 3. **D.** Incorporation of .25 μ M AF488 Efg1 into 15uM Efg1 +/- .25 μ M Ssn6/Tup1 at time zero or 24 hours. n = 3.

Tables

Supplementary Table 1: Plasmids used in this study

Plasmid	Name	Method	Starting Vector	Insert	Template	Oligos	Restriction enzymes
pSFS2a	pSFS2a	Reuss et al. 2002					
RB523	MBP vector, pRP1B-MBP/THMT	Frazer et al., 2020, gift from Nick Fawzi					
RB1207	mCherry vector	Chong et al., 2018, gift from Tjian lab					
RB1208	eYFP-LacI vector	Chong et al., 2018, gift from Tjian lab					
RB1397	pSFS2a-BsaI	Oligo insertion	pSFS2a	Oligos 6048 & 6049			Apal, XhoI
RB895	pSFS2a-mNeon vector	Subcloning	pSFS2a	mNeon	RB880 (synthesized)	n/a	insert: XhoI; vector: XhoI, CIP
RB1277	pSFS2a-Ssn6	Cloning	pSFS2a	5'-Ssn6-3'	SC5314 gDNA	5607, 5608	Apal, XhoI
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1462	eYFP-Ssn6-LacI	Cloning	RB1208	Ssn6	RB1170	6038, 6107	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB1464	eYFP-Ssn6ΔN-LacI	Cloning	RB1208	Ssn6ΔN	RB1249	6039, 6107	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB1466	eYFP-Ssn6ΔM-LacI	Cloning	RB1208	Ssn6ΔM	RB1251	6038, 6107	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB1468	eYFP-Ssn6ΔNM-LacI	Cloning	RB1208	Ssn6ΔNM	RB1253	6039, 6107	insert: BsrGI-HF & XmaI; vector: BsrGI-HF & BspEI
RB1956	eYFP-Ssn6ΔT-LacI	Cloning	RB1208	Ssn6ΔT	RB1734 (Twist)	6038, 6107	insert: BsrGI-HF & XmaI;

					Biosciences)		vector: BsrGI-HF & BspEI
RB1957	eYFP-Ssn6 Δ C-LacI	Cloning	RB1208	Ssn6 Δ C	RB1170	6038, 6106	BsrGI-HF, BspEI
RB1470	mCherry-Ssn6	Cloning	RB1207	Ssn6	RB1170	6041, 6106	BsrGI-HF, XhoI
RB1216	eYFP-Czf1-PrLD-LacI	Cloning	RB1208	Czf1 PrLD	RB516	5575, 5576	BsrGI-HF, BspEI
RB1222	eYFP-Efg1-PrLDs-LacI	Fusion PCR	RB1208	Efg1 N PrLD	RB514	5578, 5579	
				eYFP	RB1208	5580, 5581	
				Efg1 C PrLD	RB514	5582, 5583	
				N-eYFP-C	Efg1 N PrLD, eYFP, Efg1 C PrLD	5578, 5583	NheI, BspEI
RB1262	eYFP-Flo8-PrLD-LacI	Cloning	RB1208	Flo8 PrLD	RB960	5680, 5681	BsrGI-HF, BspEI
RB1410	eYFP-CmWor1-PrLD-LacI	Cloning	RB1208	CmWor1 PrLD	RB838	6117, 6118	BsrGI-HF, BspEI
RB2276	eYFP-Wor2-PrLD-LacI	Cloning	RB1208	Wor2 PrLD	RB513	6263, 6264	BsrGI-HF, BspEI
RB1218	mCherry-Czf1-PrLD	Cloning	RB1207	Czf1 PrLD	RB516	5575, 5577	BsaI-HF-v2
RB1224	mCherry-Efg1-PrLDs	Fusion PCR	RB1207	Efg1 N PrLD	RB514	5578, 5579	
				mCherry	RB1207	5580, 5581	
				Efg1 C PrLD	RB514	5582, 5584	
				N-mCherry-C	Efg1 N PrLD, mCherry, Efg1 C PrLD	5578, 5584	NheI, BspEI
RB1264	mCherry-Flo8-PrLD	Cloning	RB1207	Flo8 PrLD	RB960	5680, 5682	BsrGI-HF, BspEI
RB1268	mCherry-Wor4-PrLD	Fusion PCR	RB1207	Wor4 N PrLD	RB549	5671, 5672	
				mCherry	RB1207	5677, 5678	
				Wor4 C PrLD	RB549	5675, 5679	
				N-mCherry-C	Wor4 N PrLD, mCherry, Wor4 C PrLD	5671, 5679	NheI, BspEI

RB2277	eYFP-Full-Czf1-LacI	Cloning	RB1208	Czf1	RB516	5575, 6563	BsrGI-HF, BspEI
RB2279	eYFP-Full-Wor4-LacI	Cloning	RB1208	Wor4	RB549	6561, 5676	BsrGI-HF, BspEI
RB2278	eYFP-Full-Wor2-LacI	Cloning	RB1208	Wor2	RB572	6555, 6556	BsrGI-HF, BspEI
RB2280	eYFP-Full-Flo8-LacI	Cloning	RB1208	Flo8	RB960	7001, 7002	BsrGI-HF, BspEI
1L3, not in database	eYFP-Full-CaCmWor1-LacI	Cloning	RB1208	CaCmW or1	RB838	6552, 7000	insert: BsrGI-HF & AgeI; vector: BsrGI-HF & BspEI
RB2282	eYFP-Full-Czf1-NLS	Cloning	RB1208	Czf1	RB516	5575, 6564	BsrGI-HF, XmaI
RB2284	eYFP-Full-Wor4-NLS	Cloning	RB1208	Wor4	RB549	6561, 6562	BsrGI-HF, XmaI
RB2283	eYFP-Full-Wor2-NLS	Cloning	RB1208	Wor2	RB572	6555, 6556	BsrGI-HF, XmaI
RB2285	eYFP-Full-Flo8-NLS	Cloning	RB1208	Flo8	RB960	7001, 7003	BsrGI-HF, XmaI
1N1, not in database	eYFP-Full-CaCmWor1-NLS	Cloning	RB1208	CaCmW or1	RB838	6552, 6981	BsrGI-HF, KpnI
RB2286	eYFP-Full-Efg1-NLS	Cloning	RB1208	Efg1	RB514	6567, 6568	BsrGI-HF, XmaI
RB2287	eYFP-Ssn6-NLS	Cloning	RB1208	Ssn6	RB1170	6038, 6106	insert: BsrGI-HF & XhoI; vector: BsrGI-HF & SalI-HF
RB1966	pSFS2a-Sc-Ssn6	Golden Gate, then subcloning	pGGAselect, then pSFS2a	Ca Ssn6 5'	RB1277	7788, 7789	BsmBI-HF-v2, then Apal & XhoI
				Sc Ssn6 N	S. cerevisiae gDNA	7790, 7791	BsmBI-HF-v2, then Apal & XhoI
				Sc Ssn6 C	S. cerevisiae gDNA	7792, 7793	BsmBI-HF-v2, then Apal & XhoI
				Ca Ssn6 5'	RB1277	7794, 7795	BsmBI-HF-v2, then Apal & XhoI
		Cloning	pSFS2a	Ca Ssn6 far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1970	eYFP-Ssn6ΔC-Fus-IDR-LacI	Golden Gate, then subcloning	pGGAselect, then RB1208	Ssn6ΔC	RB1170	7725, 7726	BsmBI-HF-v2, then BsrGI-HF & BspEI
				Fus IDR	RB1209	7727, 7728	BsmBI-HF-v2, then BsrGI-HF & BspEI

RB1965	eYFP-Ssn6 Δ C-TAF15-IDR-LacI	Golden Gate, then subcloning	pGGAselect, then RB1208	Ssn6 Δ C	RB1170	7725, 7735	BsmBI-HF-v2, then BsrGI-HF & BspEI
				TAF15 IDR	RB1210	7736, 7737	BsmBI-HF-v2, then BsrGI-HF & BspEI
RB1971	eYFP-Ssn6 Δ C-CmWor1-PrLD-LacI	Golden Gate, then subcloning	pGGAselect, then RB1208	Ssn6 Δ C	RB1170	7725, 7729	BsmBI-HF-v2, then BsrGI-HF & BspEI
				CmWor1 PrLD	RB1410	7730, 7731	BsmBI-HF-v2, then BsrGI-HF & BspEI
RB1964	eYFP-Ssn6 Δ C-Sp1-IDR-LacI	Golden Gate, then subcloning	pGGAselect, then RB1208	Ssn6 Δ C	RB1170	7725, 7732	BsmBI-HF-v2, then BsrGI-HF & XmaI
				Sp1 IDR	RB1211	7733, 7734	BsmBI-HF-v2, then BsrGI-HF & XmaI
RB1277	pSFS2a-Ssn6	Cloning	pSFS2a	5'-Ssn6-3'	SC5314 gDNA	5607, 5608	Apal, XhoI
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1960	pSFS2a-Ssn6 Δ C	Golden Gate	pSFS2a-BsaI	5'-Ssn6 Δ C	RB1277	6905, 6908	BsaI-HF-v2
				3'	RB1277	6909, 6910	BsaI-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1961	pSFS2a-Ssn6 Δ C-Fus-IDR	Golden Gate	pSFS2a-BsaI	5'-Ssn6 Δ C	RB1277	7597, 7599	BsaI-HF-v2
				Fus IDR	RB1209	7600, 7601	BsaI-HF-v2
				3'	RB1277	7602, 7598	BsaI-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1963	pSFS2a-Ssn6 Δ C-TAF15-IDR	Golden Gate	pSFS2a-BsaI	5'-Ssn6 Δ C	RB1277	7597, 7611	BsaI-HF-v2
				TAF15 IDR	RB1210	7612, 7613	BsaI-HF-v2
				3'	RB1277	7614, 7598	BsaI-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB1962	pSFS2a-Ssn6 Δ C-	Golden Gate	pSFS2a-BsaI	5'-Ssn6 Δ C	RB1277	7597, 7603	BsaI-HF-v2

	CmWor1-PrLD						
				CmWor1 PrLD	RB843	7604, 7605	Bsal-HF-v2
				3'	RB1277	7606, 7598	Bsal-HF-v2
		Cloning		far 3'	SC5314 gDNA	5741, 5742	SacI-HF, SacII
RB514	MBP-Efg1	Subcloning	RB523	Efg1	synthetic	n/a	NdeI & XhoI
RB838	MBP-CaCmWor1 (original)	Fusion PCR	RB523	CaWor1 DBD	RB512	4260, 4261	
				CmWor1 PrLD	RB791	4268, 4269	
				CaCmW or1	CaWor1 DBD, CmWor1 PrLD	4260, 4269	NdeI & XhoI
RB2007	MBP-CaCmWor1 (non-slip)	CPEC cloning	RB838	7976, 7977 (oligos used as inserts)	RB838	7974, 7975	DpnI
	MBP-CaCmWor1 (pRB838) amplified with oligos 7974/7975 followed by DpnI digestion to generate an entry vector for CPEC cloning. Insert was created by annealing oligos 7976/7977. CaWor1 DBD amplified from resulting colonies using oligos 6286/3688 and the product digested with HhaI which cuts an additional site created by the substitutions in the "repaired" plasmids.			CaWor1 DBD		6286, 3688	HhaI
RB516	MBP-Czf1	Subcloning	RB523	Czf1	synthetic	n/a	NdeI & XhoI
RB636	MBP-Wor3	Cloning	RB523	Wor3	SC5314 gDNA	3909, 3935	NheI & XhoI
RB549	MBP-Wor4	Subcloning	RB523	Wor4	synthetic	n/a	NdeI & XhoI
RB1170	MBP-Ssn6	Subcloning	RB523	Ssn6	synthetic	n/a	NdeI & XhoI
RB1172	MBP-Tup1	Subcloning	RB523	Tup1	synthetic	n/a	NdeI & XhoI
RB1227	MBP-Tup1-CmR	Subcloning	RB862 (MBP chloram phenicol vector)	Tup1	RB1172	n/a	NdeI & XhoI

Supplementary Table 2: Oligonucleotides used in this study

Oligo #	Oligo name	Oligo sequence
2284	Efg1 Ko LFor V3	TTGCCCTACCCATCTACTCG
2926	Wor1 q I +2204 fwd	ACAATGGTGCTGGTGGATCT
3685	EFG1-SAT1 primer for C-term tag	ATTTGTCCCAAATAGTATAAAATTCGTTTCATGTCAATGGATTGAGAG AAGATTATGATCTATACTATTTTCAGGGAACAAAAGCTGGAGCT
3687	WOR1-SAT1 primer for C-term tag	ACAAATGGTCAAGGCGTCATCATATCATTCAATTTATCTATTTGTTGC GGCAATATTACAATTCCTTCATGAGGGAACAAAAGCTGGAGCT
3688	EcWor1-NdeI	GGCGCCCATATGAGCAATAGCAGCATTG
3909	CaWor3 to pTHMT-MBP rev XhoI	GGACCGCTCGAGTTAATTGTTTGGATACTCTTGGTGGT
3935	Wor3 to pTHMT MBP-fwd NheI	GGACCGGCTAGCGATCAAACATATTTGGACCAACAA
4260	Ca Wor1 DNA BD Fwd NdeI	GGACCGCATATGAGCAATAGCAGCATTGTTCC
4261	Ca Wor1 DNA BD Rev	GCTACCTGCAACTGAGCT
4268	C maltosa Wor1 PrLD Fwd	GCTCAGTTGCAGGTAGCAATAACGTGGCCAACAATCAA
4269	C maltosa wor1 PrLD Rev XhoI	GGACCGCTCGAGTTAATTGCTGGTTCGCATAATAACCA
4438	pSFS2 left	CTCAACCATAGCAATCATGG
4439	pSFS2 Right	GCGAAAAAGTGGGCACTAAG
4446	Efg1-Neon_scarlet fwd	CAAGGTTTCAGTTACCCCTTCACCCCAACAACATCAAGCTAATCAAT CAGCTAGCACTGTTGCCAAAGAAGAAAAGGGTGGTAGTGGTATG GTTTCTAAAG
4535	Wor1-NeonScarlet Fwd	ACTAATGGAACATTGGTTGCTGCTGGAACAGATGACGCAGTTGGA AATTCTTCTGGGTCGTATTACACCGGTACTGGTGGTAGTGGTATGG TTTCTAAAG
4887	Wor2-Neon long oligo Fwd	TATGGTCCTGTTGCAACAACACTGCTTCTGGTGGTAGTGGTGTAA ATAGAGTTTCAGTGGCTGATTTACTTAAAGGTGGTAGTGGTATGG TTCTAAAG
4888	Wor2-Neon long oligo Rev	AGTGACAGTAACAGAAAAAGGGGGCACCAGCACCAGCAGCAGCA GTAGCAGCAGCAGTAGTAACATACATACTGGCGGCCGCTCTAGA ACTAGTGGATC
4890	Wor3-Neon long oligo Fwd	TATAGTCATGCTCAACAACAACAGCAGCAGCAGCACCACAGCAA CAACCATAACCACCAAGAGTATCCAAACAATGGTGGTAGTGGTATG GTTTCTAAAG
4891	Wor3-Neon long oligo rev	TACTTCTATATATTTTGGCTACATAGTATACAATATCCATGTTGTGTA AACCTGACTATGCAAGCAAAACGTGGGCGGCCGCTCTAGAACTA GTGGATC
4892	Wor3-Neon check left fwd	TATGGAATGAACATGTGCGCATCA
4893	Wor4-Neon long oligo fwd	CAGTCTCTCTTAGGAATCAATGACGTTGATTCAAGAAACCAAATTG ACGATAACAAACCAACCCAACAGGCATTGGTGGTAGTGGTATGG TTTCTAAAG
4894	Wor4-Neon long oligo rev	CGACCAAATGCACTTGTGGCACCCCATGCAGGATCCAACCTTTACA ACAACCTTCTAAGTAATGCGTATATTGTACGGCGGCCGCTCTAGAAC TAGTGGATC
4895	Wor4-neon check left fwd	TTTCACAATACGTTTCAGCCTAG

4896	Czf1-Neon long oligo Fwd	GACGAAAATACAATTGAACATGCCGAGTTTGGTAGAATCAAAGTTT TGAGAGGTATTGTTGAATACAGAAGTAAAGGTGGTAGTGGTATGGT TTCTAAAG
4897	Czf1-Neon long oligo rev	TAAATACAATAGTAACATCAGAAAGTGGTTGCCAATAATGCTGTAA AACACTTAACATAATACAAGAGTGGTGGGCGGCCGCTCTAGAACT AGTGGATC
4898	Czf1-Neon check left fwd	TCAACCTTTGTATTCTGCTGCTG
4900	Ahr1-Neon long oligo fwd	ATGGACCCAGAACTAAAATCACAATTTTCATCATTGCTTTACCTGGA CTGTACGCTACATTCACCCAGTAAGTGATGGTGGTAGTGGTATGGT TTCTAAAG
4901	Ahr1-neon long oligo rev	GAAACCTCAACGTCAAAGCTAACGGTAGTAAAAATATATCTATATCT CAAAGCGTGGAATATATTTCCCACTCGGGCGGCCGCTCTAGAACT AGTGGATC
4902	Ahr1-neon Check left fwd	TTCGAATTTATATTCCAACCCGGA
4913	Wor2-neon check left fwd	ATTACCAAGACATGGCCAGAG
5091	Neon rev	TTTATACAATTCATCCATACCCATAACATCA
5575	eYFP-Czf1PrLD-LacI Fwd BsrGI	GGACCGTGTACAAGGGTGGTAGTGGTATGAGCAGCATTCCGAATA TT
5576	eYFP-Czf1PrLD-LacI Rev BspEI	GGACCGTCCGGAAGGGCTCGGTTGCTGCTG
5577	mCherry-Czf1PrLD Rev BspEI	GGACCGTCCGGAATACCTTTCTCTTTTGGATCAGGGCTC GGTTGCTGCTG
5578	Efg1 N-PrLD Fwd NheI	GGACCGGCTAGCGCTACCGGTCGCCACCATGAGCACCTATAGCA TTCCG
5579	Efg1 PrLD Rev	TGTCGGCTGCTGCATGGT
5580	eYFP Fwd overlaps Efg1	ACCATGCAGCAGCCGACAGGTGGTAGTGGTGTGAGCAAGGGCG AGGAG
5581	eYFP rev overlaps Efg1	CGCTAATGCTATTACCATTACCTGAACCACTACCACCCTTGTACAG CTCGTCCATG
5582	Efg1 C-PrLD Fwd	TCAGGTAATGGTAATAGCATTAGCG
5583	Efg1 C-PrLD Rev BspEI	GGACCGTCCGGATTTCTCTTTTGGCAACGGT
5584	Efg1 C-PrLD Rev	TCCGGAATATACCTTTCTCTTTTGGATCTTTCTCTTTTGG CAACGGT
5607	Ssn6 5 fwd ApaI	GGACCGGGGGCCCCAGAAAGACAAAGAGAGAGCG
5608	Ssn6 3 rev XhoI	GGACCGCTCGAGCAACTATATTCCATCCAGAAAATATTTCTATC
5611	Ssn6 5 and N-ORF Rev	ATACTTTGTCAATTGTTCTAATCTTGC
5671	Wor4 N-PrLD fwd NheI	GGACCGGCTAGCGCTACCGGTCGCCACCATGAGCAGCGATAAAC CG
5672	Wor4 N-PrLD rev	GCTATTGCTATTGCTCTGAGG
5675	Wor4 C-PrLD Fwd	ACCGCAGGCAATTATTGGTA
5676	Wor4 C-PrLD Rev BspEI	GGACCGTCCGGAATACCAGGCTGGGTGC
5677	mCherry overlaps Wor4NPrLD	CCTCAGAGCAATAGCAATAGCGGTGGTAGTGGTGTGAGCAAGGG CGAG
5678	mCherry overlaps Wor4 CPrLD	TACCAATAATTGCCTGCGGTACCACTACCACCCTTGTACAGCTCGT CCATG
5679	Wor4 C PrLD Rev BspEI	GGACCGTCCGGAATACCTTTCTCTTTTGGATCAATACCA GGCTGGGTGC
5680	Flo8PrLD_AD Fwd BsrGI	GGACCGTGTACAAGGGTGGTAGTGGTCCGCTGATTGAGCAGC

5681	Flo8PrLDAD Rev BspEI	GGACCGTCCGGAATTTTCAATCGGATCTGCGG
5682	Flo8PrLDAD-NLS REV BspEI	GGACCGTCCGGAATTTTCTCTCTTTTTTGGATCATTTTCAA TCGGATCTGCGG
5683	Ssn6-AB check left fwd	TCTTCTCTGCCCCATAAAATTCA
5684	Ssn6-AB check right rev	CACTAATACCATTACCAACAGTTTTTCAA
5740	Ssn6 tail fwd (SacII)	GGACCGCCGCGGATTTGACTATCTTGTGAATTAAAGTTTTCAA
5741	Ssn6 tail rev (SacI)	GGACCGGAGCTCTATTGCATGTTTTAAAAATGATTATTCACAAAC
5742	CaWor1 FL rev no stop Xmal	GGACCGCCCCGGGAGTACCGGTGTAATACGACC
5799	Ssn6 tail check rev	TTTCATTGGGAAAGAACAAGACAATAAAG
6038	EYFP Ssn6 fwd	ggaccgtgtacaaggggtgtagtggtatgctaccgcgcataaccattaaac
6039	EYFP Ssn6 deltaN fwd	ggaccgtgtacaaggggtgtagtggtctgccgtctagcgccgcactg
6041	mCherry Ssn6 fwd	ggaccgtgtacaaggggtgtagtggtatgctaccgcgcatac
6048	GGA Adapter for pSFS2A Fwd	CCAGTTGAGACCCATTAATGGTCTCTTACTC
6049	GGA Adapter for pSFS2A Rev	TCGAGAGTAAGAGACCATTAATGGGTCTCAACTGGGGCC
6106	mCherry ssn6 rev 2	ggaccgctcgagctataccttctctcttttttgatcttcgtcatcatagtttctgc
6107	LacI Ssn6 rev 2	ggaccgcccgggttcgtcatcatagtttctctcttc
6117	CmWor1PRLD YFPLacI Fwd BsrGI	GGACCGTGTACAAGGGTGGTAGTGGTAATAACGTGGCCAACAATC AA
6118	CmWor1 PRLD YFPLacI Rev BspEI	GGACCGTCCGGAATTGCTGGTCGCATAATAACC
6263	Wor2 PrLD EYFP Fwd BsrGI	GTAAGTGTACAAGGGTGGTAGTGGTATGACCCAGCTGCCGAGC
6264	Wor2 PrLD EYFP Rev BspEI	CAGGTATCCGGAATGATAATACACCTGGCTGTTAACATTGGCAT
6286	CaWor1 DNA BD Rev	gctacctgcaactgagctg
6342	Ssn6 N PrLD rev	tcgctgtgtgctgctgtgtgtgtgtggg
6350	Ssn6 upstream-C fusion fwd	caaagatccggaagatgattgcacctgttagac
6552	U2OS Wor1 fwd	GGACCGTGTACAAGGGTGGTAGTGGTAGCAATAGCAGCATTG GGACCGTGTACAAGGGTGGTAGTGGTACCCAGCTGCCGAGCGTT AGC
6555	U2OS Wor2 fwd	GGACCGTCCGGAATTTAGCAGATCGGCAACGC
6556	U2OS Wor2 LacI rev	GGACCGTGTACAAGGGTGGTAGTGGTAGCAGCGATAAACCGG
6561	U2OS Wor4 fwd	GGACCGCCCCGGGTCAAACCTTCCTCTTCTTTAGGAGGAATACC AGGCTGGGTCGG
6562	U2OS Wor4 NLS rev	GGACCGCCCCGGGTTTGCTGCGATATTCAACAATACC
6563	U2OS Czf1 LacI rev	GGACCGCCCCGGGTCAAACCTTCCTCTTCTTTAGGAGGTTTGCT GCGATATTCAAC
6564	U2OS Czf1 NLS rev	GGACCGTGTACAAGGGTGGTAGTGGTAGCACCTATAGCATTCCG
6567	U2OS Efg1 fwd	GGACCGCCCCGGGTCAAACCTTCCTCTTCTTTAGGAGGTTTCTC TTCTTTTGCAACGG
6568	U2OS Efg1 NLS rev	GGACCGCCCCGGGTCAAACCTTCCTCTTCTTTAGGAGGTTTCTC
6905	GG Ca Ssn6-5N fwd	GGCTACGGTCTCTCAGTCAGAAAGACAAAGAGAGAGCG
6908	GG Ca Ssn6-deltaC_rev	GGCTACGGTCTCATCTCTAATGTGGTTGTT GATATTG
6909	GG Ca Ssn6-3near_fwd	GGCTACGGTCTCAGAGATAAAGAAAAGTGG TGAAATTG

6910	GG Ca Ssn6-3near_rev	GGCTACGGTCTCCAGTACAACCTATATTCCA TCCAGAAAATATTTTC
6981	Full CaCmWor1 NLS KpnI rev	GGACCGACCGGTATTGCTGGTCGCATAATAACCA
7000	Full CaCmWor1 LacI XmaI rev	GGACCGCCCCGGGATTGCTGGTCGCATAATAACCACCACCGG
7001	Full Flo8 BsrGI fwd	GGACCGTGTACAAGGGTGGTAGTGGTATGGTTCCGAATACCACCA AACAGGTGCTG
7002	Full Flo8 LacI BspEI rev	GGACCGTCCGGAATTTTCAATCGGATCTGCGGCCAGTTCATGCC C
7003	Full Flo8 NLS Sall rev	GGACCGGTCTGACTCAAACCTTCTCTTCTTCTTAGGATTTTCAATC GGATCTGCGGCCAGTTC
7113	Ssn6 neon tag long oligo fwd	gatgacgtcaaaaaagatgaaaatccagaacctccaatgagaaagattgaagaagatgaaa attatgatgatgaaGGTGGTAGTGGTATGGTTTCTAAAG
7115	Ssn6 neon tag rev long oligo	ccccatccccctaccccgtaaatatataatattcaactatcatatacaatttcaccactttcttta tctggcgccgctctaga actagtggatc
7597	GG Ssn6deltaC fwd	ggctacggtctctcagtcagaaagacaaagagagag
7598	GG Ssn6deltaC 3 near down rev	ggctacggtctctagtacaactatattccatccagaaaatatttc
7599	GG Ssn6deltaC Fus rev	ggctacggtctccatcggttgaggcatgtggttgatattgttg
7600	GG FUS LCD fwd	ggctacggtctcagattatacccaacaagcaacc
7601	GG FUS LCD rev	ggctacggtctcctccacggtcgtctgtcc
7602	GG Ssn6deltaC Fus down fwd	ggctacggtctcgtggatagagataaagaaaagtgggtg
7603	GG Ssn6deltaC CmWor1 PrLD rev	ggctacggtctcctgatattgtgttggttg
7604	GG CmWor1 PrLD fwd	ggctacggtctccatcaacaaccacataataatgttgcaacaacc
7605	GG CmWor1 PrLD rev	ggctacggtctcatctaattgaagttgcataataacc
7606	GG Ssn6deltaC CmWor1 down fwd	ggctacggtctcatagagataaagaaaagtgggtg
7611	GG Ssn6deltaC TAF15 rev	ggctacggtctccgtaactccagaatccgaatgtggttgatattgttg
7612	GG TAF15 LCD fwd	ggctacggtctcctacggtcagctctggggg
7613	GG TAF15 LCD rev	ggctacggtctctcttatctctagtcaccaccacttgatcc
7614	GG Ssn6deltaC TAF15 down fwd	ggctacggtctctaagaaaagtggtgaaattgtatatg
7725	BsrGI U2OS Ssn6deltaC fwd	ggctaccgtctccggaggtgacaaggggtgtagtgg
7726	BsmBI U2OS Ssn6deltaC rev Fus	ggctaccgtctccttgaggcggtggtgctggtactg
7727	BsmBI U2OS FUS fwd	ggctaccgtctccaaacgattatacccaacaagcaacc
7728	BsmBI U2OS FUS rev BspEI	ggctaccgtctcgatggtccgatccacggtcctg
7729	U2OS Ssn6deltaC rev CmWor1	ggctaccgtctccgccacgttatcatgtgtggctgctggtactg
7730	BsmBI U2OS CmWor1 PrLD fwd	ggctaccgtctcctggccaacaatcaagaagtgc
7731	U2OS CmWor1 PrLD rev BspEI	ggctaccgtctccatggtccgaattgctggtcgc
7732	BsmBI U2OS Ssn6deltaC rev Sp1	ggctaccgtctcttggctcgtcatgtgtggctgctggtactg

7733	BsmBI U2OS Sp1 LCD fwd	ggctaccgtctctaagatcactccatggatgaaatgacagc
7734	BsmBI U2OS Sp1 LCD rev XmaI	ggctaccgtctcgatggcccggtgaggcaatggg
7735	U2OS Ssn6deltaC rev TAF15	ggctaccgtctctcgtaactccagaatccgagtgtggctgctggtactg
7736	BsmBI U2OS TAF15 LCD fwd	ggctaccgtctcttacggtcagtcctgggggtg
7737	BsmBI U2OS TAF15 LCD rev BspEI	ggctaccgtctccatgggtccggagtcaccaccactg
7788	Ca Ssn6 upstream fwd	ggctaccgtctcaggagggggcccgagaaagacaaag
7789	Ca Ssn6 upstream rev	ggctaccgtctcttcttccggatctttgtaatgaaaaaaattag
7790	Sc Ssn6 part 1 fwd	ggctaccgtctctaagatgaatccggcggtgaac
7791	Sc Ssn6 part 1 rev	ggctaccgtctcgTTGTGTACGCGTCTAAGGCaTCTCTGTATTGAGAAAT TTGGTAATATAAACACC
7792	Sc Ssn6 part 2 fwd	ggctaccgtctcgacaagagccataagattaaatcc
7793	Sc Ssn6 part 2 rev	ggctaccgtctcctagtcgtcgtagtttcatc
7794	Ca Ssn6 downstream fwd	ggctaccgtctccactaaagataaagaaaagtgggtaaattg
7795	Ca Ssn6 downstream rev	ggctaccgtctctatggctcgagcaactatatcc
7799	Sc Ssn6 BsmBI site mut seq rev	GGTTTTCTAACTGCTTTGTAAAGC
7974	CaWor1 DBD CPEC rev	TCCAGCTCACCATAAACCCAGAAAACGACC
7975	CaWor1 DBD CPEC fwd	GCAAATTTGGTCCGGACGATGAGTATG
7976	CaWor1 slip repair fwd	GGTCGTTTTCTGGTTTATGGTGAGCTGGACAAgAAAAACCTGATC GATAAAGATAAGAAGAAAAAgAAAAAgCGCAAATTTGGTCCGGACG ATGAGTATG
7977	CaWor1 slip repair rev	CATACTCATCGTCCGGACCAAATTTGCGcTTTTTcTTTTTCTTCTTAT CTTTATCGATCAGGTTTTTcTTGTCCAGCTCACCATAAACCCAGAAAA CGACC
8052	Ca Tup1 neon long oligo fwd	TCAAAGGGGAACCGAAGGTATCTTCGCTACAGGTAGTGCGGATTGT AAAGCCAGAATTTGGAAATGGACCAAAAAAGGTGGTAGTGGTATG GTTTCTAAAG
8053	Ca Tup1 neon long oligo rev	GTGGGTTGTTACGAAAAAATTTTTTTTTTTTGGTGGTGTTTTTTTTT CTCACATATATATACTACACACTTAaggcgccgctctagaactagtgatc
8054	Ca Tup1 neon check fwd	AGGGCCACCGCAACTCGG
8055	Ca Tup1 neon check rev	ACGTTAGTAGACATTCAATTGATGG

Supplementary Table 3: *C. albicans* strains used in this study

Strain #	name	description
CAY6342	WT white	AHY135 from Lohse, et al. 2013, <i>PNAS</i> .
CAY6343	WT opaque	AHY136 from Lohse, et al. 2013, <i>PNAS</i> .
TF121	<i>ssn6/ssn6</i>	TF121 from Lohse, et al. 2016, <i>Genetics</i> .
CAY8083	<i>ssn6/SSN6</i>	AHY337 from Hernday, et al. 2016, <i>mBio</i> .
CAY11369	Ssn6 full with 3' homology satS	RB1277 digested with Apal and SacI and transformed into TF121. 5' integration checked with oligos 5683 & 5611. 3' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY11371	Ssn6 full with 3' homology satS	RB1277 digested with Apal and SacI and transformed into TF121. 5' integration checked with oligos 5683 & 5611. 3' integration checked with oligos 5740 & 5799. SAT resistance cassette flipped out with maltose media.
CAY14367	Sc Ssn6 addback satS	RB1966 digested with Apal and SacI and transformed into TF121. 5' integration checked with oligos 5683 & 7799. 3' integration checked with oligos 4439 & 5799. SAT resistance cassette flipped out with maltose media.
CAY14368	Sc Ssn6 addback satS	RB1966 digested with Apal and SacI and transformed into TF121. 5' integration checked with oligos 5683 & 7799. 3' integration checked with oligos 4439 & 5799. SAT resistance cassette flipped out with maltose media.
CAY13594	Ssn6 Δ C with 3' homology satS	RB1960 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY13595	Ssn6 Δ C with 3' homology satS	RB1960 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14003	Ssn6 Δ C-Fus-IDR	RB1961 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14004	Ssn6 Δ C-Fus-IDR	RB1961 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14007	Ssn6 Δ C-TAF15-IDR	RB1963 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14008	Ssn6 Δ C-TAF15-IDR	RB1963 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14005	Ssn6 Δ C-CmWor1-PrLD	RB1962 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY14006	Ssn6 Δ C-CmWor1-PrLD	RB1962 digested with Apal and SacI and transformed into TF121. Integration checked with oligos 5683/6342 (5') and 4439/5799 (3'). SAT resistance cassette flipped out with maltose media.
CAY9299	Efg1-Neon	Long oligos 4446/3685 with Efg1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/2284.
CAY9300	Efg1-Neon	Long oligos 4446/3685 with Efg1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/2284.

CAY9307	Wor1-Neon	Long oligos 4535/3687 with Wor1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 2926/4438.
CAY9308	Wor1-Neon	Long oligos 4535/3687 with Wor1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 2926/4438.
CAY9645	Wor2-Neon	Long oligos 4887/4888 with Wor2 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4913.
CAY9646	Wor2-Neon	Long oligos 4887/4888 with Wor2 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4913.
CAY9647	Wor3-Neon	Long oligos 4890/4891 with Wor3 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4892.
CAY9648	Wor3-Neon	Long oligos 4890/4891 with Wor3 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4892.
CAY9649	Wor4-Neon	Long oligos 4893/4894 with Wor4 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4895.
CAY9650	Wor4-Neon	Long oligos 4893/4894 with Wor4 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4895.
CAY9653	Ahr1-Neon	Long oligos 4900/4901 with Ahr1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4902.
CAY9654	Ahr1-Neon	Long oligos 4900/4901 with Ahr1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4902.
CAY9651	Czf1-Neon	Long oligos 4896/4897 with Czf1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4898.
CAY9652	Czf1-Neon	Long oligos 4896/4897 with Czf1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into RB1173. Integration checked with oligos 4438/4898.
CAY14362	Tup1-Neon	Long oligos 8052/8053 with Tup1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY6342. Integration checked with oligos 8054/5091 (5') and 4439/8055 (3'). SAT resistance cassette flipped out with maltose media.
CAY14363	Tup1-Neon	Long oligos 8052/8053 with Tup1 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY6342. Integration checked with oligos 8054/5091 (5') and 4439/8055 (3'). SAT resistance cassette flipped out with maltose media.
CAY13555	Ssn6-Neon	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11369. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.
CAY13556	Ssn6-Neon	Long oligos 7113/7115 with Ssn6 homology used to amplify Neon-SAT cassette sequence from plasmid RB895. PCR product transformed into CAY11369. Integration checked with oligos 6350/5091 (5') and 4439/5684 (3'). SAT resistance cassette flipped out with maltose media.

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Appendix: Abstracts and contributions for co-authored papers

Epigenetic cell fate in *Candida albicans* is controlled by transcription factor condensates acting at super-enhancer-like elements.

Frazer C, Staples MI, Kim Y, Hirakawa M, **Dowell MA**, Johnson NV, Hernday AD, Ryan VH, Fawzi NL, Finkelstein IJ, Bennett RJ.

Nature Microbiology, 2020.

Abstract: Cell identity in eukaryotes is controlled by transcriptional regulatory networks that define cell-type-specific gene expression. In the opportunistic fungal pathogen *Candida albicans*, transcriptional regulatory networks regulate epigenetic switching between two alternative cell states, ‘white’ and ‘opaque’, that exhibit distinct host interactions. In the present study, we reveal that the transcription factors (TFs) regulating cell identity contain prion-like domains (PrLDs) that enable liquid–liquid demixing and the formation of phase-separated condensates. Multiple white–opaque TFs can co-assemble into complex condensates as observed on single DNA molecules. Moreover, heterotypic interactions between PrLDs support the assembly of multifactorial condensates at a synthetic locus within live eukaryotic cells. Mutation of the Wor1 TF revealed that substitution of acidic residues in the PrLD blocked its ability to phase separate and co-recruit other TFs in live cells, as well as its function in *C. albicans* cell fate determination. Together, these studies reveal that PrLDs support the assembly of TF complexes that control fungal cell identity and highlight parallels with the ‘super-enhancers’ that regulate mammalian cell fate.

Contributions: I contributed to this study by cloning the plasmids used for U2OS cell transfection.

Filamentation and biofilm formation are regulated by the phase-separation capacity of network transcription factors in *Candida albicans*.

Ganser C, Staples MI, **Dowell M**, Frazer C, Dainis J, Sircaik S, Bennett RJ.

Plos Pathogens, 2023.

Abstract: The ability of the fungus *Candida albicans* to filament and form biofilms contributes to its burden as a leading cause of hospital-acquired infections. Biofilm development involves an interconnected transcriptional regulatory network (TRN) consisting of nine transcription factors (TFs) that bind both to their own regulatory regions and to those of the other network TFs. Here, we show that seven of the nine TFs in the *C. albicans* biofilm network contain prion-like domains (PrLDs) that have been linked to the ability to form phase-separated condensates. Construction of PrLD mutants in four biofilm TFs reveals that these domains are essential for filamentation and biofilm formation in *C. albicans*. Moreover, biofilm PrLDs promote the formation of phase-separated condensates in the nuclei of live cells, and PrLD mutations that abolish phase separation (such as the removal of aromatic residues) also prevent biofilm formation. Biofilm TF condensates can selectively recruit other TFs through PrLD-PrLD interactions and can co-recruit RNA polymerase II, implicating condensate formation in the assembly of active transcriptional complexes. Finally, we show that PrLD mutations that block the phase separation of biofilm TFs also prevent filamentation in an in vivo model of gastrointestinal colonization. Together, these studies associate transcriptional condensates with the regulation of filamentation and biofilm formation in *C. albicans*, and highlight how targeting of PrLD-PrLD interactions could prevent pathogenesis by this species.

Contributions: I contributed to this study by cloning plasmids used for U2OS cell transfection.

