Investigating the molecular mechanism of cohesin loading in *Candida albicans*, and its role in antifungal drug resistance

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Introduction

Candida albicans, a prevalent commensal human fungal pathogen, has an incredibly plastic genome with high rates of aneuploidy (incorrect number of chromosomes) in response to stresses such as antifungal drugs. This genomic plasticity allows for rapid evolution of resistance, posing a significant threat for healthcare which is further enhanced by limited antifungal therapeutics. The mechanisms by which *Candida albicans* controls this aneuploidy are not well understood. A key molecular machine regulating aneuploidy is the cohesin complex, regulated by a kinase-driven conserved process [2]. Centromeric cohesin is essential for chromosome cohesion during chromosome biorientation during metaphase. This process is crucial in ensuring accurate chromosome segregation, and errors can lead to aneuploidy [1]. A conserved region on the Scc4 protein in the Scc2-Scc4 cohesin loading complex which binds the kinetochore component, Ctf19, when phosphorylated directs cohesin loading to centromeres [2]. When this interaction is interrupted, centromeric cohesin is not enriched at centromeres. Previous work in the lab predicted 2 conserved residues in *C. albicans* involved in the Scc4-Ctf19 interaction, corresponding with the residues identified in *S. cerevisiae* [3]. We hypothesize that point mutations of these 2 residues could deplete centromeric cohesin, leading to aneuploidy when *Candida* experience stresses, such as antifungal treatment, and that this aneuploidy is critical for antifungal drug resistance. Furthering the molecular understanding of cohesin loading and contribution to resistance could reveal novel antifungal targets and therapeutic strategies.

This project aims to: (1) Generate *scc4-2A* and *scc4-2E* mutant strains to disrupt the Scc4-Ctf19 interaction and carry an epitope tagged Scc1-3HA subunit; (2) use these strains to assess loading of cohesin at the centromere by Chromatin Immunoprecipitation/qPCR and (3) phenotypically characterize the mutant strains following treatment with Fluconazole (FLC).

Methods

CRISPR-Cas9 point mutations: Double alanine (Scc4-2A) and glutamic acid (Scc4-2E) point mutations were generated by introducing point mutations as described in Nguyen et al. (2017) with modifications [3]. Successful mutants were detected by enzymatic digestion and confirmed by sequencing. Successful mutants were saved as glycerol stocks (VM330 - VM332; VM346, VM347).

Epitope tagging of Scc1: 3xHA fragments with homology to Scc1 were transformed into Scc4-2A (VM330) and Scc4-2E (VM346) [3]. Colony PCRs determined successful integration using Scc1 and 3xHA specific primers. 3xHA expression was determined by Western blot.

Western Blot: Protein samples were run on SDS-PAGE and transferred on nitrocellulose (90V, 90min). Mouse Anti-HA11 (Sigma; 1:1000 dilution) was used with anti-mouse secondary antibody (1:2000), as previously described [2].

ChIP-qPCR: Cells were arrested in metaphase using nocodazole to depolymerize microtubules and cell cycle stage checked by microscope. Protein and DNA were cross linked by formaldehyde, cells were lysed, and DNA sheared by sonication. Chromatin Immunoprecipitation (ChIP) was performed using anti-HA (12CA5; Sigma) antibody, and samples were saved for western blot. Cross linking was reversed, and DNA concentration determined for the ratio of Input to IP (1:1 ratio used). Quantitative qPCR was performed using primers targeting Chromosome 7 centromere (Cen7) and Arm (Arm7) on untagged (SC5314), wild type Scc1-3xHA, and double mutant Scc1-3xHA *scc4-2A* strains.

Spotting: 5-fold dilutions of strains were spotted onto plates with fluconazole. Plates were incubated at 30□c and imaged at 24 hours and 48 hours.

***In vitro* evolution under antifungal drugs**: Triplicates of wild type and Scc4-2A mutants were passaged at 2x MIC of SC5314 (WT strain) of FLC (1□g/ml) or no FLC for 8 days. Every 24 hours an aliquot of culture was re-cultured in fresh media (1:1000 dilution). Samples were saved every 48 hours (~24 generations). Growth curves were done on day 8 (~96 generations), with change in absorbance measured every hour for 24 hours. Varying concentrations of FLC were tested to assess resistance.

Results and outcomes

To test whether perturbation of the conserved patch (scc4-2A) reduces association of the cohesin subunit, Scc1, with the centromere of Chromosome 7, centromeric cohesin loading in wild type and scc4-2AS mutant strains were assessed by ChIP qPCR. Preliminary ChIP-qPCR experiments showed no difference in the localization of Scc1 subunit in mutant versus wild type strain (Fig 1A). No difference in the expression of Scc1 was observed between strains (Fig 1A). Tagged strains showed no enrichment with respect to untagged in Cen7 (Fig 1A) despite the ChIP, suggesting that the method needs further optimization to obtain conclusive results. Spotting of the strains showed no significant resistance, which aligned with previous work in the lab. Fluconazole is a fungistatic drug, which slows growth but does not kill. As such, the spotting may not be a sensitive enough method to show any difference in

response to Fluconazole treatment. This is compounded by resistance emerging as an evolutionary response to prolonged exposure to the drug, which further suggests that 48 hours of exposure is likely not sufficient to see any significant evolution of resistance. This led to the development of the in vitro evolution experiment. The 24-hour growth curve showed no significant increase in resistance after 96 generations passaged in 1 □g/ml FLC between the scc4-2A double mutants (DM) and the wild type (WT). However, the FLC conditions tested (5 and 10 □g/ml) were likely too high of a concentration and limited any growth. It is likely that if any resistance evolved, it is low level tolerance to concentrations nearer to 1 □g/ml.

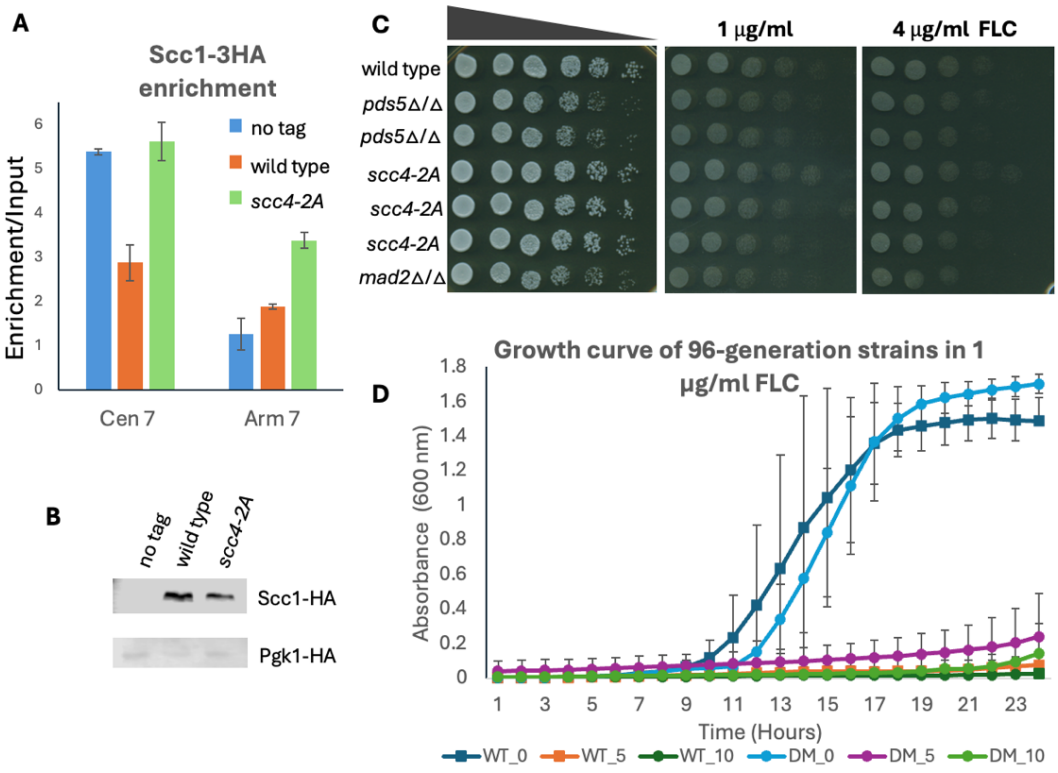

Figure 1. **Characterization of scc4-2A mutant in Candida albicans**. A) ChIP/qPCR using primers for Cen7 (Chromosome 7 centromere) and Arm7 primers (Chromosome 7 arms) with standard deviation error bars. B) Western blot of no tag (SC5314), wild type (Scc1-3xHA) and Scc1-3HA scc4-2A strains. Pgk1 expression shown as a loading control. C) Spotting assays using Fluconazole, incubated for 24 hours in 30□c. Scc4-2A double alanine mutants used are different isolates as biological triplicate. A spindle assembly mutant, mad2 deletion strains was used as a control. The cohesin complex accessory protein, Pds5 knockout mutant was also a control. D) 24-hour growth curve of samples cultured for 96 generations in 1□g/ml Fluconazole. Tested under 0, 5 and 10 □g/ml. Average of 2 duplicate conditions with error bars are shown. WT = SC5314. DM = VM330 (Scc4 double Alanine mutant)

<u>Departure from original plan and Future directions</u>

Initial western blots were unsuccessful which delayed strains construction. However, this allowed time for development and planning of the in vitro evolution experiment. There was also time to create strains using CRISPR for double guanine mutations to complement the resulting phenotype by ChIP/qPCR. Further testing of the in vitro evolution experiment is needed to assess the methods use in developing resistance and testing differences in rates of resistance. This includes passaging in both higher concentrations of FLC and increasing FLC concentrations. Optimization of the ChIP/qPCR is needed to answer the original question of the report. Strains generated in this report are important tools that will be needed to characterize Scc4's role in C. albicans. The In vitro evolution experiment when optimized will be an important tool for the lab to assess resistance and for comparative analysis with clinical isolates resistant to Fluconazole.

<u>Value of studentship:</u> This summer project has enabled me to learn yeast genetics and develop important laboratory skills, such as CRISPR-Cas9, ChIP-qPCR, western blot, essential for a career in research. I have also learnt how to adapt to a professional setting, multitask and optimize time management skills. Importantly, this research project has also been key in learning how to design and plan and experiment and critically analyze the resulting data.

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