Characterization of a novel cell cycle regulator Csa6 in the human fungal pathogen *Candida tropicalis*

A thesis submitted for the partial fulfillment of the degree of

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by

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DECLARATION

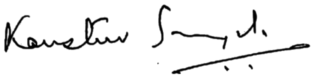
I hereby declare that the work described here in this thesis titled '**Characterization of a novel cell cycle regulator Csa6 in the human fungal pathogen *Candida tropicalis***' has originally been carried out by myself under the guidance and supervision of Prof. Kaustuv Sanyal, Professor, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru-560064, India.

Harshit Arya

Date: 28.04.2022

CERTIFICATE

This is to certify that this thesis entitled '**Characterization of a novel cell cycle regulator Csa6 in the human fungal pathogen *Candida tropicalis'*** submitted by Harshit Arya, as part of the project for the Master of Science degree at Jawaharlal Nehru Centre for Advanced Scientific Research, was based on the studies carried out by him under my supervision and guidance.



Kaustuv Sanyal

Date: 28/04/2022

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Harshit Arya

ABBREVIATIONS

ºC	Degree Celsius
hrs	Hours
rpm	Revolution per minute
Ca	*Candida albicans*
Ct	*Candida tropicalis*
Cd	*Candida dubliniensis*
OD_{600}	Optical density at 600 nm
O/N	Overnight
SPB	Spindle pole body
kb	Kilo base pair
bp	Base pair
mM	Milli molar
gDNA	Genomic DNA
PCR	Polymerase chain reaction
NAT	Nourseothricin acyl transferase
ml	Milli litre
ORF	Open reading frame
GFP	Green fluorescent protein
µl	Micro litre
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis

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INTRODUCTION

Cell division

Cell division is a process by which a cell divides and segregates its genomic and cytoplasmic contents to the daughter cell. Cell division is a crucial phase in every organism's life cycle. It serves as a mode of reproduction for unicellular organisms such as bacteria, fungi, protists, etc. While for multicellular organisms, cell division plays crucial roles in growth, development, repair, healing, maintenance, and renewal. The series of events a cell undergoes before and during cell division is called the cell cycle. The cell cycle reflects the temporal segregation of events and phases during cell division and can be broadly categorized into four phases G1 phase, S phase, G2, and M phase.

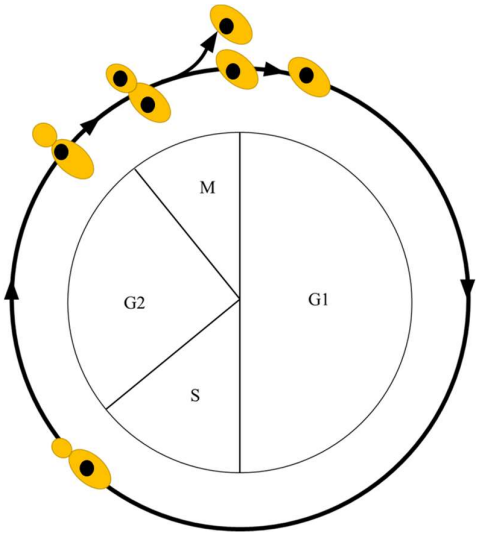


Figure 1: Cell cycle phases in budding yeast. An unbudded cell enters G1 phase and buds as it progresses through the cell cycle. During subsequent stages, nucleus divides and segregates between the two cells. Nuclear division is accompanied with cytokinesis to form two daughter cells. Orange colored ovals represent budding yeasts. Black colored circles represent nucleus.

Cells grow larger in size in the G1 phase. In the S phase, cells undergo DNA replication. In the G2 phase, cells prepare themselves for chromatin and cytoplasm segregation. G1, S, and G2 phases together represent interphase, a phase between two mitotic divisions. Post-G2 phase, the cell enters M-phase. M-phase generally refers to the mitotic phase, an equational division wherein a nucleus is equally divided between the two cells with chromosome content as is in the mother cell. In germ cells, M-phase is often represented by the meiotic phase, a reductional division wherein a nucleus is divided between four cells with chromosome content half as is in the mother cell. Two division phases accompany meiotic divisions called meiosis I and meiosis II.

Though meiosis is restricted to germ cells, mitosis is a general characteristic of living cells. The M phase in mitosis can be primarily divided into four phases prophase, metaphase, anaphase, and telophase. In prophase, the loosely packed chromatin condenses prior to its segregation. During

metaphase, microtubules emanated from microtubule organizing centers (MTOCs) get attached to kinetochores surrounding the centromeres. Proper biorientation of the mitotic spindle marks the end of metaphase and the beginning of anaphase, wherein the duplicated chromosomes are segregated between the two daughter cells. Once the chromosomes reach opposite poles, the chromosomes start to decondense and mark the last stage of the M-phase, the telophase. Nuclear segregation is followed by cytoplasmic separation during cytokinesis. During cytokinesis, two cells are separated as distinct cytoplasmic compartments and are accompanied by plasma membrane and cell wall (if present) synthesis at the division junction.

Regulation of cell cycle

Cell division is a crucial phase and any alteration in genome duplication or chromosome segregation can lead to erroneous effects like aneuploidy mediated by chromosome gain or loss, genome instability events. Therefore, the cell cycle is tightly regulated. The central control of the cell cycle is mediated by cyclins and cyclin-dependent kinases (CDKs). The cyclic levels of cyclins activate their cognate CDK, which are serine/threonine kinases that phosphorylate nuclear lamins, microtubules, and other regulatory proteins (Arellano and Moreno, 1997; KA, 1998). CDK- cyclin complex is the active state of a CDK and is guided towards its substrate via localization signal present on cyclins. Rapid degradation of cyclins by ubiquitin- proteasome pathway ensures CDK activity only at the required phase of the cell cycle (Arellano and Moreno, 1997; KA, 1998).

Other than cyclins and CDKs, cell cycle checkpoints exist that act as a halt if any process during the cell cycle is error prone. Cell cycle checkpoints are defined as “biochemical pathways that ensure dependence of one process upon another process that is biochemically unrelated”(McGowan, 1996). As an example, improper genome duplication can lead to a prolonged S phase or an S phase arrest.

Once the DNA replication checkpoints are crossed, cells enter the M phase, wherein the chromosomes segregate. Chromosomes segregation is a highly complex and delicate phenomenon. The majority of budding yeasts show an arrangement of chromosomes wherein centromeres of all the chromosomes cluster together. This is called kinetochore clustering. Kinetochores are clustered throughout the cell cycle in *Saccharomyces cerevisiae, Candida sp.,* etc. However, they can be clustered only in large budded cells with a budding index of 0.4 – 0.55 in *Cryptococcus neoformans* (Kozubowski *et al.*, 2013). Centromeres can be identified by specific loading of a histone H3 variant CENP-A (Cse4 in yeasts). These sites marked by CENP-A serve as the region where kinetochore is assembled. The kinetochore is a multiprotein subunit complex comprising inner and outer kinetochore. Inner kinetochore lies closest to the centromere and is represented by the constitutive centromeric associated protein (CCAN) network. The CCAN network consists of 16 CENP proteins in *S. cerevisiae* and links the centromeric chromatin to the outer kinetochore. The outer kinetochore consists of the KMN complex and the Dam1 complex. The KMN complex comprises three sub-complexes Knl1 complex, Mtw1 complex, and

Ndc80 complex. The KMN complex helps in the attachment of microtubules to the kinetochore by the ability of the Knl1 complex and Ndc80 complex to bind to the microtubules. The Dam1 complex is a ring-like complex through which microtubules attach to the KMN complex (Musacchio and Desai, 2017).

Kinetochores are attached to the microtubules emanated from spindle pole bodies, the functional homolog of metazoan centrosomes in yeast, to form a stable mitotic spindle. SPBs movement towards the opposite poles help in the proper segregation of the chromosomes. Other than segregating the chromosomes apart, kinetochores and SPBs are also the site of spindle assembly checkpoint (SAC) and spindle positioning checkpoint (SPOC) (Musacchio and Desai, 2017). Both SAC and SPOC guard metaphase to anaphase transition, wherein SAC checks for correct microtubule-kinetochore attachments and SPOC checks for correct positioning of the mitotic spindle during mitosis. The spatial proximity of SPBs to the kinetochores helps recognize spindle assembly defects. Errors in any of the two can lead to a metaphase arrest.

Strict cell cycle surveillance ensures faithful chromosome segregation as any error in chromosome segregation can be disastrous. Chromosome mis-segregation events lead to genome instability. Genome instability is often reported by the loss or gain of chromosomes leading to aneuploidy, whole-genome or segmental duplications, cell cycle arrest, or chromosome instability (CIN) (Diogo *et al.*, 2009). Chromosome instability is often associated with catastrophic effects such as cell cycle arrest, increase in ploidy, delayed growth, or loss of viability. It can also be a leading cause of increasing resistance to particular drugs, especially in *C. albicans* or *C. tropicalis* (Guin *et al.*, 2020; Sankaranarayanan *et al.*, 2020).

Budding yeast as a model system

Budding yeast serves as an excellent model to study cell cycle-related phenomena. Being a unicellular eukaryote, yeast cell cycle stages are relevant and similar to higher metazoans, including humans. Each cell cycle stage in budding yeasts depicts a clear morphological attribute peculiar to that stage (Legrand *et al.*, 2019). Cell cycle stages can be roughly identified in budding yeasts by calculating their budding index. The budding index is defined as the ratio of the diameter of the daughter bud to the diameter of the mother bud. When budding yeast starts to enter mitosis, the equational division, a small bud appears on one end of the cell and demarcates the G1/S phase of yeast with a budding index of ≥ 0.2 (Sreekumar *et al.*, 2021). During the S phase, the genome duplicates and is ready to be divided between the two buds. During the onset of the M phase, the bud grows bigger in size with a budding index of 0.1 and can reach up to 0.9 during the late M phase stage. The duplicated chromosome gets attached to the microtubules emanating from the spindle pole bodies through the kinetochore, and the nucleus appears to be present at the bud neck. This stage can be recognized as prophase/metaphase with a budding index of 0.2 to 0.6. The onset of anaphase is marked by the segregation of the duplicated chromosomes

between the two buds and a budding index of 0.4 to 0.9 (Sreekumar *et al.*, 2021). Once the segregated chromosomes reach both the buds, this marks the beginning of cytokinesis and the separation of the daughter bud from the mother cell. The divided cells can then enter a new round of mitotic cell division.

Candida sp.

Candida sp. is an ascomycetous fungi belonging to the CTG-Ser1 clade of fungi. Fungi belonging to CTG-Ser1 clade code for Serine instead of leucine for CUG codon. *Candida sp.* are human commensals often found in mucosal linings of healthy individuals like urinogenital tract and gut. However, they can become invasive in immunocompromised individuals and cause oral thrush, candidiasis, and other *Candida* infections (Legrand *et al.*, 2019). Most nosocomial fungal infections are represented by this group of ascomycetous fungi, especially *C. albicans*.

Along with clinical relevance, *Candida* sp. genome is highly plastic and can tolerate genome level rearrangements and loss of heterozygosity. Loss of heterozygosity events are caused by loss of one of the homologous chromosomes and subsequent duplication of the retained chromosome to achieve homozygosity. Other than *C. albicans, C. tropicalis* is also an increasing concern due to its remarkable anti-fungal drug resistance and prevalence in tropical countries like India. Though several factors may contribute to its gain of resistance, the aneuploidy-mediated drug resistance induced by chromosomal instability events remains the principal suspect. Since *Candida* genome is plastic and can tolerate fluctuations in copy number and genome-level rearrangements, copy number variations in drug resistance genes have been identified to cause drug resistance in these organisms (Guin *et al.*, 2020). Therefore, it becomes vital to identify regulators of genome stability in these fungal pathogens, which can be used as potential drug targets.

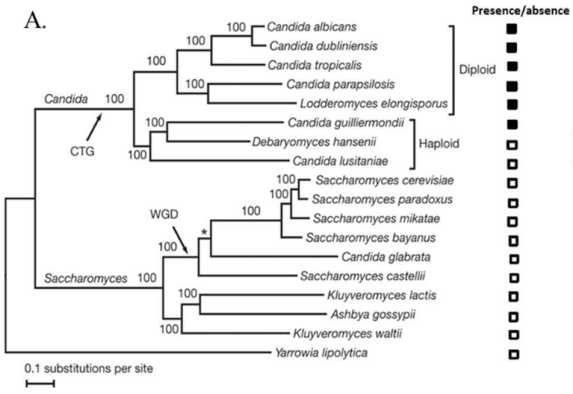
Csa6 as a regulator of genome stability

Jaitly et al., 2021 (BioRxiv, 2021) identified six regulators of genome stability in a high throughput screening. The overexpression of these genes led to an increase in chromosomal instability events, which could be identified using a reporter strain. In this reporter strain, GFP and BFP genes were integrated at the same intergenic locus on chromosome 4a and chromosome 4b, respectively. Loss of either BFP or GFP would represent an LOH event. The genes, overexpression of which were responsible for such LOH events, were termed chromosome stability (CSA) genes. Of the six CSA genes (*CSA1*^{CLB4}, *CSA2*^{ASE1}, *CSA3*^{KIP2}, *CSA4*^{MCM7}, *CSA5*^{BFA1,} *and CSA6*), *CSA6* was unique without any known orthologs in *S. cerevisiae* and is restricted to only a few members of the CTG-Ser1 clade of ascomycetes. *CSA6* is an essential gene for viability and localizes to spindle pole bodies. The overexpression of *CSA6* led to a *MAD2*-dependent metaphase arrest, while its depletion led to anaphase/telophase arrest due to spindle assembly defects. Interestingly, the expression of *C. dubliniensis* ortholog of *CSA6* could rescue

cell cycle arrest in a *CSA6*-depleted *C. albicans* strain. Along with functional complementation, *CdCSA6* also colocalizes to spindle pole bodies similar to *CaCSA6*.

Rationale of the current study

A critical regulator of the cell cycle, Csa6, was phylogenetically restricted which intrigued us to search for homology and synteny between different orthologs. BLAST analysis revealed only limited sequence similarity between various orthologs. *C. dubliniensis* Csa6 (CdCsa6) was most similar to *C. albicans* Csa6 (CaCsa6) with ~79% similarity, followed by *C. tropicalis* (~25%), *C. parapsilosis* (~14%), *C. orthopsilosis* (~14%), *Lodderomyces elongisporus* (~15%), *Debaromyces hansenii* (~12%), and *C. guillermondii* (~14%).



B.

			cov	pid
C. albicans	1	C2_01420C_A	100.0%	100.0%
	2	CAWG_03915	100.0%	97.4%
C. dubliniensis	3	Cd36_16290	98.7%	79.6%
C. tropicalis	4	CTRG_01235	88.7%	25.0%
C. parapsilosis	5	CPAR2_214050	95.0%	14.4%
C. orthopsilosis	6	CORT_0A13370	93.5%	14.5%
L. elongisporus	7	LELG_01137	98.3%	15.0%
D. hansenii	8	DEHA2A01738g	86.4%	12.6%
C. guillermondii	9	PGUG_04951	67.1%	14.6%

Figure 2. Csa6 is a phylogenetically restricted protein. A. Phylogenetic tree indicating the presence or absence of orthologs of Csa6 (adapted from Butler et al. 2009). Csa6 orthologs are only present in a few members of the CTG-Ser1 clade. B. Percentage similarity between orthologs of Csa6 (from Candida genome database) cov: Coverage; pid: percentage identity.

C. dubliniensis is closest to *C. albicans* on the phylogenetic tree and shares the most similarity in the Csa6 sequence. We aimed to address if ectopic expression of the next closest homolog would also be able to rescue Csa6 depletion mediated cell cycle arrest. As mentioned, CtCsa6 is only ~25% similar to CaCsa6, and therefore it would be interesting to see if a protein with limited sequence similarity can also serve the same purpose.

RESULTS

1. **Csa6 is essential in *C. albicans*, and its depletion can be rescued by *C. tropicalis* Csa6**

To assess whether Csa6 orthologs can functionally complement the depletion of Csa6 in *C. albicans*, *CaPJ301* (*SN148 csa6Δ/PmetCSA6 TUB4/TUB4 mCherry*) was used. MET3 promoter (represented by Pmet) is a repressible promoter (Care *et al.*, 1999). The addition of methionine and cysteine in the media represses the gene under MET3 promoter. Since Csa6 is an essential gene, CaPJ301 fails to grow in the repressible medium containing 5mM methionine and 5mM cysteine. Still, it can grow in permissive medium lacking methionine and cysteine, as seen in Fig. 3C.

Csa6 ortholog in *C. tropicalis* was identified as CTRG_01235 in FungiDB. HA004 and HA005 were used to amplify CtCsa6 with 1kb upstream of the start site, including a putative promoter region. The amplicon was then cloned in pTP16, and the final construct pHA001 was digested by StuI and transformed in CaPJ301 to obtain CaHA001, CaHA002, and CaHA003, wherein CtCsa6 was C-terminally tagged with GFP and integrated into the RPS-1 locus (Fig. 3A). The obtained transformants were confirmed by PCR (Fig. 3B)

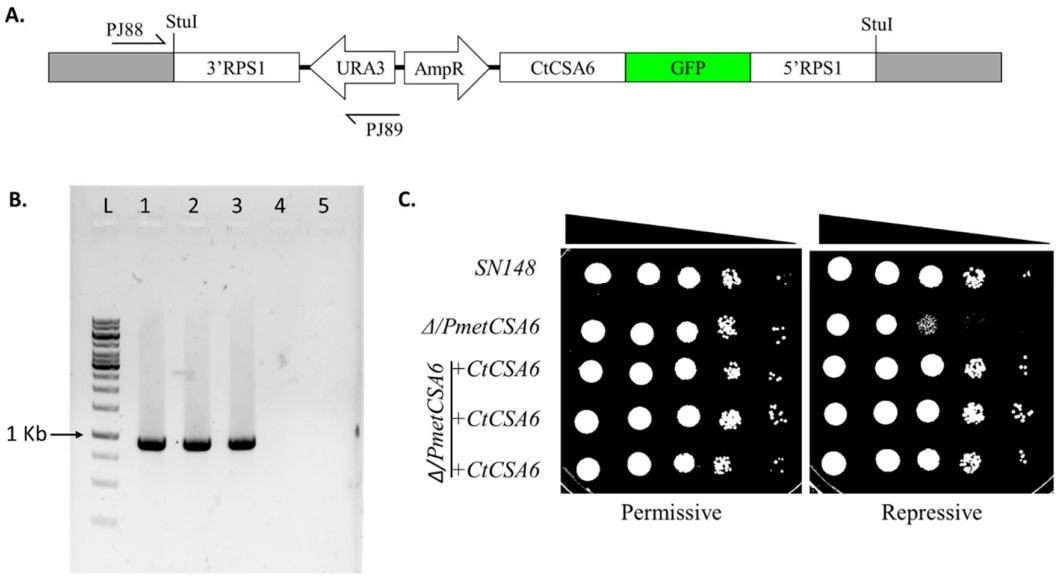


Figure 3. Ectopic expression of CtCsa6 is sufficient to rescue CaCsa6 depletion mediated growth defect. A) genomic integration of CtCSA6-GFP in PJ301 and primer pair for strain confirmation. B) PCR confirmation of strains with primer pair PJ88 and PJ89. Lane L represents ladder, Lane 1 to 3 represents three independent biological transformants, Lane 4 represents parent strain CaPJ301, and Lane 5 represents No template control. An expected band of ~ 1kb confirms the correct transformants as seen in lanes 1, 2, and 3. C) Spotting dilution assay to check for functional complementation of CaCsa6 repression by CtCsa6 in permissive and repressive media (5mM methionine and 5mM cysteine). The delay in growth in promoter shut down mutant was rescued upon expression of CtCsa6.

Overnight grown cultures of SN148, CaPJ301, CaHA001, CaHA002 and CaHA003 were diluted and spotted on CM Met- Cys- Ura+ agar plates (permissive conditions) and CM Met+ Cys+ Ura+ agar plates (repressive conditions). *C. albicans* fails to grow upon repression of CaCsa6 but can grow upon integration of CtCsa6, as shown in Fig. 3C. This indicates that Csa6 is essential in *C. albicans* and CtCsa6 can rescue growth defects caused by CaCsa6 depletion.

2. CtCsa6 expression rescues large bud arrest induced upon CaCsa6 depletion

Repression of CaCsa6 was shown to arrest cells in the large-budded stage (Jaitly and Sanyal, 2021). To test whether the expression of CtCsa6 rescues the cell cycle arrest induced upon CaCsa6 depletion, PJ301, CaHA001, CaHA002 and CaHA003 were grown overnight in YPD + uridine and then inoculated in 5ml permissive (YPD uridine) and repressive (YPD uridine + met + cys) for 6 hours. The cells were observed under the microscope. As seen in Fig. 4A, unbudded cells were identified as cells lacking any bud, small-budded cells were identified as cells with a budding index of 0.1 to 0.4, large-budded cells were identified as cells with a budding index of >0.4 (Sreekumar *et al.*, 2021) and chain of cells were identified as an array of more than two cells.

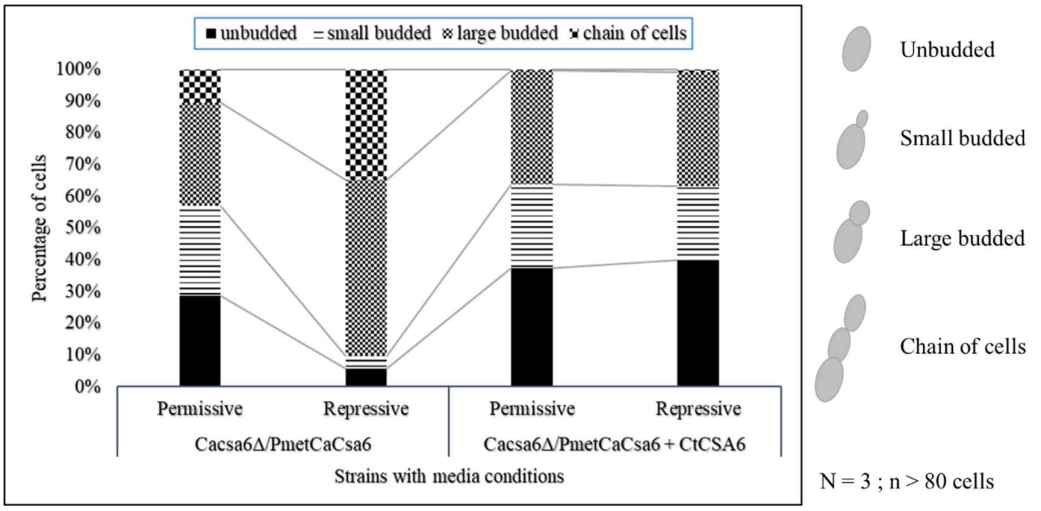


Figure 4. Microscopic profile of CaCsa6 promoter shut down mutant vs. when complemented by CtCsa6. Repression of CaCsa6 leads to an increased population of large-budded cells and chain of cells. However, a recycling population of all cell cycle stages can be seen after the integration of CtCsa6.

As shown in Fig. 4, Csa6 depletion arrests *C. albicans* in the large-budded stage, wherein around 90% of cells were either large-budded or in chains. Expression of CtCsa6, however, rescues the cell cycle arrest caused by CaCsa6 depletion resulting in a regular cycling population of all cell cycle stages around 30% each (N = 3, n ≥ 100).

3. **CtCsa6 localizes to spindle pole bodies at all stages of the cell cycle**

To check for the localization of CtCsa6 in *C. albicans*, CaHA001, CaHA002 and CaHA003 were grown overnight in YPD uridine and further inoculated in YPD uridine to achieve a final OD_{600} of 0.2OD/ml cells. The cells were grown for 6 hours at 30ºC at 180rpm. Cells were then observed under the fluorescent microscope. Captured images were analyzed with ImageJ software, and >100 cells were checked for localization of GFP and mCherry signal. Representative images in Fig. 5 show that CtCsa6 localizes to spindle pole bodies at all cell cycle stages.

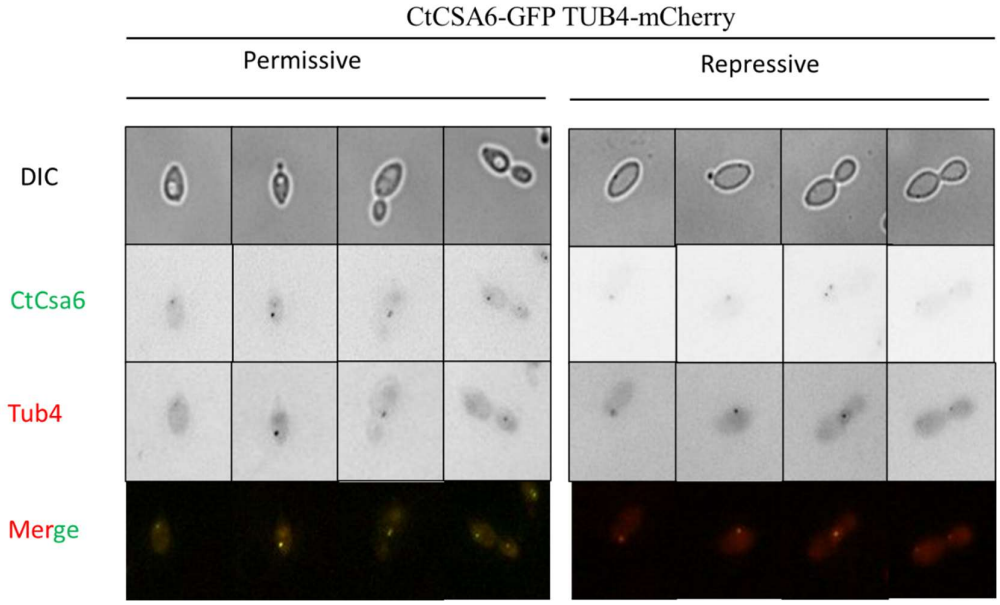


Figure 5. Subcellular localization of CtCsa6 in *C. albicans*. Fluorescence microscopy of CtCsa6 tagged to GFP and Tub4 tagged to mCherry in both CaCsa6 expression and CaCsa6 repression, respectively. Both the GFP and m-Cherry signal colocalizes at all stages of the cell cycle in *C. albicans*. Permissive media lacks methionine and cysteine whereas repressive media is supplemented with methionine and cysteine.

4. **First copy deletion of Csa6 in *C. tropicalis***

To understand the role of Csa6 in *C. tropicalis*, we first deleted one copy of Csa6 by using the construct described below. 360 bp region upstream of CtCsa6 was cloned with the primer pair HA018 and HA019 between KpnI and XhoI in pSFS2a plasmid to get pHA002. 600 bp region from the start site was cloned with the primer pair HA020 and HA021 between SacI and SacII site in pHA002. The final construct, pHA003, was transformed in UHA103, a triple auxotroph of *C. tropicalis*, after digesting with KpnI and SacI (Fig.6B). The obtained transformant CtHA011 was confirmed by PCR with the primer pair HA022 and PJ3 (Fig. 6A and C). The NAT marker was recycled by growing CtHA011 in YP + 2% maltose and then patching on YPD and YPD+NAT plates. The obtained strains CtHA012, CtHA013, and CtHA014, grows

comparable to the parent strain UHA103 indicating the gene is haplo-sufficient or non-essential.

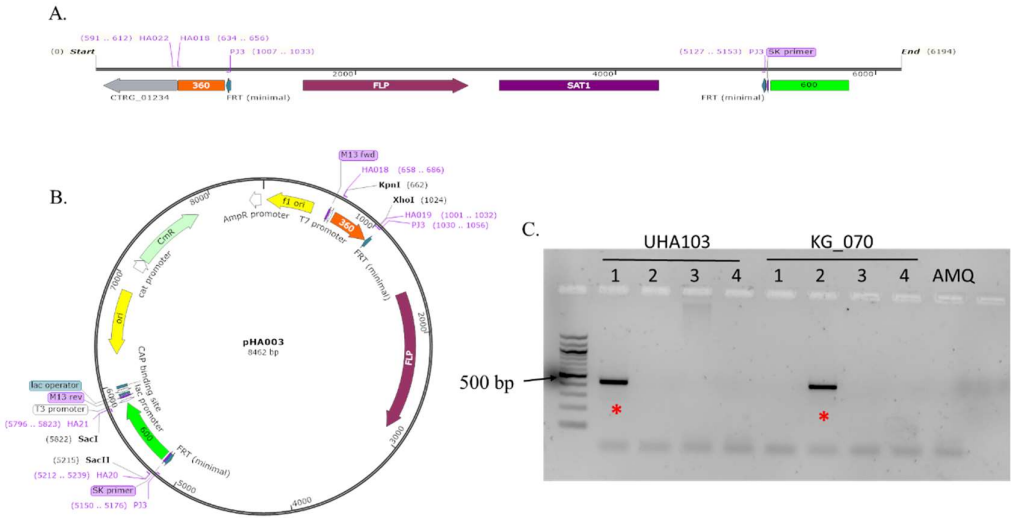


Figure 6. Construction of CtCsa6 first copy deletion strain. A) Map showing genomic integration of pHA003 where 360 represents 360 bp upstream region, FLP- FRT represents the flippase cassette, SAT1 represents nourseothricin acyl transferase, and 600 represents 600 bp downstream region. B) Plasmid map for pHA003 used for transformation after KpnI and SacI digestion. C) PCR confirmation of pHA003 integration by primer pair HA022 and PJ3. Lane 1 to 4 represents independent biological transformants in two different genetic backgrounds UHA103 and KG_070 (NUF2/NUF2-GFP), respectively, and lane AMQ represents no template control. A band of around 450 bp represents the correct integration. One positive transformant in each UHA103 and KG_070 was used for marker recycling.

5. GAL1 promoter mediated overexpression of CtCsa6.

To check whether overexpression of CtCsa6 causes cell cycle arrest as mediated by CaCsa6 overexpression, we aimed to construct a strain where one copy of CtCsa6 is under the control of galactose promoter in wild type genomic background. pGAL-TAP-HIS1 was used to clone 360 bp of upstream region amplified by primer pair HA023 and HA024 between SacI and SacII sites to form pHA004. 500 bp of ORF homology region was amplified by primer pair HA025 and HA026 and cloned between KpnI and ApaI site in pHA004 to form pHA005. The final cassette was released by KpnI and SacI digestion and transformed in UHA103. The obtained transformants were confirmed by PCR (Materials and methods), and positive strain HA051 was inoculated overnight in YPDU, followed by secondary inoculation in YPU + 2% galactose. Cells were collected every 2 hrs, and protein levels were determined by western blot analysis. The protein started to overexpress after 6 hrs of galactose induction.

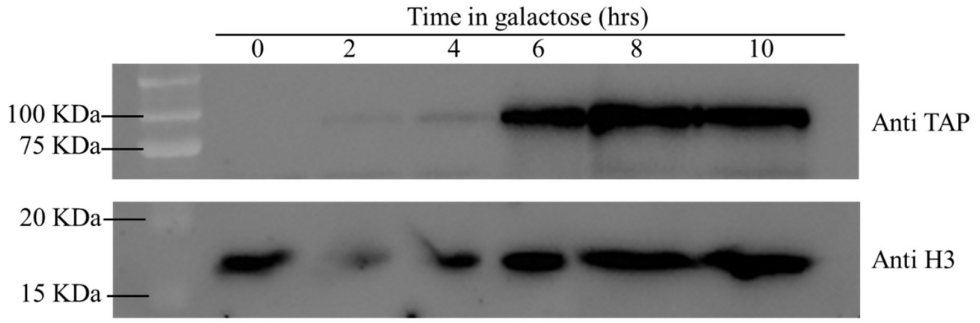


Figure 7. CtCsa6 levels increase after 6 hours of galactose induction. Western blot with anti-TAP antibodies and histone H3 as the loading control. The signal intensity increases upon incubation in galactose post 6 hours, and the signal is completely absent when cells are grown in the presence of dextrose.

6. **Overexpression of CtCsa6 might not affect cell cycle progression in *C. tropicalis*.**

To score for overexpression mediated cell cycle effect, cells were collected after 6 hrs of growth in YPGU. After Hoechst staining, cells were visualized under a fluorescent microscope and categorized as unbudded, small budded, and large budded, as previously described. Post 6 hrs of galactose induction, cells appear to be cycling as wild type as shown in Fig. 6. Since this experiment has been done with only one transformant, at least two more transformants are required to support the data. However, there is no observed cell cycle defect when done in three technical replicates. This indicates that the overexpression of CtCsa6 mediated by the GAL1 promoter might not cause any cell cycle-related arrest. The cells grow comparable to wild type on both dextrose and galactose-containing media (data not shown).

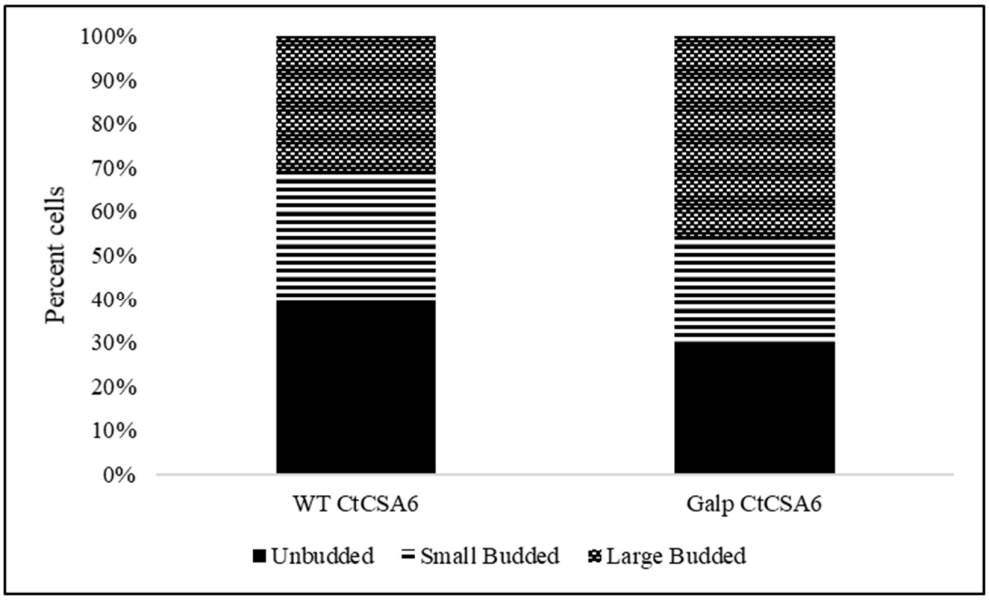


Figure 6. Graphical distribution of different categories of cells observed upon incubation in galactose for 6 hours. Both wild-type and GAL1 promoter-mediated CtCsa6 overexpressing *C. tropicalis* cells could be identified at all cell cycle stages without any nuclear segregation defects. (N = 3, n ≥ 100)

DISCUSSION

Csa6 is an essential gene for viability in *Candida albicans*. The repression of which leads to large bud arrest with segregated nuclei. Expression of orthologous Csa6 from *C. tropicalis* could functionally complement knockdown of CaCsa6 in a promoter shut down mutant. This observation also holds true for another closely related species, *C. dubliniensis*. However, whether similar functional conservation would be observed in distantly related species is still a question that needs to be addressed.

CaCsa6 localizes to spindle pole bodies at all cell cycle stages in *C. albicans*. A similar subcellular localization is also seen for CtCsa6 when expressed in *C. albicans*. This indicates along with the functional conservation, the site of the Csa6 function might also be the same. However, whether the native CtCsa6 also localizes to spindle pole bodies in *C. tropicalis* has not been yet investigated.

In an attempt to address the role of CtCsa6 in cell cycle progression in *C. tropicalis*, one copy of CtCsa6 was deleted. First copy deletion strains grow normally, which can now be used to place the second copy under a repressible promoter like galactose promoter. Galactose promoter is shut down in dextrose but is overexpressed in the presence of galactose. Since overexpression of CaCsa6 also causes a cell cycle arrest, it was important to know whether overexpression of CtCsa6 also causes any cell cycle arrest. One copy was placed under the GAL1 promoter in a wild-type background, followed by a tap tag, and the other was intact. After six hours of galactose induction, CtCsa6 levels increases, as observed by western blot. However, there is no visible cell cycle arrest as seen by microscopy. Since only one transformant was obtained, this experiment needs to be repeated by at least two more transformants to strengthen the data. Another question that remains unaddressed is the strength of the GAL1 promoter. CaCsa6 was overexpressed by the tetracycline promoter. Whether the GAL1 promoter is also overexpressed to a comparable level in *C. tropicalis* remains to be investigated.

To summarize, both *C. dubliniensis* and *C. tropicalis* are phylogenetically related to *C. albicans*. Ectopic expression of both orthologs can rescue cell cycle arrest mediated by CaCsa6 depletion. Ectopic expression of GFP tagged orthologs localizes them to spindle pole bodies. Unlike CaCsa6, GAL1 promoter-mediated overexpression of CtCsa6 does not cause any cell cycle arrest at least in one transformant observed with three technical replicates. This might hint toward the divergence of the protein in terms of function across distant orthologs of CaCsa6.

Materials and methods

Media and growth conditions

All fungal cultures were grown at 30ºC at 180 rpm in 1% yeast extract, 2% peptone, 2% dextrose, and 10mg/100ml uracil (for *C. tropicalis*) and uridine (for *C. albicans*) unless specified.

5mM methionine and 5 mM cysteine were added to YPD or CM for repression with the met promoter.

For repression by GAL1 promoter, standard YPD media was used, and for overexpression, 2% galactose was added instead of dextrose.

gDNA isolation

The overnight grown culture was pelleted down and washed with water. The pellet thus obtained was mixed in 200µl extraction buffer (2ml Triton X, 10 ml 10% SDS, 2 ml 5M NaCl, 1ml 1M Tris-Cl pH8.0, and 0.2 ml 0.5M EDTA raised to 100ml with distilled water), 0.3g acid-washed glass beads and phenol: chloroform: isoamyl-alcohol (25:24:1), vortexed for 2 minutes and centrifuged for 5 mins at 13,000 rpm. The supernatant obtained was invert mixed with 1 ml 100% ethanol followed by 70% ethanol wash and finally resuspending in 50µl 1XTE or AMQ.

Plasmid isolation

Overnight grown (in suitable antibiotic) culture was pelleted down and resuspended in 350µl STET buffer (20ml 0.5M EDTA pH8.0, 2ml 1M Tris-Cl pH8.0, 1ml Triton X, 16g Sucrose raised to 200 ml with distilled water) followed by addition of 25µl 10mg/ml lysozyme and boiling for 40 seconds. The suspension was then pelleted down, and the pellet was removed with a sterile toothpick. 2µl of 10mg/ml RNase was added to the supernatant and incubated at 37ºC for 1 hour. The supernatant was then mixed with phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 13,000 rpm for 10 minutes, and the supernatant was mixed with 500µl 100% isopropanol followed by washing with 70% ethanol and finally resuspending in 50µl 1XTE or AMQ

Candida transformation

0.2 OD/ml cells were inoculated in 50ml YPDU from an overnight grown culture. After reaching an OD between 0.8 and 1.0, the cells were pelleted down and washed with water and lithium acetate solution (500µl lithium acetate, 500µl 10X TE, and 4ml AMQ). Pellet obtained was resuspended in 3 times the volume of pellet lithium acetate solution and stored in ice. In a fresh centrifuge tube, 10µl single-stranded salmon sperm DNA, 30µl DNA to be transformed, 100µl cells in lithium acetate solution, and six times 50% PEG 3350 were added and incubated at 30ºC for 6-16 hours. Following incubation, the mix was incubated at 44ºC for 15 mins, and 1 ml YPDU was added for recovery for 1 hour. Cells were then pelleted down and plated on a selection plate.

Strains, plasmids, and primers.

Strains used are listed in table 1, plasmids used in table 2, and primers used in table 3

Construction of CaHA001, CaHA002, and CaHA003 strain

C. tropicalis Csa6 ortholog (CTRG_01235) with its native promoter was cloned in pTP16 (native promoter of CaCsa6 with GFP in Cip10 plasmid) by replacing native promoter CaCsa6 with native promoter CtCsa6 and the cassette (pHA001; primer pair: HA004 and HA005; amplicon size: 2.6kb) after linearizing with StuI was transformed in CaPJ301to obtain CaHA001, CaHA002, and CaHA003.

Construction of CtHA011,CtHA031.

Upstream (360 bp; Primer pair: HA018 and HA019) and downstream (600 bp; Primer pair: HA020 and HA021) were cloned between KpnI/ApaI and SacI/SacII, respectively, in pSFS2a. The obtained cassette (pHA003) was linearized by KpnI/SacI double digestion and transformed in UHA103 and KG_070 to obtain CtHA011 and CtHA031, respectively.

Construction of CtHA051.

Upstream (360 bp, Primer pair: HA023 and HA024) and ORF homology region (500 bp; Primer pair: HA025 and HA026) were cloned between SacI/SacII and KpnI/ApaI respectively in pGAL TAP HIS1. The obtained construct (pHA005) was linearized by KpnI and SacI digestion and transformed in UHA103 to obtain CtHA051.

Construction of CtHA061, CtHA062 and CtHA063.

pHA001 was digested by AflII and transformed into CtHA012. Transformants obtained were screened by fluorescence microscopy.

NAT marker recycle

CtHA011 and CtHA031 were grown overnight in 2% maltose, 1% yeast extract, 2% peptone and 10mg/ml uracil. One hundred cells were then plated on YPDU, and colonies obtained were patched on YPDU and YPDU+NAT (100µg/ml) plate. The colonies that failed to grow on YPDU+ NAT (100µg/ml) were streaked on YPDU plates and named as CtHA012, CtHA013 and CtHA014 when obtained from CtHA011 and CtHA032, CtHA033 and CtHA034 when obtained from CtHA031.

Fluorescence microscopy

CaHA001, CaHA002, CaHA003 and PJ301 were grown overnight in permissive media (YPD uridine) and 0.2OD/ml cells were inoculated in permissive (YPD uridine) and repressive (YPD uridine + 5mM methionine + 5mM cysteine) for 6 hours at 30ºC. Cells were washed with 1X PBS and imaged at

100X by a fluorescence microscope (Zeiss Axio Observer 7 equipped with Colibri 7 as the LED light source)

Complementation assay

Single colonies from SN148, PJ301, HA001, HA002, and HA003 were streaked and spotted on permissive (CM Met- Cys-) and repressive agar plates (CM Met+ Cys+) and imaged.

Western blot analysis

CtHA051 was grown overnight in YPDU, followed by secondary inoculation in YPGU with a starting OD_{600} of 0.2 OD/ml. 3 OD cells were collected every 2 hrs up to 10 hrs and stored in 16% trichloroacetic acid at -20ºC overnight. The cells were then thawed on ice, washed with acetone, and finally boiled in lysis buffer (1% SDS + 0.1 N NaOH). 5µl of the supernatant was loaded on a 10% SDS-PAGE gel.

The protein samples were then transferred on a nitrocellulose membrane and blocked by 5% skim milk for 1 hr. The blot was kept overnight in anti-TAP antibodies (1:5000 in 2.5% skim milk) and anti-H3 antibodies (separately) at 4ºC on a rocker. Following primary antibody incubation, the blot was washed with 1X PBST (1X PBS + 0.05% Tween 20) and incubated with secondary antibody anti-rabbit antibody. The blot was then developed by adding equal volumes of chromogenic substrates (Biorad Luminol/enhancer and peroxide substrate) and imaged by gel doc.

Hoechst staining

1µl of 1mg/ml Hoechst stain was added to 100µl of cell suspension, appropriately mixed, and incubated for 5 mins at room temperature.

Primers used in this study:

Primer	Sequence (5'-3')	Description
HA004	ATATGGTACCTACTTGGCATGTTATTGTCGG	CtCsa6 Fwd primer with KpnI
HA005	ATATCTCGAGTTTGACTAAGGAAATGCGAAA CTGTG	CtCsa6 rev primer with XhoI
PJ88	ATACTACTGAAAATTTCCTGACTTTC	RP10 integration confirmation fwd primer
PJ89	ATTACTATTTACAATCAAAGGTGGTC	RP10 integration confirmation rev primer
HA018	ATTGGGTACCTGTGTGTTTTAGAATAATATCT C	CtCsa6 deletion fwd primer US KpnI
HA019	ATTCCTCGAGTATGAAAAAAAAAATGCAATT G	CtCsa6 deletion rev primer US XhoI
HA020	AGCTCCGCGGTATACCATTGGTGATGCGGAT G	CtCsa6 deletion fwd primer DS SacII
HA021	GCCAGAGCTCTATCATGAAACTGCATCGTTT C	CtCsa6 deletion rev primer DS SacI
HA022	TAGCCAAAGCGTTAATTTCTTG	CtCsa6 deletion confirmation fwd primer
PJ3	CTATTCTCTAGAAAGTATAGGAACTTC	CtCsa6 deletion confirmation rev primer
HA023	ATTGGAGCTCTGTGTGTTTTAGAATAATATCT C	GALpr-CtCsa6 US fwd primer SacI
HA024	ATTCCCGCGGGTATGAAAAAAAAAATGCAAT TG	GALpr-CtCsa6 US rev primer SacII
HA025	ATGCGGGCCCGCTGACGCTACAGAAG	GALpr-CtCsa6 ORF fwd primer (without ATG)ApaI
HA026	ATGCGGTACCCACTGGTTAATTCAATATTTTC TTTCATTATTTCATC	GALpr-CtCsa6 ORF rev primer KpnI

HA027	GATCTGATTTTGTTCATAG	GALpr Confirmation fwd primer in GALpr
HA029	CTTTCAAACTTTCTCTTGAG	GALpr Confirmation rev primer outside cassette

Plasmids used in this study:

Name	**Construct**	**Description**
pHA001	pTP16-CaCsa6+CtCsa6	CtCsa6 cloned with native promoter between KpnI and XhoI in pTP16
pHA002	pSFS2a+ CtCsa6 US (360bp)	US cloned between KpnI and XhoI in pSFS2a
pHA003	pHA002 + CtCsa6 DS (600bp)	DS cloned betweem SacI and SacII in pHA002
pHA004	pBS GAL1 TAP HIS1 +ORF (500 bp)	500 bp homology region of ORF cloned between ApaI and KpnI in pBS GAL1 TAL HIS1
pHA005	pBS GAL1 TAP HIS1 +US (360 bp)	360 bp US cloned between SacI and SacII in pHA004

Strains used in this study:

Name	**Genotype**	**Reference**
SN148	*Δura3::imm434/Δura3::imm434, Δhis1::hisG/Δhis1::hisG, Δarg4::hisG/Δarg4::hisG, Δleu2::hisG/Δleu2::hisG*	Noble and Johnson, 2005
CaPJ301	*SN148 csa6::FRT/MET3prCSA6 (HIS1), TUB4/TUB4-mCherry(ARG4)*	Jaitly et al., 2021
CaHA001	*CaPJ301 RPS1/RPS1::CtCSA6-GFP::URA3*	This study
CaHA002	*CaPJ301 RPS1/RPS1::CtCSA6-GFP::URA3*	This study
CaHA003	*CaPJ301 RPS1/RPS1::CtCSA6-GFP::URA3*	This study

UHA103	*ura3::FRT/ura3::FRT his1::FRT/his1::FRT arg4::FRT/arg4::FRT*	Chatterjee et al., 2016.
CtHA011	*UHA103 CSA6/csa6::NAT*	This study
CtHA012	*UHA103 CSA6/csa6::FRT*	This study
CtHA013	*UHA103 CSA6/csa6::FRT*	This study
CtKG070	*UHA103 NUF2/NUF2 GFP (HIS1)*	unpublished
CtHA031	*KG070 CSA6/csa6::NAT*	This study
CtHA032	*KG070 CSA6/csa6::FRT*	This study
CtHA033	*KG070 CSA6/csa6::FRT*	This study
CtHA034	*KG070 CSA6/csa6::FRT*	This study
CtHA051	*UHA103 Gal7p-CSA6 (URA3) /CSA6*	This study
CtHA052	*UHA103 Gal7p-CSA6 (URA3) /CSA6*	This study
CtHA053	*UHA103 Gal7p-CSA6 (URA3) /CSA6*	This study
CtHA061	*HA013 csa6::FRT/CSA6::CSA6-GFP (URA3)*	This study
CtHA062	*HA013 csa6::FRT/CSA6::CSA6-GFP (URA3)*	This study
CtHA063	*HA013 csa6::FRT/CSA6::CSA6-GFP (URA3)*	This study

References

1. KA, S. (1998). The cell cycle: a review. In *Veterinary Pathology* (Vol. 35, Issue 6, pp. 461–478).
2. Winey, M., & O'Toole, E. T. (2001). The spindle cycle in budding yeast. *Nature Cell Biology*, *3*(1), 23–27. https://doi.org/10.1038/35050663
3. Westermann, S., Drubin, D. G., & Barnes, G. (2007). Structures and functions of yeast kinetochore complexes. *Annual Review of Biochemistry*, *76*, 563–591. https://doi.org/10.1146/annurev.biochem.76.052705.160607
4. Burrack, L. S., & Berman, J. (2012). Flexibility of centromere and kinetochore structures. *Trends in Genetics*, *28*(5), 204–212. https://doi.org/10.1016/j.tig.2012.02.003
5. Ben-Abdallah, M., Sturny-Leclère, A., Avé, P., Louise, A., Moyrand, F., Weih, F., Janbon, G., & Mémet, S. (2012). Fungal-induced cell cycle impairment, chromosome instability and apoptosis via differential activation of NF-κB. *PLoS Pathogens*, *8*(3). https://doi.org/10.1371/journal.ppat.1002555
6. Loll-Krippleber, R., Feri, A., Nguyen, M., Maufrais, C., Yansouni, J., D'Enfert, C., & Legrand, M. (2015). A FACS-optimized screen identifies regulators of genome stability in Candida albicans. *Eukaryotic Cell*, *14*(3), 311–322. https://doi.org/10.1128/EC.00286-14
7. Legrand, M., Jaitly, P., Feri, A., d'Enfert, C., & Sanyal, K. (2019). Candida albicans: An Emerging Yeast Model to Study Eukaryotic Genome Plasticity. *Trends in Genetics*, *35*(4), 292–307. https://doi.org/10.1016/j.tig.2019.01.005
8. Diogo, D., Bouchier, C., d'Enfert, C., & Bougnoux, M. E. (2009). Loss of heterozygosity in commensal isolates of the asexual diploid yeast Candida albicans. *Fungal Genetics and Biology*, *46*(2), 159–168. https://doi.org/10.1016/j.fgb.2008.11.005
9. Maity, A., McKenna, W. G., & Muschel, R. J. (1994). The molecular basis for cell cycle delays following ionizing radiation: a review. *Radiotherapy and Oncology*, *31*(1), 1–13. https://doi.org/10.1016/0167-8140(94)90408-1
10. Kozubowski, L., Yadav, V., Chatterjee, G., Sridhar, S., Yamaguchi, M., Kawamoto, S., Bose, I., Heitman, J., & Sanyal, K. (2013). Ordered kinetochore assembly in the human-pathogenic basidiomycetous yeast Cryptococcus neoformans. *MBio*, *4*(5). https://doi.org/10.1128/mBio.00614-13
11. Musacchio, A., & Desai, A. (2017). A molecular view of kinetochore assembly and function. *Biology*, *6*(1). https://doi.org/10.3390/biology6010005

12. Guin, K., Chen, Y., Mishra, R., Muzaki, S. R. B. M., Thimmappa, B. C., O'brien, C. E., Butler, G., Sanyal, A., & Sanyal, K. (2020). Spatial inter-centromeric interactions facilitated the emergence of evolutionary new centromeres. *ELife*, *9*, 1–28. https://doi.org/10.7554/eLife.58556
13. Sankaranarayanan, S. R., Ianiri, G., Coelho, M. A., Reza, M. H., Thimmappa, B. C., Ganguly, P., Vadnala, R. N., Sun, S., Siddharthan, R., Tellgren-Roth, C., Dawson, T. L., Heitman, J., & Sanyal, K. (2020). Loss of centromere function drives karyotype evolution in closely related Malassezia species. *ELife*, *9*, 1–33. https://doi.org/10.7554/eLife.53944
14. Sreekumar, L., Kumari, K., Guin, K., Bakshi, A., Varshney, N., Thimmappa, B. C., Narlikar, L., Padinhateeri, R., Siddharthan, R., & Sanyal, K. (2021). Orc4 spatiotemporally stabilizes centromeric chromatin. *Genome Research*, *31*(4), 607–621. https://doi.org/10.1101/GR.265900.120
15. Jaitly, P., & Sanyal, K. (2021). *A phylogenetically-restricted essential cell cycle progression factor in the A phylogenetically-restricted essential cell cycle progression factor in the human pathogen Candida albicans Molecular Mycology Laboratory , Molecular Biology and Genetics Unit ,. September*.
16. Arellano, M., & Moreno, S. (1997). Regulation of CDK/cyclin complexes during the cell cycle. *International Journal of Biochemistry and Cell Biology*, *29*(4), 559–573. https://doi.org/10.1016/S1357-2725(96)00178-1