Aneuploidy of Chromosome R Regulates *Candida albicans* Colony Color Variations on CHROMagar Media

By

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Thesis

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Abstract:

Candidiasis is caused by fungi of the *Candida* genus and can range from mild to life-threatening systemic infections. In the United States, *Candida* bloodstream infections rank as the fourth most common cause of invasive bloodstream infections. Assessment of *Candida* virulence mechanisms could advance current understanding of *Candida* infections and improve diagnosis and treatment. CHROMagar medium has been used for the quick assessment of *Candida* species due to colonies forming species-specific colors. *Candida albicans* usually exhibit a standard white color, but previous studies from our lab showed that *C. albicans* variants can alter the color on CHROMagar, resulting in differences within a single *Candida* species. It had been further demonstrated that *C. albicans* white-opaque cell states, or *C. albicans* mutants such as those lacking *EFG1* were distinguishable on CHROMagar. Understanding the mechanisms underlying such color variations could broaden the potential applications of CHROMagar medium and further enable the assessment of different *C. albicans* cell states. In this thesis, the genetic mechanisms behind colony color variation on CHROMagar was investigated by analysis of a collection of clinical *C. albicans* isolates.

Whole-genome-sequencing revealed that a number of clinical isolates could adopt a purple colony color and that a number of these had gained an extra copy of chromosome R. Color change was also observed in euploid strains with two strains associated with homozygous *EFG1* mutations resulting in a distinct phenotypic state. Crispr-Cas9 editing was employed to delete the left or right arm of the one copy of chromosome R (chrR) in strains trisomic for this chromosome. We further narrowed down the region on chrR associated with the color change on CHROMagar, specifically a region composed of 279 genes on the left arm of chR. In line with previous studies, we observed that chromosome R trisomy led to increased triazole resistance,

and identified a region on chromosome R associated with this resistance. Together, these results demonstrate that changes to chromosome R copy number regulate *C. albicans* CHROMagar color variation and fluconazole resistance in multiple clinical isolates. These findings provide insights into *C. albicans* aneuploidy, phenotypic plasticity, and antifungal drug susceptibility.

Chapter1: Introduction

1.1 Overview of *Candida* Species and Infections

Some fungal pathogens are commensal microorganisms that colonize healthy hosts but turn pathogenic in immunocompetent populations, causing infections that can be minor or leading to systemic infections with high morbidity [1]. Invasive bloodstream infections have risen with the increasing numbers of immunodeficient individuals, including those undergoing immunosuppressive therapies [2]. *Candida* species are the most common human fungal pathogens in nosocomial environments, and candidiasis ranks as the fourth most common cause of invasive bloodstream infections in the United States [3]. 170 species belong to the *Candida* genus but 90% of infections are caused by five species: *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis,* and *Candida krusei* [4]. Among the five, *C. albicans* is the most commonly isolated species and remains the leading cause of candidiasis [4]. Understanding the underlying mechanisms responsible for commensal or pathogenic growth is important as this can enable further insights into *C. albicans* biology and potential treatment strategies.

1.2 *C. albicans*: Cell Types, Morphotypes, and Aneuploidy

Candida albicans can be found in the gastrointestinal (GI) tract [5], mouth [6], skin, and female reproductive tract in humans and animals. As the most common pathogen in the *Candida* genus, it is recognized as causing superficial and systemic fungal infections, particularly in immunocompromised individuals [7]. As a part of human gut microbiota composition, *C. albicans* usually initializes benign colonization during infancy and maintains a presence in more than 70% of adults with a high adaptation in various host conditions [8]. And when hosts

develop immunodeficiency, colonization can become pathogenic and lead to infections. *C. albicans* exhibits phenotypic heterogeneity through reversible morphological transitions such as the yeast-hyphal and white-opaque switches, and these can enable adaptation to different host environments [8, 9]. Yeast, hyphae, and pseudohyphae are the classic morphologies in *C. albicans* and directly impact virulence, while white-opaque switching involves alternative yeast-like morphotypes that can alter mating, virulence, and biofilm formation [8]. The ability to undergo white-opaque switching is determined by mating-type locus, with cells expressing both *MTLa* and *MTL alpha* inhibited in forming the opaque state. Cells in the opaque state are specialized for mating whereas white cells undergo only very low levels of mating [8].

White-to-opaque switching is inhibited in *MTL*a/alpha cells via the a1-α2 complex.

Ploidy changes and loss of heterozygosity (LOH) are common genetic events that can significantly impact basic cellular properties and contribute to phenotypic plasticity [10]. For example, yeast species have been shown to increase their fitness in response to stressful environments by gaining additional copies of certain chromosomes. *C. albicans* cells are diploid and contain 8 heterozygous chromosomes which exhibit a certain level of instability. A number of forms of aneuploidy have been identified in *C. albicans*, such as trisomy of chromosome 7 which decreases filamentation [10], and trisomy of chromosome R, which confers resistance to antifungal drugs [11]. These findings have established that aneuploidy can alter *C. albicans* fitness, morphology, and other adaptive traits.

1.3 CHROMagar Usage and Prior Work in the Bennett Lab

PCR, Mass Spectrometry, and CHROMagar *Candida* are three commonly used methods to quickly identify yeast species in the clinic [12]. CHROMagar for *Candida* identification is

straightforward and is used in both clinical and laboratory settings. CHROMagar is a solute media for distinguishing different *Candida* species infections by exhibiting species-unique colony colors [13]. This chromogenic media contains a chemical chromogen that can be hydrolyzed by different *Candida* enzymes. CHROMagar medium is composed of at least one chromogen as well as carbohydrate, alcohol, and additional components often present in a standard yeast medium (e.g. antibiotics, agar, sodium chloride). As discussed, the presence of *Candida* species on CHROMagar results in hydrolysis of the chromogen, generating a chromophore of derived colors. Chromogens present in CHROMagar can comprise an X-GluNac (Glu is glucose, NAc is an N-acetyl group and X is a chromophore), X-GalNAc (wherein Gal is galactose, NAc is an N-acetyl group and X is a chromophore), and phosphate group (Appendix Table 1), and different enzymes from *Candida* species can hydrolyze those chromogens in different manners [13].

The enzymic hydrolysis of chromogens leads to CHROMagar exhibiting species-specific colors. *C. albicans* can exhibit different colony colors on CHROMagar medium at 23°C, including white and green colors at room temperature [14]. Previous studies from the Bennett lab recognized color variations within *C. albicans* strains on CHROMagar medium, highlighted by white-opaque switching (white cells form light green colonies and opaque cells form dark green colonies). Deletion of transcriptional factors regulating the white-opaque switching also lead to altered colors: deletion of efg1 results in cells adopting the “gray” state which form green colonies on CHROMagar [9]. This finding indicated that *C. albicans* cell morphological states and gene mutations can be associated with CHROMagar colors, indicating that cellular mechanisms can affect this phenotypic and the potential application of CHROMagar for accessing *C. albicans* cell state information. Although CHROMagar's potential applications have

been demonstrated, studies have yet to investigate the mechanisms behind species/mutant color differences.

1.4 Clinical Isolates and Phenotypic Variations

Currently there are gaps in understanding *C. albicans* growth on CHROMagar, and the mechanisms determining color differences [9]. In this work, we examined the impact of genetic variants and aneuploidy on the color variations exhibited by *C. albicans* on CHROMagar. We show that *C. albicans* isolates can transition between white, green and purple states (as well as other colors) on CHROMagar, and investigate these changes in this paper. White and purple colonies from the same isolates were used as comparison groups. After filtering out strains with unstable phenotypes, the comparison groups exhibiting stable color variations were further assessed. Genome sequencing was used to analyze these strains and showed that loss of *EFG1* was one mechanism driving a shift from white to green colonies. Furthermore, we demonstrated changes in chrR copy number resulted in white to blue/purple color shifts. CRISPR-Cas9 was used to make chromosomal deletions in these strains, and showed that removal of the chrR left arm or a truncation composed of 279 genes on the left arm shifted purple colonies back to white. This study therefore reveals a novel link between aneuploidy of chromosome R and color changes on CHROMagar, and further investigations will address which genes are driving this change.

Chapter 2: Methods

Strains and culture conditions:

Candida albicans clinical isolates were obtained from MSKCC (Memorial Sloan Kettering Cancer Center) and struck onto YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and CHROMagar Candida medium (Fisher#NC9514149; Drg International Inc #CA222). Strains were cultured at room temperature or at 30°C. CHROMagar plates were incubated for up to 15 days before phenotypic data collection.

Whole Genome Sequencing (WGS) and variant analysis:

Genomic DNA was extracted from the *C. albicans* strains grown on CHROMagar for ~15 days. DNA from fifty strains was purified with a QiaAmp Mini DNA kit (QIAGEN). DNA concentrations were measured with Nanodrop and Qubit (Thermo Fisher) following the manufacturer's protocols. Whole genome sequencing libraries were prepared and sequenced by SeqCenter. Raw sequencing reads were processed using a Conda pipeline created by Iuliana Ene (Institut Pasteur, France) in the Pilon package. Briefly, Illumina paired-end reads were converted from BAM to FASTQ format using BEDTools BamToFastq (2.31.0). FASTQ files underwent quality control and trimming with FastQC and Trimmomatic before alignment to the *C. albicans* SC5314 reference genome (version A22) using BWA-MEM (v0.7.17). Variants were called using GATK 4.0 and Pilon, generating VCF files. Individual VCFs were merged into a multi-sample VCF and genotyped using GATK CombineGVCFs and GenotypeGVCFs. Following manual filtering, variants were annotated with the SC5314 reference genome and C_albicans_SC5314_A22_current_features.gff (version A22) using snpEff.

Phylogenetic Tree:

Seventeen *C. albicans* strains from different subclades were retrieved from NCBI Project PRJNA432884 and analyzed using a custom Nextflow-based WGS pipeline (modified from the

Conda workflow by Paul Cao – Brown Center for Computation and Visualization). The resulting VCF files were merged and genotyped with those from MSKCC clinical isolates using GATK CombineGVCFs and GenotypeGVCFs. The combined VCF file was converted into a FASTA file using the vcf2fasta Python package with the parameters '-e CDs -b -r' and annotated with C_albicans_SC5314_A22_current_features.gff. Individual FASTA sequences were concatenated and converted into PHY format using catfasta2phyml. Phylogenetic tree construction was performed using IQ-TREE v1.6.12 with 1,000 bootstrap replicates.

Coverage plots:

Genome coverage was calculated using a python script by Iuliana Ene (Institut Pasteur) incorporating BEDTools coverage function which subdivided the genome into non-overlapping 10 kb windows then the number of reads in each window was tallied. Coverage plots were visualized using GraphPad Prism.

Strain Construction:

Chromosome R deletions were generated using CRISPR-Cas9 with plasmids cloned using Golden Gate Assembly. To delete Mbp scale chromosome fragments two gRNAs were used to target Cas9 to within 1000 bp from the ends of the desired deletions. A repair template with gDNA homology arms adjacent to deleted fragments was used to seal the gap and introduce a SatR selectable marker.

PCR was used to amplify repair fragments including the SatR resistance marker and *C. albicans* homolog pairs. SatR was amplified from pSFS2A using oligos 8893/8894 for chrR left/right-arm deletion and 9540/9541 for chrR segmental deletion. *C. albicans* homologs pairs were amplified from SC5314 genome using following oligo pairs: chrR left distal (oligos

8903/8904), chrR left proximal (oligos 8905/8906), chrR right proximal (oligos 8907/8908), and chrR right distal (oligos 8909/8910). For segmental deletions, the SC5314 homologous pairs DelN distal (oligos 9326/9327), DelN proximal (oligos 9328/9329), DelO distal (oligos 9330/9331), DelO proximal (oligos 9332/9333), DelP distal (oligos 9334/9335), DelP proximal (oligos 9336/9337), DelQ distal (oligos 9338/9339), and DelQ proximal (oligos 9340/9341) were amplified. Golden Gate Assembly (GGA) was used to construct repair templates by digesting plasmid pGGA Select (New England Biolabs) with BsaI or BsmbI and inserting the SatR and individual homologous pairs.

gRNAs were generated with by annealed forward and reverse oligos such that they made a double stranded molecule encoding the guide sequence, along with single stranded ends that were complementary to BsmBI digested pRB476 [15], chrR left distal (oligos 8885/8886), chrR left proximal (oligos 8887/8888), chrR right proximal (oligos 8889/8890), and chrR right distal (oligos 8891/8892). gRNAs were cloned into plasmid pRB476 digested with BsmBI, and assembled via GGA. The repair template and gRNA plasmids were transformed into *E. coli* JM109 (Promega), grown in LB, LB + carbenicillin (50 μg/mL), or LB + chloramphenicol(25 ug/mL), and then sequences examined confirmed by Sanger sequencing (Genewiz) and Oxford Nanopore long read sequencing (Plasmidsaurus).

gRNAs and Cas9 were introduced transiently into *C. albicans*. gRNA fragments were PCR amplified from cloned gRNA plasmids using oligos 8145/8146. Cas 9 was PCR amplified from gRNA plasmids using oligos 8143/8144. Repair templates were excised from repair template plasmids by PacI digestion. Strains with chromosome R deletions were generated by transforming 30 μg of DNA (9 μg gRNA1 product, 9 μg gRNA2 product, 9 μg Cas9 product, and 3 μg repair template plasmid) into CAY12801, CAY12902, and CAY12837.

Fluconazole susceptibility testing:

Fluconazole stocks were prepared in ethanol (20 mg/ml) and diluted 1:10 in water. YPD plates were supplemented with fluconazole to final concentrations of 8 μg/mL and 32 μg/mL. Spot assays were performed in 5x dilutions under different fluconazole concentrations. Plates were incubated at room temperature for three days before assessment.

Chapter 3: Results

3.1 Clinical Isolates Exhibit Color Phenotypic Variation on CHROMagar

138 *C. albicans* clinical isolates received from MSKCC were plated on CHROMagar media. We observed phenotypic variations after isolates were plated on CHROMagar for 7-15 d at room temperature. In 55 samples, purple or pink sectors or colonies arose on the CHROMagar plate, while some colonies formed the light green color previously associated with the "gray" *efg1*Δ/Δ state (Figure 1a, 1b). Purple colonies were replated along with the white colonies from the same sample for pairwise comparisons. We observed that most phenotypic variants were stable, with some colonies or sectors displaying mixed colors (Appendix Fig 1). 50 replated MSKCC isolates (including 19 pairwise comparison sets) were then selected for their stable phenotypic colors and used for further investigation. PCR analysis established that these strains were *MTL*a/alpha strains consistent with previous studies demonstrating that most *C. albicans* strains are *MTL* heterozygous [24].

We sequenced the 50 MSKCC-derived isolates with stable phenotypic colors and aligned these genomes to those of the SC5314 reference genome [23]. *C. albicans* strains have been divided into 17 genetic clusters [16]. Characteristics such as geographical origin and genotype distribution were applied to classify strains and define clade divisions, facilitating the construction of a clade map [17]. Phylogenetic analysis indicated that the 50 MSKCC strains fell into seven clades, representing a broad cross section of *C. albicans* phylogeny (Figure 1c). 27 strains fell into clade 1 along with SC5314, which is the most frequently encountered clade among *C. albicans* isolated from human infections [18]. Other strains fell into clades 2, 4, 8, 10, 12, and 16. This result demonstrates that the 50 MSKCC strains represent diverse lineages across

the *C. albicans* population and that similar color variations can arise independently in different genetic backgrounds.

3.2 Loss of Heterozygosity and Ploidy Changes

Loss of Heterozygosity (LOH) events frequently occur in diploid *C. albicans* strains and can drive phenotypic diversity [19]. We observed LOH sections in the 50 sequenced strains on chromosome 1, 3, 6, 7, and R relative to SC5314, but LOH events were shared between white-purple pairs, suggesting that LOH might not be the primary driver of the observed color variation in our study.

Aneuploidy has been commonly observed in *C. albicans* isolates. For example, *C. albicans* can adapt its fitness under environmental stress conditions and adapt through the gain of additional chromosomes [20]. Coverage analysis showed that aneuploidy changes were repeatedly associated with white-purple color changes (Figure 2a). Thus, 11 MSKCC lineages that grew as darker colors (blue/pink/purple) had become aneuploid with a complete extra chromosome or segmental aneuploidy. The majority of cases showed chromosome gains in Chr7 and ChrR, with gaining an additional copy of chromosome R the most common occurrence (Figure 2b, 2c). By contrast, the other seven comparison sets showed no ploidy changes. The potential correlation between aneuploidy in chromosome R, genetic variants, and color variation was further investigated.

3.3 *EFG1* Mutations Occur and Drive the White-to-Gray Transitions

The diversity observed indicates that phenotypic color variation can arise by different mechanisms in different genetic backgrounds. We examined (1) the genomic changes that occur in euploid strains, i.e., no karyotypic shift upon color change, and (2) the link between aneuploid formation and color change. In the first category, we note that mutations in transcription factors associated with *C. albicans* virulence and filamentation such as *EFG1* can result in color variations on CHROMagar plates [6]. We hypothesized that the color variation observed in MSKCC was driven by such genetic mutations and tested this idea in the seven euploid pairs. Variant analysis identified 1,023 SNP mutations in total that were not shared between white and derived isolates, which can be either different in SNP position or heterozygosity. We filtered the variant pool manually based on the impact-of-mutation/mutation positions and refined it by integrating results from Pilon and GATK workflows, resulting in 32 candidate mutations (Figure 3b). Notably, we identified two mutations in *EFG1* located near the start of the ORF. Those two mutations appeared in both the white parental strain and in derived progeny but showed differences in heterozygosity. A frameshift mutation located at the position encoding amino acid (aa) 38 and a nonsense mutation at the position encoding aa 62 were present on one allele in the white parental strain but on both alleles in progenies (Fig 3a). Our lab previously showed that loss of EFG1 function could lead to formation of the gray state – which appears green on CHROMagar [6]. Similarly, our result shows that the *EFG1* ORF was disrupted in one allele in the parental isolate and then underwent LOH to form a homozygous *efg1* null on forming the green colonies on CHROMagar.

3.4 Aneuploidy of Chromosome R Drives Color Variations

Our analyses showed that the white-to-purple shift in a number of strains when grown on CHROMagar was associated with the gain of one extra copy of chrR. We therefore hypothesized that a trisomy of chrR was responsible for the change in CHROMagar phenotypes. To determine whether specific arms of ChrR impact color variations, we performed arm deletions using a CRISPR-Cas9 system [15, 21]. This system uses Cas9 to introduce dual breaks into the chromosome at defined locations and then a repair template is present to fuse the breaks together, resulting in a defined deletion that can encompass large sections of a chromosome arm.

CAY12902, CAY12801, and CAY12837 are three strains from different clades that exhibit purple color on CHROMagar and all carry a trisomy of ChrR. We constructed plasmids for 1) homologous DNA repair templates and 2) guide RNAs (gRNAs) through Golden Gate Assembly and then introduced these constructs together with a construct expressing Cas9 (Figure 4a, 4b). PCR checks were used to establish that the targeted region had been deleted in transformants.

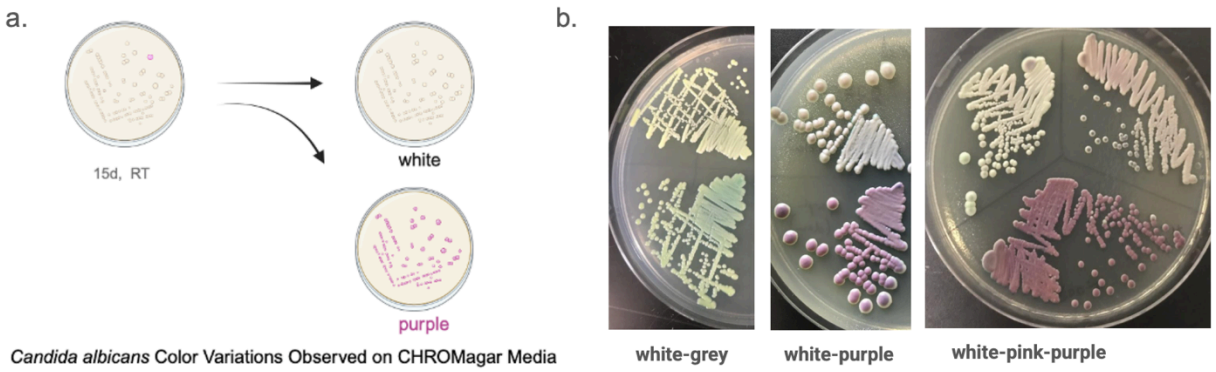
A phenotypic analysis of these recombinant strains grown on CHROMagar was conducted. Most strains with a left-arm deletion exhibited a white color whereas strains with a right-arm deletion still exhibited a purple color. We sequenced multiple strains and results verified that strains with a reversed color on CHROMagar either successfully lost one copy of the chrR left arm or had lost one copy of the whole chrR (Figure 4c, 4d). One strain showed a gain of additional copy chrR right arm with a chrR left arm depletion while exhibiting a white color. Strains that had maintained purple colors were also selected and sequenced, and we verified that these strains had retained the extra copy of chrR. These analyses linked the gene dosage on the left arm of ChrR to the purple color of colonies on CHROMagar. Furthermore, one strain derived from clinical isolate CAY12902 was found to have an incomplete segmental

deletion (~ 200 genes, 628 kb) of chrR (Figure 4e). This strain exhibited a white-ish colony phenotype on CHROMagar similar to strains lacking the entire left arm of chrR. These results implicate the deleted region on chrR as being a major driver in determining the CHROMagar color.

3.5 Chromosome R Region Associated with Fluconazole Resistance

Previous studies have linked aneuploidies of chr5 and chrR in *C. albicans* to resistance to the azole class of antifungal drugs [11, 13]. We therefore evaluated the response to fluconazole in strains harboring a trisomy of chrR versus parental euploid controls. Drug susceptibility tests were performed on isogenic versions of CAY12902 including: 1) CRISPR edited version, 2) 3xchrR version, and 3) the parental white euploid strain. Our results revealed that 3xchrR strains showed higher resistance towards fluconazole than the isogenic 2xchrR strain. Upon loss of the left arm of chR, this resistance phenotype was lost. The segmental deleted strain, however, largely maintained the resistance advantage, demonstrating the factors that contribute to the fluconazole resistance phenotype are outside of this deleted segment. This result indicates that aneuploidy of chrR can increase fluconazole resistance in the clinical MSKCC isolate and begins to narrow the region(s) responsible for this phenotype.

Figure 1



Candida albicans Color Variations Observed on CHROMagar Media

c.

Figure1: Representative for color variations derived from *C. albicans* clinical isolates on CHROMagar. The 138 MSKCC clinical isolates were directly plated on CHROMagar medium for 14 d at room temperature and 55 strains chosen for further analysis based on color variations.
a) Schematic of clonal color variants identified on CHROMagar plates. Two colonies exhibiting varied colors (white parental vs purple progeny) were subcloned to obtain pure populations. Colonies from the same parental strain form a comparison group for further analyses.
b) Different colony colors can be stably maintained on CHROMagar.
c) Phylogenetic tree indicated the relationship across 50 clinical isolates used in this study along with 17 representative clade genomes downloaded from NCBI. Samples were subdivided based on the current established clades. Clusters established in the first version were annotated with the number 1-18 while the newly added clusters were named with A-Z.

Figure 2

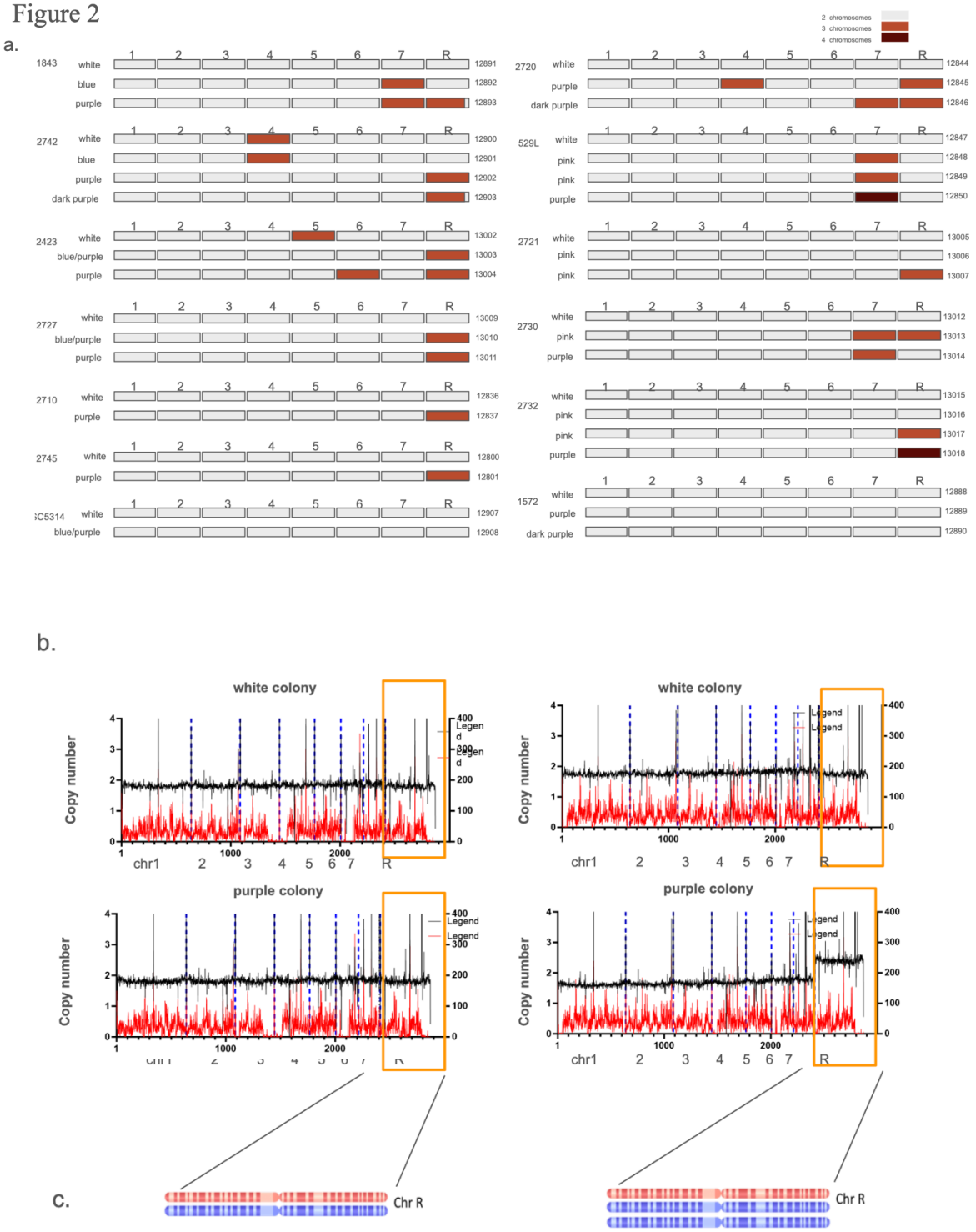
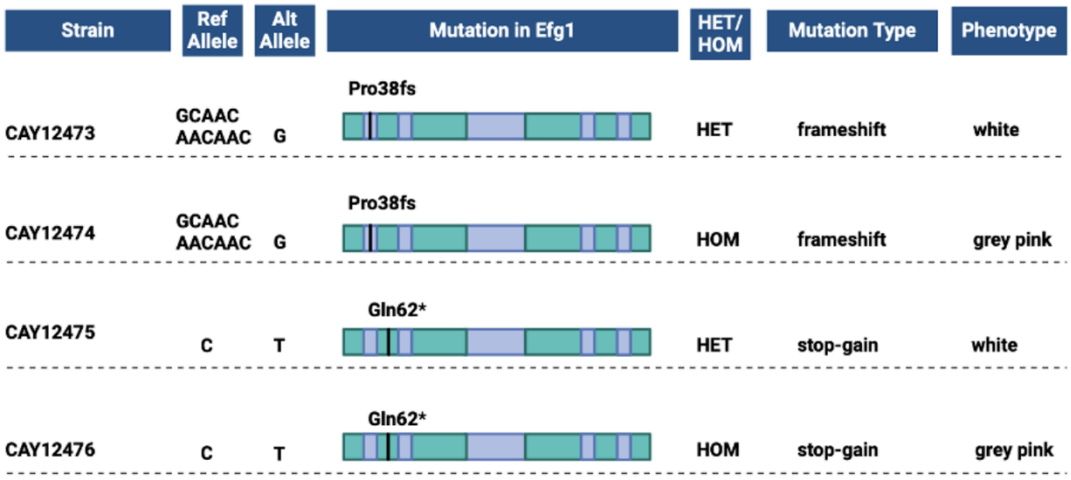


Figure2: Genomic analysis of the *C. albicans* clinical isolates strains. Whole genome sequencing analysis was performed on the strains to detect the changes in ploidy, LOH and genomic variants.

a) Summary for estimated chromosome numbers in the clinical strains calculated from in coverage plots with the reads mapped to the 10 kb windows.
b) Coverage and heterozygosity plots of the two genotypes found in most of the strains: euploidy strains and aneuploidy chrR.
c) An illustration showing that purple variants arose that were associated with aneuploidy of ChrR or no change in karyotype.

Figure 3

a.



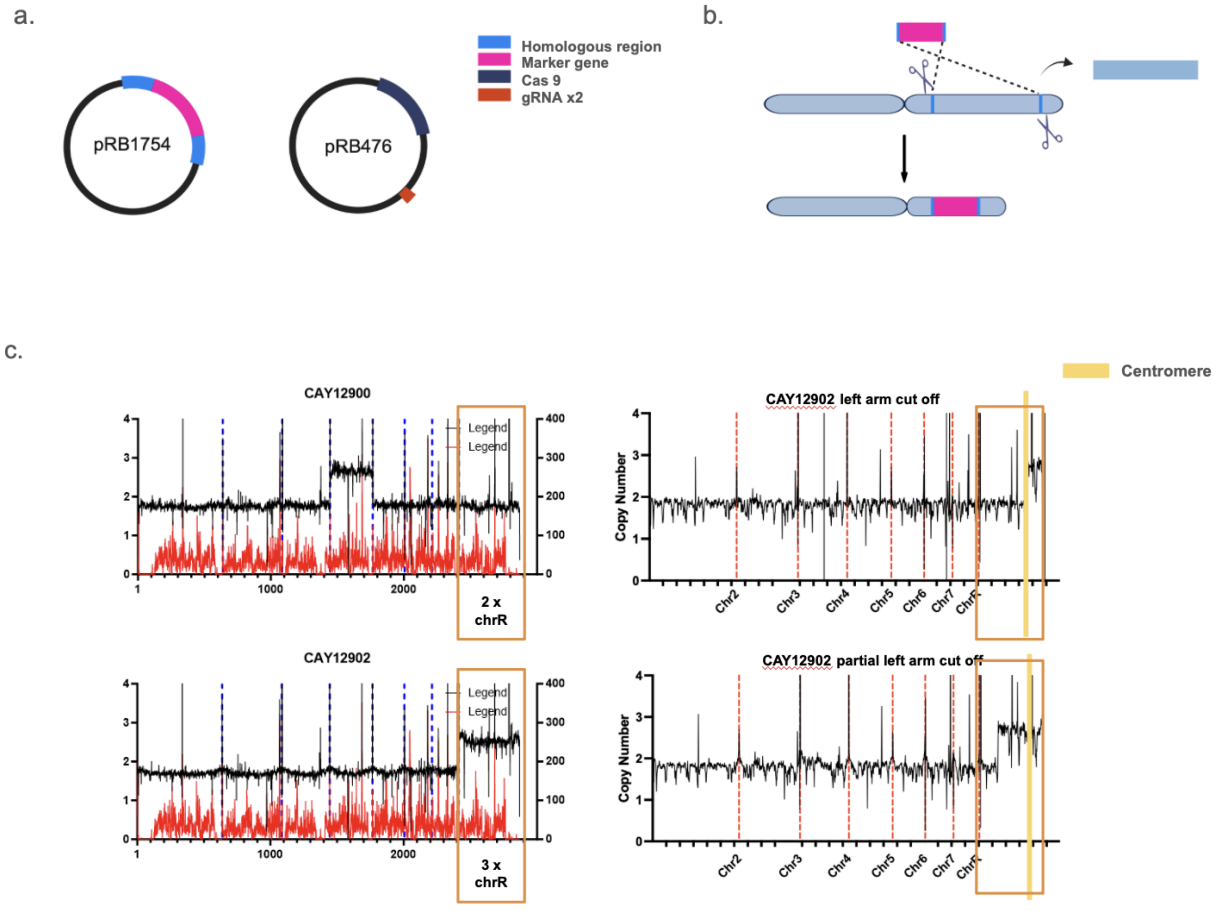
b.

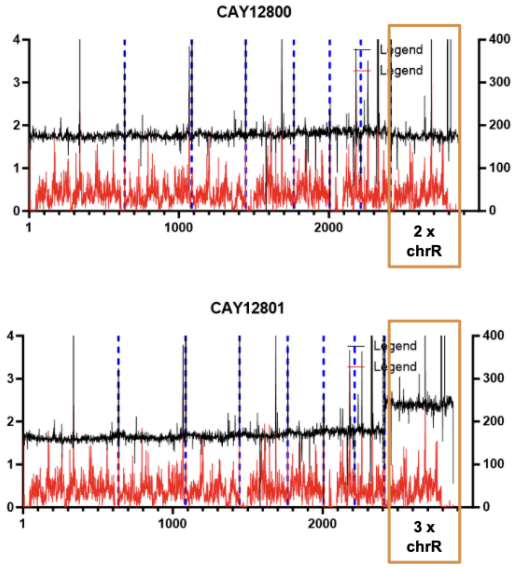
orf19.6115	**TLO34**
orf19.2449	**FGR 6-3**
orf19.2061	**RPN1**
orf19.4511	**APL1**
orf19.1893	**FGR23**
orf19.4636	**FGR28**
orf19.1935	**FGR 6-1**
orf19.4055	**SPT6**
orf19.104	**MIS12**
orf19.105	**OPT1**
orf19.6911	**SRP40**
orf19.6547	**EFG1**
orf19.6463	orf19.6644
orf19.2539	orf19.3713
orf19.2847.1	orf19.609

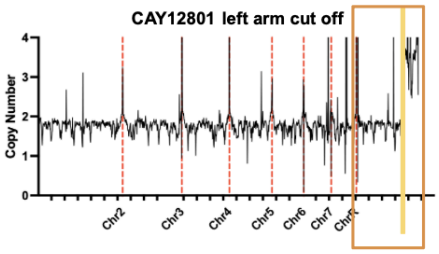
Figure3: *EFG1* variants identified for the color variations in euploidy pairs. Sequencings were screening for variant changes across white-gray(light green) strain pairs.

a) Table for *EFG1* mutations found in two pairs of comparison groups in the euploidy group, including information in the following: strain number, ref genome, alt genome, amino acid position in *EFG1* genome, mutation type, and phenotypes displayed on CHROMagar.
b) 32 mutations after manual filters in different strains pairs that haven't been fully investigated nor discussed in this study. (More to say about this data?)

Figure 4

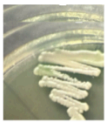


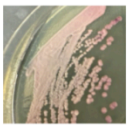




d.

RT, 17 d CHORMagar

Strains	12900: 2 x chrR	12902: 3 x chrR	chrR left arm cut off	chrR partial left arm cut off
Phenotypes				

Strains	12800: 2 x chrR	12801: 3 x chrR	chrR left arm cut off
Phenotypes			

e.

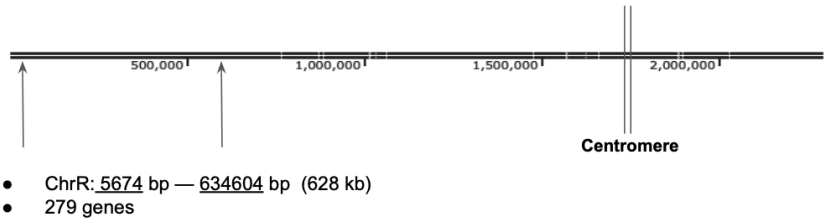


Figure4: Deletions in chrR alter phenotypes on CHROMagar. Illustration of the chrR

deletion experiments in strains with three copies of chrR.

a) Schematic of the constructs for the repair template and gRNAs. The repair template plasmid contained a 5375 bp fragment insertion consisting of the SatR marker (4375 bp) along with SC5314 homology (500 bp) on each side. Two gRNA plasmids and a plasmid expressing Cas9 were also used.
b) Brief workflow of plasmid construction using golden gate assembly, and chromosome editing using CRISPR Cas9. The lower panel shows the expected genotypes after arm deletion.
c,d) Phenotypes of the control and edited strains after 17 d on CHROMagar along with coverage plots showing the chromosome complements.
e) A deletion involving 279 genes caused color variation on CHROMagar.

Figure 5

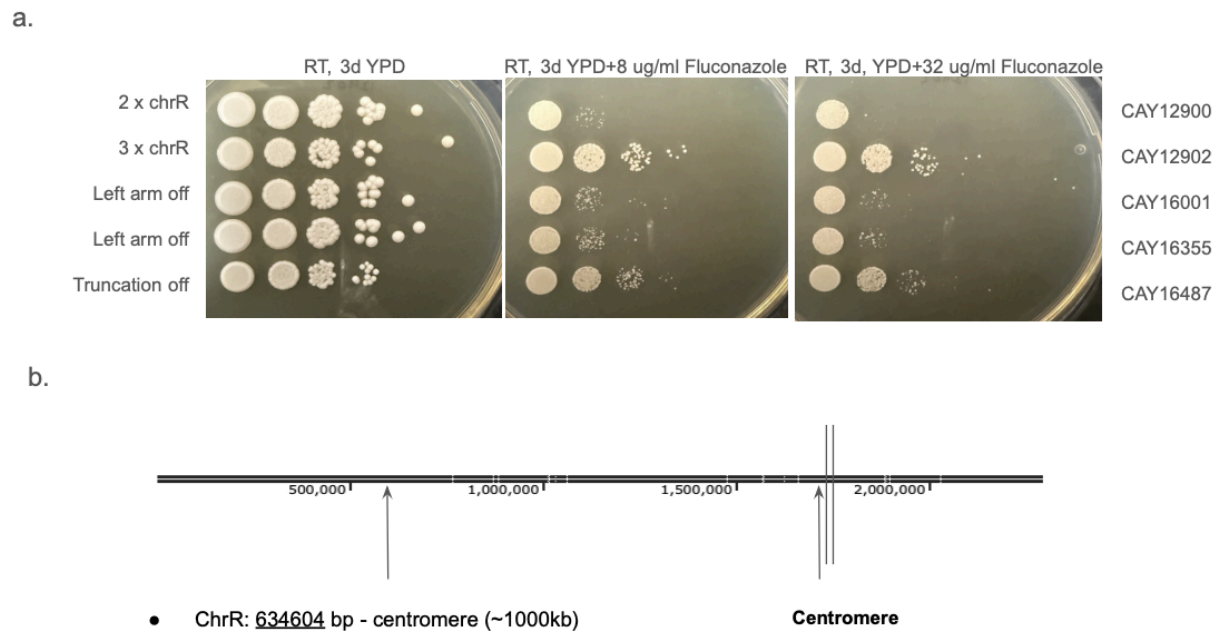


Figure5: The CAY12900/12902 series of strains showed a difference in sensitivity to fluconazole.
a) Dilution spot assays were performed on CAY12900/CAY12902 and derived strains +/- fluconazole: YPD + 0 µg/ml fluconazole, YPD + 8 µg/ml fluconazole, and YPD + 32 µg/ml fluconazole, along with chromosome R ploidy numbers. Plates were grown for 3 days at room temperature.
b) Schematic of the truncation on chrR that its presence caused the fluconazole resistance difference.

Chapter 4 Discussion and Conclusion

CHROMagar media have been used in clinical trials to distinguish between infectious species since the early 2000s [13]. The mechanisms exhibiting species-specific derived color have been studied although less is known about CHROMagar variation within a single species. Previous studies expand the usage of CHROMagar to represent *C. albicans* species-specific morphology transitions (white-opaque states), and gene mutations including *EFG1* demonstrated mechanisms on *C. albicans* can alter color variations of colonies over CHROMagar, and

reversely can also be distinguishable well on CHROMagar [9]. We note that we used CHROMagar Candida while other labs have used other Chromagar formulations to examine *C. albicans* variation including that due to deletion of transcription factors [22].

In this study, we screened a large set of MSKCC isolates to evaluate their starting phenotypes on CHROMagar and whether variants arise. We found two strains harboring heterozygous *EFG1* mutations and that these strains could undergo LOH to become *efg1* null mutants and adopt green colonies on CHROMagar. This state has been referred to as the "gray" state due to the color of colonies on standard lab media [9]. Gray cells exhibit increased fitness over control strains in certain host niches including the antibiotic-treated mouse GI tract, as they lack the ability to filament [8]. Loss of filamentation itself leads to increased fitness in this niche. EFG1 has been described as a hotspot for mutations/adaptation due to frequent occurrence of polymorphisms in this gene and how loss of function can enable gut adaptation [25].

Much of our focus revolved around the acquisition of extra copies of chrR that were linked to adoption of a pink/purple coloration on CHROMagar. We found that strains harboring 3xchrR (or even 4xchrR) formed purple colonies whereas their euploid parents formed white colonies on this medium. Using a CRISPR-Cas9 methodology we were successful in deleting the left or right arm of chrR and linked the purple coloration to genes on the left arm. One strain with a narrower deletion on chrR also lacked the purple color thereby identifying a specific region on the left arm as being critical to this coloration. Interestingly, we found that extra copies of chrR were also linked to decreased susceptibility to fluconazole, in agreement with previous studies [8]. This phenotype was also driven by genes on the left arm of chrR but were not linked to the region responsible for purple coloration, indicating different genes impact these two phenotypes.

Future Directions and Limitations

Segmental deletions of chromosome R will further define the key regions responsible for purple coloration and fluconazole resistance/tolerance. However, one complication has been the instability in color variations and ploidy numbers in *C. albicans* strains. We narrowed down the instability in color pigmentations by selecting strains with stable phenotypes, but the ploidy instability scenario was maintained through the experiments based on WGS information, and the frequency of aneuploidy loss increased after chrR arm deletion. Chromosome copy number changes can be regulated by parasexual production, and environmental stress, and can be commonly found in euploidy fungi organisms [20]. Aneuploidy regulation in *C. albicans* can occur commonly, especially associated with environmental stressors and antifungal drugs. To accelerate our ability to verify aneuploid states without a need for WGS, the Bennett lab has begun testing whether fluorescence markers can be used to monitor target chromosomes. For this approach, constitutive red and green fluorescent reporters can be integrated onto target chromosomes (typically next to the centromere) and used to estimate the chromosome configurations. This approach appears valid and will potentially speed up our analysis of aneuploid/segmented chromosomes, or at least narrow down the strains to be further analyzed by WGS. Eventually the goal will be to identify the genes that are causative on chrR for driving changes in CHROMagar coloration and azole resistance, as currently it is unknown what are the *C. albicans* factors on chrR that are contributing to these phenotypes.

Appendix:

Table1: Chromogens used in CHROMagar and their compositions.

Chromogens	Composition
Glucosaminide Chromogens	X-GluNAc (Glu is glucose, NAc is an N-acetyl group and X is a chromophore) - 5-bromo-4-chloro-3-indolyl N-acetyl B-D-glucosaminide - 5-bromo-6-chloro-3-indolyl N-acetyl B-D-glucosaminide,
Galactosaminide Chromogens	X-GalNAc (wherein Gal is galactose, NAc is an N-acetyl group and X is a chromophore)
phosphate-based Chromogens	Phosphate group: - 5-bromo-6-chloro-3-indolyl phosphate p toluidine salt - 5-bromo-4-chloro-3-indolyl phosphate p toluidine salt - 6-chloro-3-indoxylphosphate.

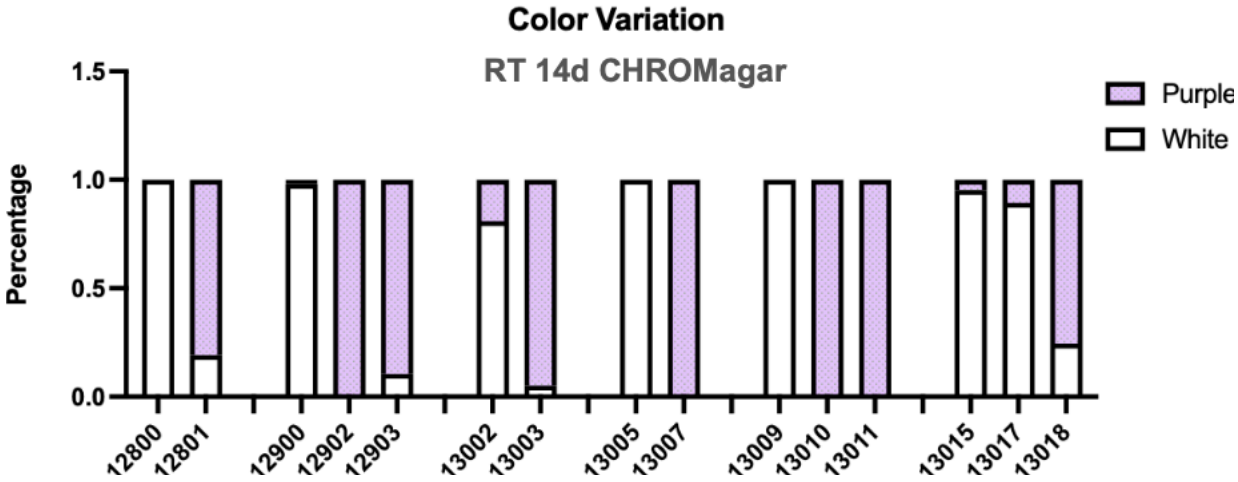


Figure 1: Chromosomal Instability in *C. albicans* as Measured by Colony Color Variation. Instability was observed in *C. albicans* strains, as reflected by the number of colonies displaying distinct colors after 100 days of incubation. The data are presented as bar graphs, representing the frequency of each colony color

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