Role of a putative CENP-A specific E3 ubiquitin ligase Psh1 in human pathogenic yeast *Candida albicans*

A thesis submitted for the partial fulfillment of the degree of

Master of Science

(Biological sciences)

as part of Integrated Ph.D. program

by

Priya Jaitly

Molecular Biology and Genetics Unit

Jawaharlal Nehru Center for Advanced Scientific Research

(A deemed university)

Jakkur, Bangalore 560064.

March 2016

DECLARATION

I hereby declare that the work described here in this thesis entitled **'Role of a putative CENP-A specific E3 ubiquitin ligase Psh1 in human pathogenic yeast *Candida albicans'*** has originally been carried out by myself under the guidance and supervision of Dr. Kaustuv Sanyal, Associate Professor, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India.

Priya Jaitly

Date:

CERTIFICATE

This is to certify that this thesis entitled '**Role of a putative CENP-A specific E3 ubiquitin ligase Psh1 in human pathogenic yeast *Candida albicans***' submitted by Priya Jaitly towards the Integrated Ph.D Program, as part of Project for MS, at Jawaharlal Nehru Centre for Advanced Scientific Research, was based on the studies carried out by her under my supervision and guidance.

Prof. Kaustuv Sanyal

Date:

ACKNOWLEDGEMENT

First and foremost, I have to thank my research supervisor Prof. Kaustuv Sanyal. I thank him sincerely for instilling confidence in me to pursue my research problem. His enthusiasm for the topic made a strong impression on me. His scientific temperament and approach towards the subject helped me understand my scientific problem better. His valuable insights, support, scholarly guidance and constant encouragement has given this story the necessary impetus that it needed.

I would like to express my gratitude towards our past and present presidents, Prof. M.R.S Rao and Prof. V. Nagaraja respectively, for providing all the necessary facilities in the department, making it easier for us to perform the experiments. Similarly, I am grateful to our chairman Prof. Anuranjan Anand who has always encouraged all the students, including me, with cheerful words and kind advice. The various courses taken during the M.S. program have been very useful to me towards understanding the basic concepts and techniques of molecular biology. Hence I thank all MBGU faculties Prof. Anuranjan Anand, Prof. M.R.S Rao, Prof. Tapas Kundu, Prof. Ranga Uday Kumar, Prof. Namita Surolia, Prof. Hemalatha Balaram, Prof. Maneesha Inamdar, Dr. Ravi Manjithaya, Dr. James Chelliah and Dr. G.R. Ramesh.

My special thanks and appreciations go to Prof. Maneesha Inamdar under whom I did my first lab rotation. She taught me the preliminary concept of developmental biology. Discussions with her have taught me a lot about how to approach a scientific question.

I thank B. Suma of confocal facility for the imaging and discussions about image processing. Similarly, I am grateful to Anitha mam for sequence analysis.

Members of Molecular Mycology lab have always lent a helping hand during the lab work. I would like to thank Laxmi and Neha for patiently teaching me the initial basic techniques related to the yeast genetics. I thank Gautam da for his valuable suggestions during the experiment. My thanks and appreciations also go to other members of the lab, Vikas, Lakshmi, Sundar, Shreyas, Rima, Krishnendu, Radha and Aditi for their

stimulating suggestions and encouragement. They all were always there to troubleshoot my work related problems. Special thanks to Nagraja for making lab reagents available on time.

I have been blessed with good friends in JNCASR. I would like to thank Ronak Ji, Deeti, Simi, Diana, Divyesh and Saloni di. Talking to them was the biggest stress buster for me. They have helped immensely during my stay at JNCASR. I personally felt lucky to have Jigyasa and Sarika as my juniors. Very special thanks to Payel di, who was always there with me during my bad and good times. I will always cherish the time we had spent. I would also like to express my gratitude to Shashank bhaiya for his valuable suggestions.

I am extremely grateful for having such a talented group of batchmates, Veena, Shubhangini, Pallabi, Siddharth and Bhavana.

I thank all my MBGU seniors and juniors, who are too numerous to be named, but who, nevertheless, have inspired me, at some moment or the other through their work or personal interactions.

Most importantly none of this could have happened without my family. To my mother, father and brother- everytime I was sad or depressed, you were there to make me smile and have always supported me. I am forever grateful to your unconditional love and encouragement.

-Priya

TABLE OF CONTENTS

List of figures and tables

1. Introduction

2. Results

4. Materials and methods

ABBREVIATIONS

$O.D_{600}$	Optical density at 600nm
bp	base pair
DAPI	4',6-Diamino-2-phenylindole
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
h	hour
kb	kilo basepairs
Mb	Mega basepairs
Min	Minutes
µg	micrograms
ng	nanograms
ml	milli litre
µl	micro litre
mM	milli molar
ORF	Open reading frame
PCR	Polymerase chain reaction
S	Seconds
˚C	Degree celsius

“A river cuts through a rock not because of its power but its persistence”

Introduction

1.1 Cell cycle and chromosome segregation

An ordered set of events by which a cell divides to produce two daughter cells, each containing the same number of chromosomes as the parent cell, constitute the mitotic cell cycle. In eukaryotes, the cell cycle events are broadly divided into two discrete phases, interphase and mitotic (M) phase.

During interphase, a cell accumulates necessary components required for its division. Interphase is further subdivided into G1, S and G2 phases. In S phase, chromosomes are duplicated. In G1 and G2 phases, a cell prepares itself for the subsequent stages. Mitotic (M) phase is the stage when a single cell is divided into two separate daughter cells. M phase consists of mitosis followed by cytokinesis. In mitosis, the duplicated sister chromatids are separated from each other. Mitosis is further subdivided into prophase, metaphase, anaphase and telophase. In cytokinesis, cytoplasm, organelles and cell membrane are divided, to finally give rise to two identical cells. This event marks the end of the cell cycle.

Proper execution of a number of coordinated events during cell cycle is crucial for maintaining genomic stability. Defects in the process of chromosome segregation can result in aneuploidy – a hallmark of cancers and various other human diseases. To prevent this catastrophe, a cell possesses machineries which take care of accurate chromosome segregation. It includes assembly of the centromere/kinetochore (KT), kinetochore–microtubule (KT-MT) interactions and a surveillance mechanism by the spindle assembly checkpoint.

1.2 The kinetochore

The KT is a multi-protein structure that assembles on centromere and is essential for chromosome segregation. A KT acts as a platform for MT attachment to the sister chromatids, so that they can be pulled apart and segregated equally between the daughter cells. The KT consists of several proteins that can be divided into inner and outer domains (YAMAGISHI *et al.* 2014) (Figure 1).

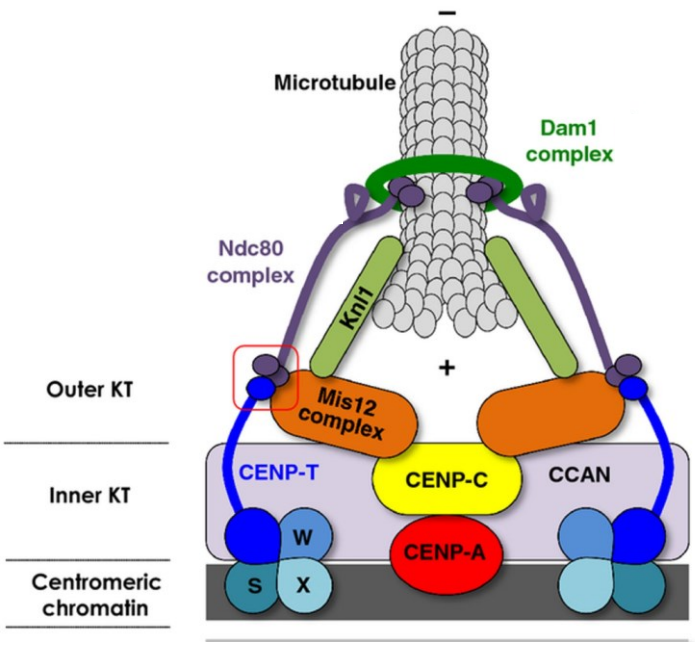


Figure 1. Schematic of a KT. The inner KT consists of CENP-A, CENP-C and CCAN, of which one sub-complex (CENP-T/W/S/X) is shown. The outer KT contains the DAM1 and KNL complex. The Ndc80 complex, KNL1 and the Dam1 complex have MT binding activity. The figure has been adapted and modified from (YAMAGISHI *et al.* 2014).

Inner kinetochore: The inner KT proteins bind to the centromere DNA. It consists of several proteins, including CENP-A, CENP-C and a group of about 16 proteins that are collectively known as the constitutively centromere associated network (CCAN). The CCAN proteins assemble as distinct sub-complexes; CENP-H/I/K, CENP-L/M/N, CENP-O/P/Q/R/U and CENP-T/W/S/X (Figure 1).

Outer kinetochore: The outer KT acts as a bridge between inner KT and the plus end of the MTs. It mainly comprises of two complexes namely the KMN complex and the Dam1 complex. The KMN complex further consists of three sub-complexes; the KNL1 (Spc105) complex, the Mis12/MIND complex and the Ndc80 complex. The Ndc80 complex, KNL1 and the Dam1 complex are responsible for binding to MTs.

1.3 Centromeric chromatin

Centromere is a specialised locus on a chromosome that provides a foundation for KT assembly and serves as a site for sister chromatid attachment. Although this function of centromere is conserved from yeast to humans, the DNA sequence composition and size of the centromere are poorly conserved across the eukaryotes. The budding yeast *S. cerevisiae* contains a defined centromere DNA sequence of ~125bp. The short point centromere of *S. cerevisiae* contains three conserved DNA elements (CDEs) -CDEI, CDEII and CDEIII. In contrast, centromeres of most organisms ranging from fission yeast to humans do not contain any defined sequence. These organisms have regional centromeres of larger sizes (40-4000 kb), with repetitive and AT-rich sequences. The fission yeast centromere contains a non-repetitive central core, flanked by inverted repeat regions while centromeres in metazoans such as *D. melanogaster* and *H. sapiens* are composed of highly repetitive DNA sequences (VERDAASDONK AND BLOOM 2011).

Since, centromeres are so diverse, there are epigenetic mechanisms involved in its specification. A hallmark of most of the eukaryotic active centromere is the presence of centromere-specific histone H3 variant - CENP-A in budding yeast, Cnp1 in fission yeast, CID in fruit fly and CENP-A in humans.

1.4 CENP-A: The centomere-specific histone H3 variant

CENP-A is a centromere specific histone H3 variant which replaces the canonical histone H3 in the nucleosomes of functional centromeres and is the hallmark of centromere identity in most of the organisms. CENP-A, similar to the core histone H3, consists of a C-terminal domain, containing the typical and much conserved histone-fold domain and an N-terminal domain. The N-terminal tail, however, is strikingly unique from that of the histone H3 in terms of amino acid sequence and length.

Within the histone-fold domain of CENP-A, Loop 1 (L1) and alpha 2 (α2) helix together constitute the centromere targeting domain (CATD) which is necessary and sufficient to target a histone H3 chimera to the centromere (BLACK *et al.* 2007) (Figure 2). This domain also binds to the chaperone Scm3 in budding yeast or HJURP in humans. Since Scm3/HJURP is required for CENP-A deposition at the centromere (FOLTZ *et al.* 2009; SHIVARAJU *et al.* 2011), a potential mechanism to explain the role of CATD in CENP-A localization can be envisaged.

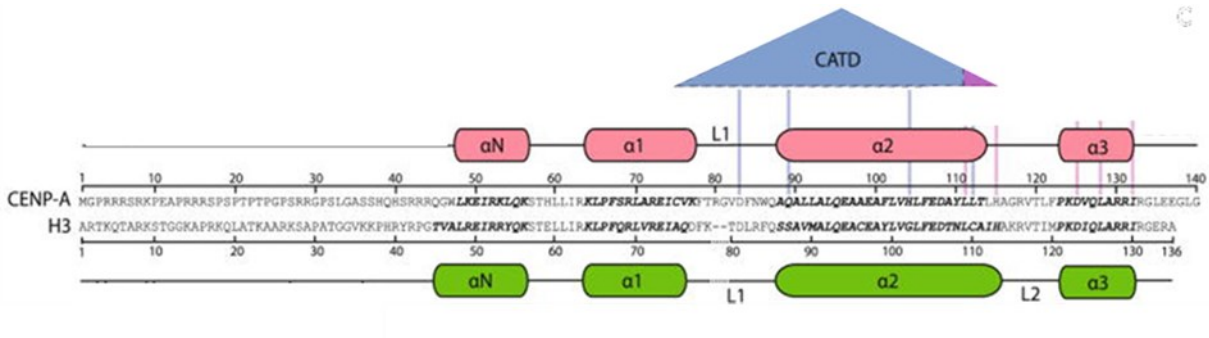


Figure 2. Comparison of CENP-A and canonical histone H3 of humans. The L1 and α2 of CENP-A together makes the CATD domain which is required for its localisation at the centromere. The figure has been adapted and modified from (STELLFOX *et al.* 2013).

1.5 Ensuring CENP-A specificity at the centromere

Over-expression of CENP-A in several systems leads to its mis-incorporation onto other ectopic sites within the genome (HEUN *et al.* 2006; VAN HOOSER *et al.* 2001). In addition, CENP-A can be detected at sites of DNA double strand breaks (DSBs) prior to removal at the time of DNA repair (ZEITLIN *et al.* 2009). These data suggest that while CENP-A is found specifically at the centromeres, it retains the ability to localize the rest of the genome. Mis-localisation of CENP-A on general chromatin by over-expression, or to specific non-centromeric loci via targeted deposition can lead to genomic instability and has been shown to occur in numerous cancers (AMATO *et al.* 2009; HEUN *et al.* 2006; HU *et al.* 2010; LI *et al.* 2011; TOMONAGA *et al.* 2003). Thus, to ensure one centromere per monocentric chromosome, a cell must employ mechanisms to deposit CENP-A specifically at the centromere and also mechanisms to prevent its non-centromeric deposition.

1.5.1 Depositing CENP-A at the centromere

The direct recruitment of CENP-A at the centromere is the major way to ensure stable centromere identity. However, there are no conserved mechanisms for recruiting CENP-A at the centromere. In *S. cerevisiae*, with short point centromeres, Scm3 and Ndc10 together binds to CDEIII via Ndc 10's specificity for *CEN* DNA and recruit CENP-A to the centromere (CAMAHORT *et al.* 2007). In *S. pombe* which has regional centromeres, Mis16 and Mis18 are required for deposition of CENP-A containing nucleosomes (HAYASHI *et al.* 2004), by controlling the localisation of Scm3 at the centromere (WILLIAMS *et al.* 2009). In addition, heterochromatin formed by the inverted repeats flanking the central core at *S. pombe* centromere, is also required to promote CENP-A-mediated KT assembly over the central domain (FOLCO *et al.* 2008). More recently, in

Drosophila, it has been shown that a long non-coding RNA from the satellite repeats of the centromere of chromosome X recruit CENP-C and CENP-C interacting factors such as CENP-A at the centromere (ROSIC *et al.* 2014). CENP-A incorporation in humans occurs by the recruitment of the Mis18 complex, CENP-H, CENP-I, RbAp46/48, followed by recruitment of HJURP that finally loads CENP-A at the centromere (VERDAASDONK and BLOOM 2011). Thus, there are distinct mechanisms operating in different organisms to load CENP-A specifically at the centromere.

1.5.2 Removing or degrading CENP-A from the ectopic loci

Insights into the mechanisms that prevent CENP-A mis-incorporation have come from the studies in *S. cerevisiae* and *Drosophila*. In *S. cerevisiae*, mis-incorporation of CENP-A is prevented with the help of the SWI/SNF chromatin remodeller complex (GKIKOPOULOS *et al.* 2011). Absence of Snf2, the catalytic subunit of the SWI/SNF complex, causes accumulation of CENP-A at non-centromeric loci. Furthermore, *in vitro* experiments demonstrated that Snf2 specifically disassembles CENP-A-containing nucleosomes, suggesting non-centromeric CENP-A nucleosomes are sensitive to destabilization by chromatin remodellers.

CENP-A localisation is also regulated by proteasome-dependent ubiquitin-mediated proteolysis (COLLINS *et al.* 2004; MORENO-MORENO *et al.* 2006). In *Drosophila*, the F-box protein Ppa (protein partner of paired) regulates CID/CENP-A stability (MORENO-MORENO *et al.* 2011). Ppa is a component of the SCF complex, a primary multimeric E3 ubiquitin ligase that incorporates different F-box proteins as variable subunits for specific recognition of the substrate (CARDOZO AND PAGANO 2004). Depletion of Ppa results in increased CENP-A levels and mis-localisation throughout chromatin (MORENO-MORENO *et al.* 2011). This phenomenon of proteasomal degradation of CENP-A

is also conserved in *S. cerevisiae* where a specific E3 ubiquitin ligase called Psh1, is responsible for degrading CENP-A at non-centromeric loci (HEWAWASAM *et al.* 2010; RANJITKAR *et al.* 2010).

CENP-A proteolysis has also been detected in human cells undergoing senescence or infection with herpes simplex virus 1 (LOMONTE *et al.* 2001; MAEHARA *et al.* 2010) suggesting that CENP-A proteolysis is a conserved mechanism. However, a host ubiquitin ligase that is coupled to target CENP-degradation in humans remains to be discovered.

1.6 Psh1 (Pob3/Spt16 histone associated)

Psh1 is an E3 ubiquitin ligase in *S. cerevisiae*. Psh1 specifically ubiquitinates CENP-A by recognizing its CATD domain (RANJITKAR *et al.* 2010). Deletion of Psh1 prevents CENP-A from degradation and increases its association at non-centromeric loci under CENP-A over-expression (HEWAWASAM *et al.* 2010; RANJITKAR *et al.* 2010). Structurally, Psh1 consists of a C4 type zinc finger domain and a RING domain, a type of zinc finger which has been shown to possess ubiquitin ligase activity. Whereas the RING domain is required for binding to CATD of CENP-A (RANJITKAR *et al.* 2010), both the RING and zinc finger domains are required for CENP-A degradation (HEWAWASAM *et al.* 2010). Together, Psh1-mediated degradation prevents CENP-A from accumulating in euchromatin.

It has been proposed that CENP-A at the centromere is prevented from Psh1-mediated degradation by the CENP-A chaperone Scm3. In budding yeast, Scm3 is present at the centromere (CAMAHORT *et al.* 2007) throughout the cell cycle (XIAO *et al.* 2011) and Psh1 fails to ubiquitinate CENP-A in the presence of Scm3 *in vitro* (HEWAWASAM et al. 2010). Interestingly, Scm3 also recognizes CENP-A using the CATD

domain (SHIVARAJU *et al.* 2011). Taken together, these data suggest that Scm3 binding to the CATD domain of CENP-A at the centromere might prevent CENP-A from Psh1-mediated proteolysis. However, in absence of Scm3, CENP-A is only de-localised from the centromere, but not degraded (CAMAHORT *et al.* 2007). The inability of Psh1 to degrade CENP-A in the absence of Scm3 explains the complexity of the mechanisms by which CENP-A levels are regulated.

Deletion of *PSH1* does not show a phenotype unless CENP-A is over-expressed. In agreement to this, CENP-A is not completely stabilised when Psh1 is deleted (HEWAWASAM *et al.* 2010; RANJITKAR *et al.* 2010) and a lysine-free mutant of CENP-A is still degradable (COLLINS *et al.* 2004), suggesting additional mechanisms are responsible for degrading CENP-A in vivo.

1.7 E3 ligases and the RING domain

Ubiquitination begins with the activation of the ubiquitin monomer by an E1 activating enzyme. This is followed by transfer of the activated ubiquitin to an E2 conjugating enzyme. Subsequently, E3 ligase enzyme brings the target protein and E2 enzyme in close proximity and transfers ubiquitin monomer onto the target protein (Figure 3).

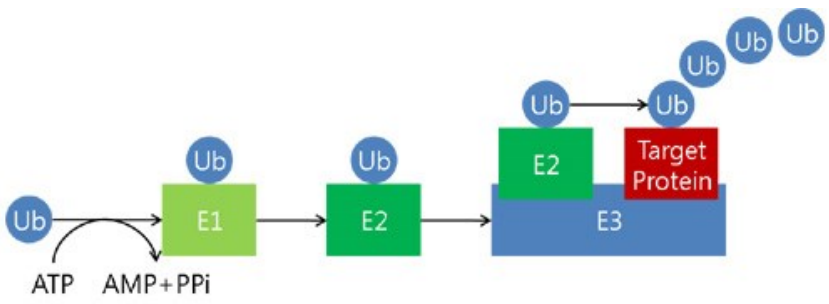


Figure 3. The ubiquitination pathway. Ubiquitination is an enzymatic process in which a small protein called ubiquitin is attached to a protein substrate. The process

requires sequential activity by three enzymes, E1, E2 and E3 (http://e3miner.biopathway.org/help_intro.html).

The E3s are a large, diverse group of proteins which confer specificity to ubiquitination reaction by recognizing target substrates and mediating transfer of ubiquitin from an E2 ubiquitin conjugating enzyme to substrate. These enzymes are characterized by one of the several defining motifs. These include a HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene) or an U-box domain. Whereas, HECT E3s transfer ubiquitin directly to the substrate, the latter two require presence of E2 for conjugating ubiquitin to the substrate. The U-box domain, however, differs from that of the RING domain in lacking the key residues required for metal chelation. Instead, it utilizes salt bridges to maintain its structure (ARDLEY AND ROBINSON 2005).

The RING finger family potentially represents the largest group of E3 ligases. The RING finger is a type of zinc finger; a small protein motif that binds to one or more zinc atoms and contains finger-like protrusions to bind to target molecule. The RING domain is a C_3HC_4 type zinc finger which binds to two zinc cations and promotes both protein–protein and protein–DNA interactions (Figure 4). The consensus sequence of the RING domain is defined as Cys^1-Xaa_2-Cys^2-$Xaa_{9–39}$-$Cys^3Xaa_{1–3}$-His^4-$Xaa_{2–3}$-Cys/His^4-Xaa_2-Cys^5-$Xaa_{4–48}$-Cys^6-Xaa_2-Cys^7 (where Xaa can be any amino acid residue) (ARDLEY AND ROBINSON 2005). Structurally, the RING domain binds to an E2 ubiquitin conjugated complex and the substrate-docking site is 50–60 Å away from it (METZGER *et al.* 2012) .

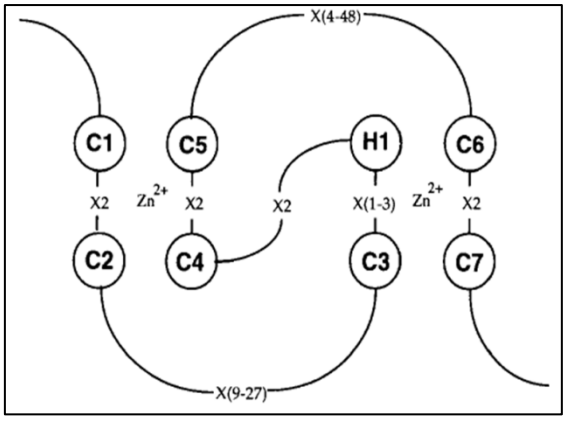


Figure 4. Structure of the RING domain. The RING domain consists of C_3HC_4 motif which binds to two zinc ions. The zinc binding amino acids are shown in circles. The sequence between the conserved cysteines and histidine is also mentioned. X is any amino acid (BORDEN AND FREEMONT 1996).

Members of the RING finger ubiquitin ligase family can function as monomers, dimers or multi-subunit complexes (METZGER *et al.* 2012)(Figure 5). Both Psh1 and F-box protein Ppa are RING domain containing E3 ligases. The former is, however, a monomer whereas the latter is a multi-subunit complex.

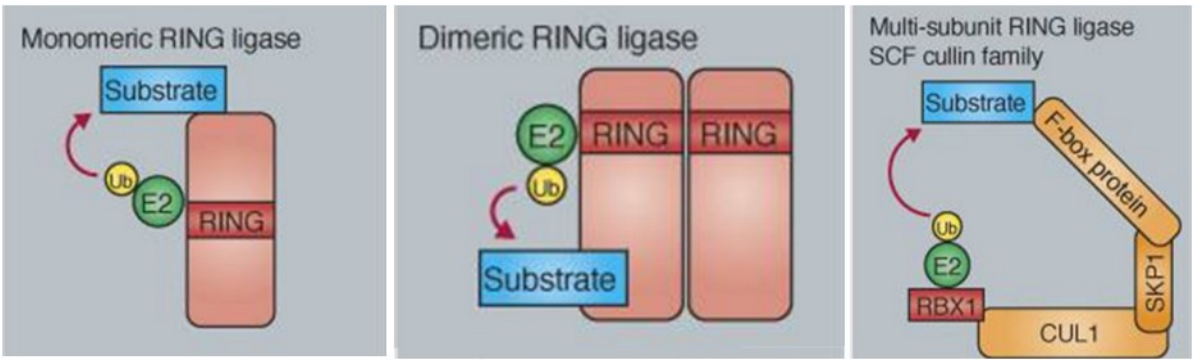


Figure 5. Types of RING E3 ligases. RING E3 ligases can be monomeric, dimeric or multimeric. The RING domain binds to E2 and the substrate binding site is 50 Å away from it. The figure has been adapted and modified from (METZGER *et al.* 2012).

1.8 *Candida albicans*

Candida albicans is a diploid ascomycetous yeast which lives as a harmless commensal in mouth, skin, nails, mucous membranes of respiratory, gastrointestinal, female genital tract etc. of healthy individuals. It is polymorphic, as it grows both in yeast as well as in filamentous forms. However, under certain circumstances, especially in immune-suppressed individuals, *C. albicans* can overgrow and behave as an opportunistic pathogen causing candidiasis, one of the most common human fungal diseases. Several factors and activities have been identified which contribute to the pathogenic potential of this fungus. Among them, are the molecules which mediate adhesion to and invasion into host cells, the secretion of hydrolases, the yeast-to-hypha transition, biofilm formation and phenotypic switching (MAYER *et al.* 2013).

The genome size of *C. albicans* is about 16 Mb with eight pairs of chromosomes. The CENP-A-rich centromeres in this organism are of ~3 - 5 kb in size, intermediate between point and regional centromeres and lack any repetitive sequence except some of the chromosome-specific pericentric repeats (present on chromosome 4 and 5). The centromere sequences on each of the eight chromosomes are unique and different (SANYAL *et al.* 2004).

CENP-A chromatin establishment in *C. albicans* is mediated by homologous recombination (HR) proteins, Rad51 and Rad52. Centromeres are the earliest replicating chromosomal regions in the respective chromosomes in *C. albican*s (KOREN *et al.*) and KTs remain clustered throughout the cell cycle (SANYAL AND CARBON 2002). As a result, the replication fork stalls at the centromere because clustered KT acts as a barrier to replication fork movement (MITRA *et al.* 2014). The HR proteins, Rad51 and Rad52, have been shown to play a role in fork restart and CENP-A recruitment at the centromere. Earlier we have shown an essential function of Scm3 in CENP-A

recruitment at the centromere. However, in contrast to *S. cerevisiae*, depletion of Scm3 in *C. albicans* leads to both de-localisation as well as degradation of CENP-A, suggesting CENP-A levels are regulated differently in different organisms (Varshney & Sanyal, unpubslished). An interesting feature of *C. albicans* is that the structural stability of the KT is a determinant of CENP-A stability. Depletion of any of the essential KT proteins results in disintegration of the KT and degradation of CENP-A (THAKUR and SANYAL 2012).

1.9 Basis of this study

C. albicans is responsible for the majority of Candida infections and is the most prevalent human fungal pathogen in immuno-compromised patients. Increasing resistance of *C. albicans* towards antifungals is becoming a major concern. In many of the clinical isolates, aneuploidy has been observed and is associated with antifungal resistance (SELMECKI *et al.* 2006). Moreover, the efficiency of neocentromere formation in *C. albicans* is remarkably high (THAKUR and SANYAL 2013). As discussed, mis-incorporation of CENP-A at the ectopic loci often leads to aneupoidy. In *C. albicans*, HR proteins, Rad51 and Rad52, along with the CENP-A chaperone Scm3, maintain kinetochore integrity which has been shown to be required for *CEN*-specific localisation of CENP-A. However, the mechanism by which CENP-A is prevented from mis-incorporation has not been fully understood. Furthermore, over-expression of CENP-A in *C. albicans* leads to its increased binding at the centromere without causing any lethality (BURRACK *et al.* 2011). The underlying pathway which restricted CENP-A to the 3 kb regional centromere upon over-expression is a mystery. As discussed, proteasomal degradation is one of the mechanisms by which a cell regulates levels and localisation of

CENP-A. CENP-A gets degraded by proteasomal machinery in *C. albicans* (THAKUR and SANYAL 2012). However, the associated E3 ubiquitin ligase is not known. In this study, we sought to identify and characterize a putative homolog of Psh1 in *C. albicans*. Preliminary results on the role of Psh1 on CENP-A stability has been discussed.

Results and Discussion

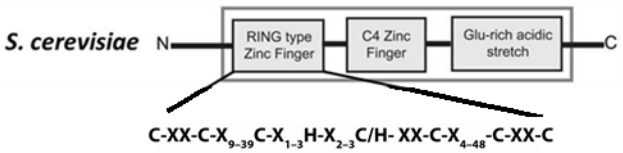
2.1 Psh1 is non-essential for viability in *C. albicans*

2.1.1 Identification of putative homolog of Psh1 in *C. albicans*

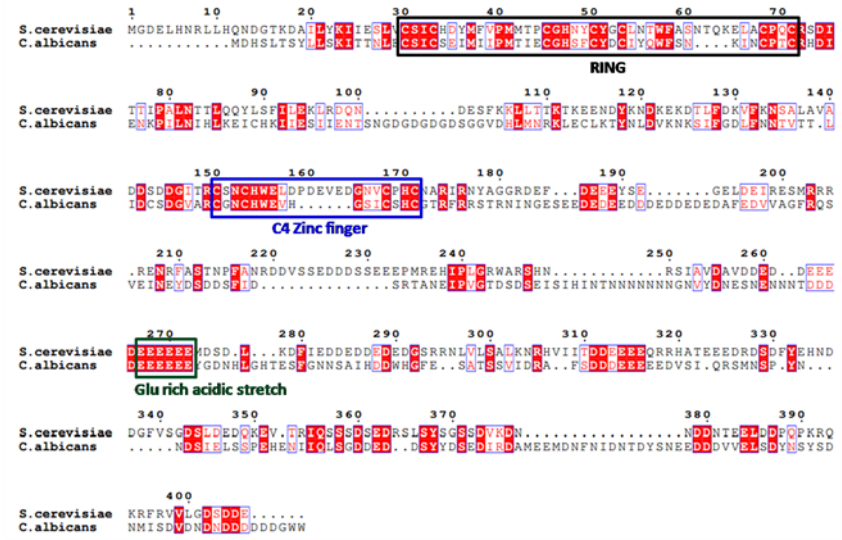
The amino acid sequence of *C. albicans* Psh1 (*Ca*Psh1) was retrieved from the Candida Genome Database. *Ca*Psh1 is encoded by ORF19.229 (Ch3 coordinates 527117-528651C). The ORF is 1535 bp long that codes for a putative 47.3 kDa protein containing 417 amino acids. To further confirm the retrieved sequence, we wanted to identify specific domains in *Ca*Psh1. Since, structural information of *D. melanogaster* Ppa (*Dm*Ppa) is not available, except that it contains the F-box domain, we used *S. cerevisiae* Psh1 (*Sc*Psh1) sequence to identify domains in *Ca*Psh1. *Sc*Psh1 contains a RING domain, a C4 type zinc finger and a glutamate rich acidic domain (HEWAWASAM *et al.* 2010)(Figure 6A). We aligned *Sc*Psh1 with *Ca*Psh1 using Clustal omega and Espript 3.0 software and found that *Ca*Psh1 also contains the RING, C4 zinc finger and glutamate rich acidic domain (Figure 6B).

The class Ascomycota includes a number of non-pathogenic fungi such as *S. cerevisiae*, *S. pombe* as well as pathogenic ones including many species of *Candida* including *C. albicans.* To see if Psh1 exists in other ascomycetes as well, we performed a BLASTP search in the public database (National Center for Biotechnology Information) taking the amino acid sequence of *Sc*Psh1 as the query and aligned them using Gene Doc software (Figure 6C). We found putative homologs of Psh1 in other ascomycetes, suggesting Psh1 can be a conserved protein throughout the ascomycetes.

A



B



C

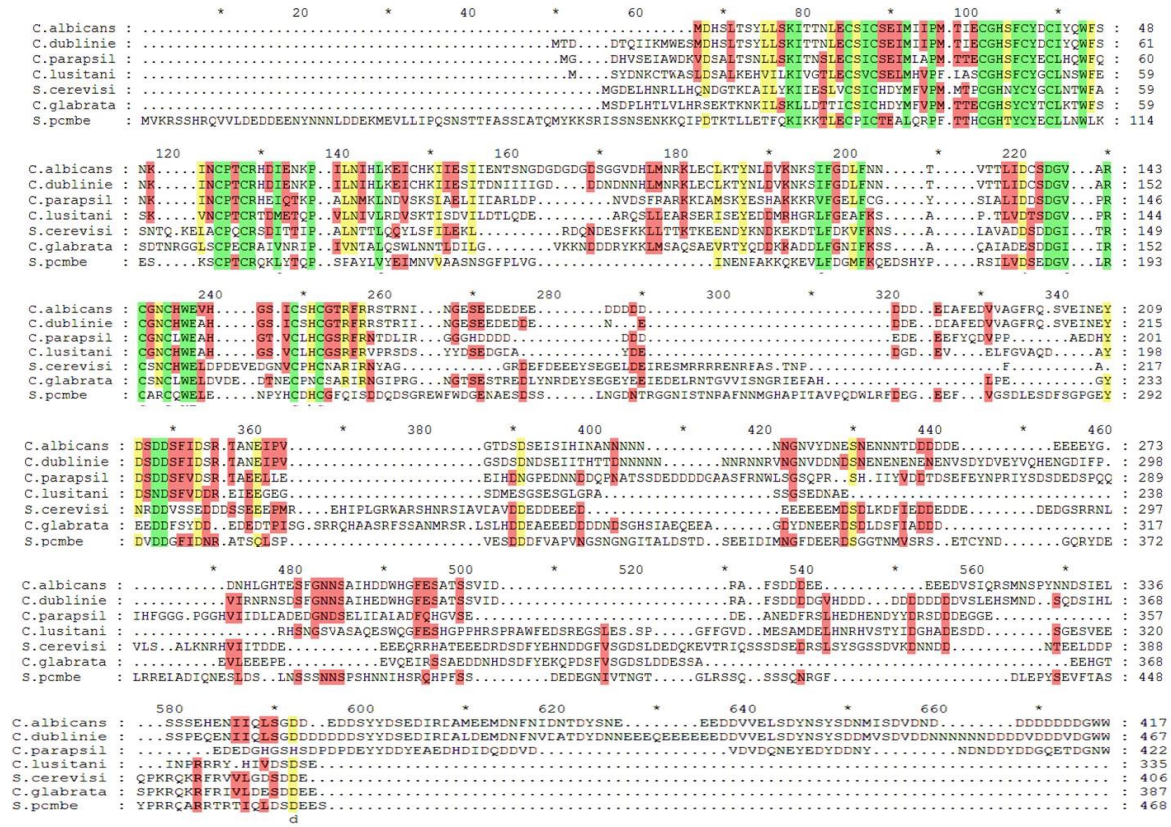


Figure 6. Psh1 in ascomycetes including *C. albicans.* **(A)** Schematic of the domains of *Sc*Psh1 (HEWAWASAM *et al.* 2010). **(B)** Sequence alignment of *Sc*Psh1 and *Ca*Psh1 showing the RING, C4 Zinc finger and glutamate rich domains. **(C)** Homologs of Psh1 in other ascomycetes.

2.2. Construction of *psh1/psh1* null mutants

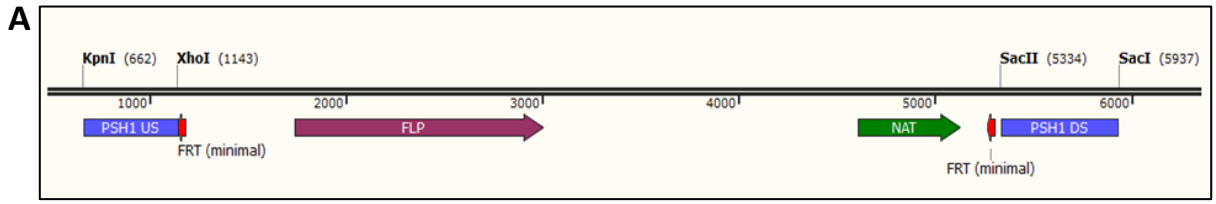
Since *C. albicans* is diploid, both the alleles have to be deleted to create mutants lacking *PSH1*. *psh1/psh1* null mutants were constructed using the *SAT1* flipper cassette (pSFS2a) (REUSS *et al.* 2004). The flipper cassette uses the principle of homologous recombination to replace the desired ORF with the marker *NAT* (Nourseothricin Acetyl Transferase) based on the sequences flanking the cassette. In addition, the cassette possesses the *FLP* recombinase coding region which is under an inducible maltose promoter (MAL2p) and two *FRT* (*FLP* Recombinase Target sequence) sites. Induction of the MAL2p in a maltose containing medium allows expression of the *FLP* recombinase that brings about recombination between the *FRT* sites, thus excising the *NAT* marker used to replace the gene of interest. The recombination consequently allows excision of the marker, retaining one of the *FRT* sites (34 bp in length). The same cassette can then be used for the second copy deletion of the desired gene. Two rounds of recycling of the cassette (in diploid organisms) generate the deletion strain free of the *NAT* resistance marker gene.

To delete *PSH1*, upstream (US) and downstream (DS) sequences of *PSH1* were amplified from the genome. They were cloned in pSFS2a as *Kpn*I/*Xho*I and *Sac*II/*Sac*I fragments respectively to obtain the plasmid pPJ1. The clones were confirmed using restriction analysis. Plasmid pPJ1 was digested by *Kpn*I and *Sac*I to release the desired deletion cassette of 5275 bp (Figure 7A - B).

For the first copy deletion of *PSH1*, the *Kpn*I/*Sac*I digested pPJ1 was transformed into *C. albicans,* using the standard lithium acetate transformation procedure as described before (Gietz and Woods 2002). For the selection of *NAT*-resistant (NAT^R) transformants, 100 µg/ml of nourseothricin (Werner Bioagents, Jena, Germany) was added to YPDU agar plates. Deletion of one copy of *PSH1* was confirmed by PCR. The

resulting strain was *PSH1/psh1::FRT-NAT-FRT*. To obtain *NAT* sensitive derivatives (NAT^S), transformants were grown in presence of 2% maltose to obtain the strain *PSH1/psh1::FRT*. The desired heterozygous *PSH1/psh1* (one copy deleted) NAT^S transformants were used to delete the remaining copy of *PSH1* by releasing the deletion cassette from pPJ1 to get the final strain *psh1::FRT-NAT-FRT/psh1::FRT*. In this way, homozygous *psh1/psh1* null mutants were generated in two different genetic backgrounds. The resulting strains were PJ100 and PJ102 (see Materials and methods for the genotype). Homozygous and heterozygous null mutants of Psh1 were confirmed by Southern blot analysis (Figure 7C-E). Growth of these mutants on YPDU plates indicates that Psh1 is not essential for viability in *C. albicans* (Figure 7F).

In *C. albicans*, over-expression of CENP-A does not cause lethality (SANYAL and CARBON 2002). Thus, an increase in CENP-A levels that might happen upon Psh1 deletion should keep the organism viable. This can explain why Psh1 is non-essential for viability in *C. albicans*. Growth of a strain lacking Psh1 also suggests the existence of additional pathways for regulating CENP-A levels. Psh1 is non-essential for viability in *S. cerevisiae* also. Only when CENP-A was overexpressed in the *PSH1* deletion background, the organism becomes inviable (HEWAWASAM *et al.* 2010; RANJITKAR *et al.* 2010). Thus, it will be interesting to analyse the phenotype of *psh1/psh1* null mutants in CENP-A over-expressed condition in *C. albicans* as well.



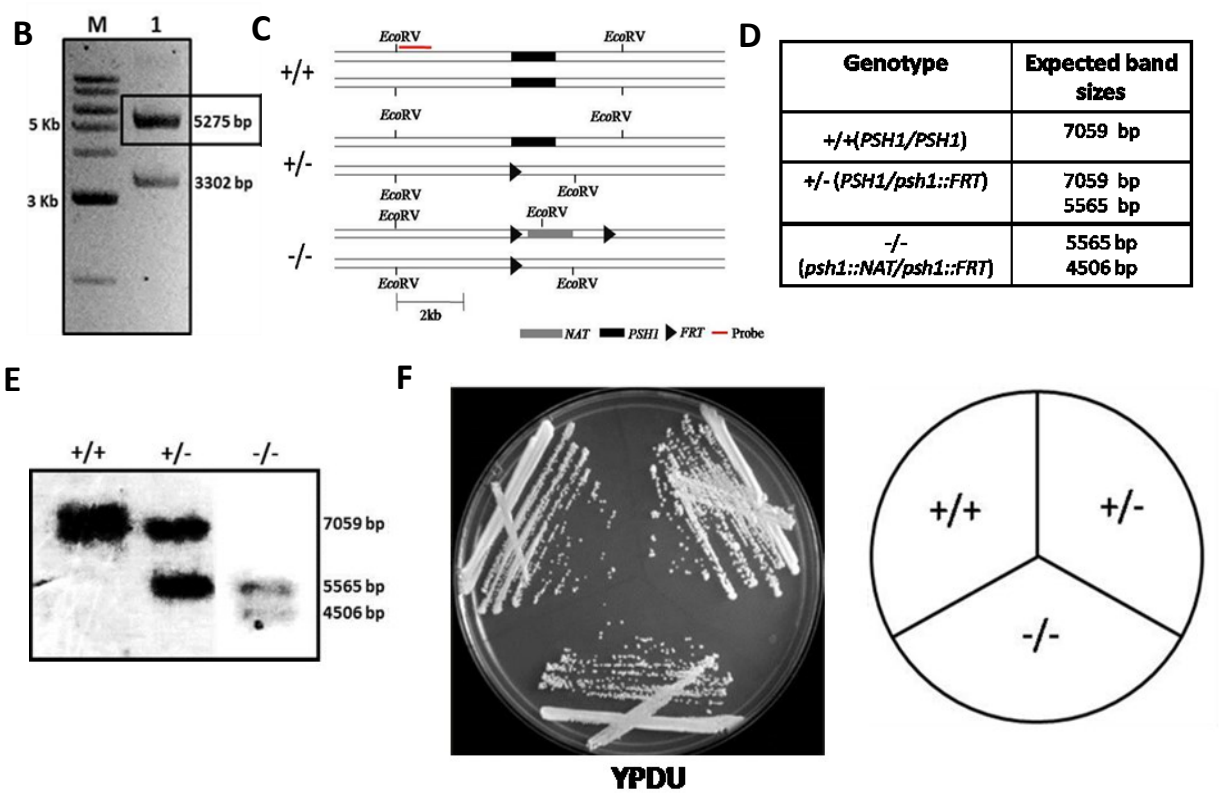


Figure 7. Psh1 is non-essential for viability in *C. albicans.* (A) Schematic of the *PSH1* deletion cassette*.* **(B)** Agarose gel showing Psh1 deletion cassette. M: 1 kb ladder (NEB), 1: the *Kpn*I/*Sac*I double digested pPJ1 releasing the desired 5275 bp cassette. **(C)** Line diagrams for Southern blot confirmation of *PSH1* deletion. The genomic DNA was digested with *Eco*RV and hybridized with an upstream sequence (marked as a red line) as the probe. **(D)** A table showing expected band sizes on Southern blot corresponding to the genotypes of the strains. **(E)** Southern hybridization analysis for the confirmation of *psh1/psh1* null mutants. **(F)** Strains were streaked on YPDU plates and grown at 30°C. Photographs were taken after 3 days. +/+, wild type; +/-, heterozygous *psh1/PSH1* mutant; -/-, homozygous *psh1/psh1* null mutant.

2.2. Psh1 associates with chromatin including centromeres

Psh1 has been shown to degrade CENP-A from the ectopic loci in *S. cerevisiae*. Since CENP-A is a DNA binding protein, we speculated Psh1 should also be present in the close proximity to chromatin. Hence, we wanted to determine if Psh1 associates with chromatin in *C. albicans*.

2.2.1 Construction of *PSH1-GFP* tagged strain.

Psh1 was tagged with GFP at its C-terminus using pGFP-HIS (CHATTERJEE *et al.* 2016). For this, a C-terminal part of *PSH1* ORF was PCR amplified from the genome and cloned between *Spe*I and *Sac*II sites of pGFP-HIS to obtain pPsh1GFP. The clones were confirmed by restriction analysis (Figure 8 A-B). The plasmid was then linearised by *Nsi*I and used to transform *C. albicans* by the standard lithium acetate transformation procedure (see Materials and methods). The transformants were selected on CM-His plates and desired transformants were confirmed by PCR and DNA sequencing (Figure 8C). The resultant strain obtained was PJ10 (*PSH1-GFP (HIS1)/PSH1*).

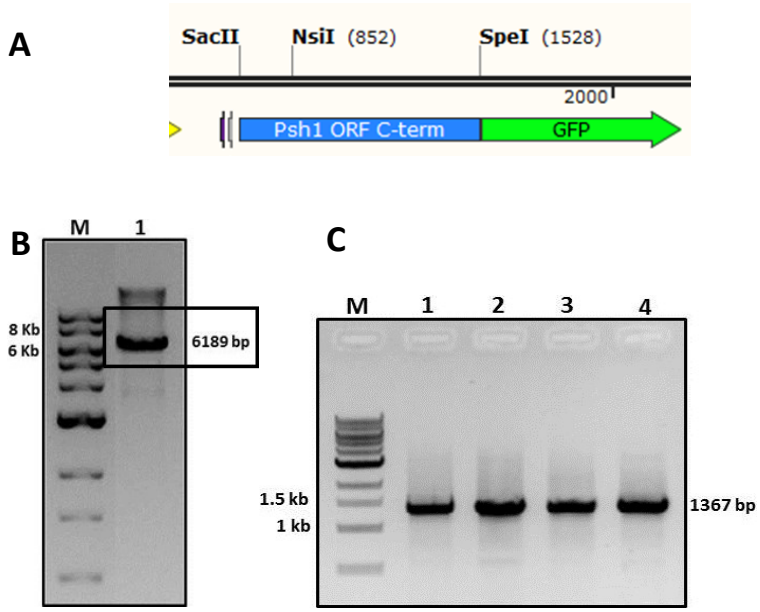


Figure 8. Tagging Psh1 with GFP. (A) Schematic of the Psh1-GFP tagging cassette. **(B)** Agarose gel showing confirmation of the Psh1-GFP tagging cassette. M: 1 kb ladder (NEB), 1: the *Nsi*I linearized pPsh1GFP showing the desired 6189 bp cassette. **(C)** PCR confirmation of *PSH1-GFP* tagged *C. albicans* strains. M: 1 Kb ladder (NEB), 1-4: *PSH1 GFP* tagged transformants.

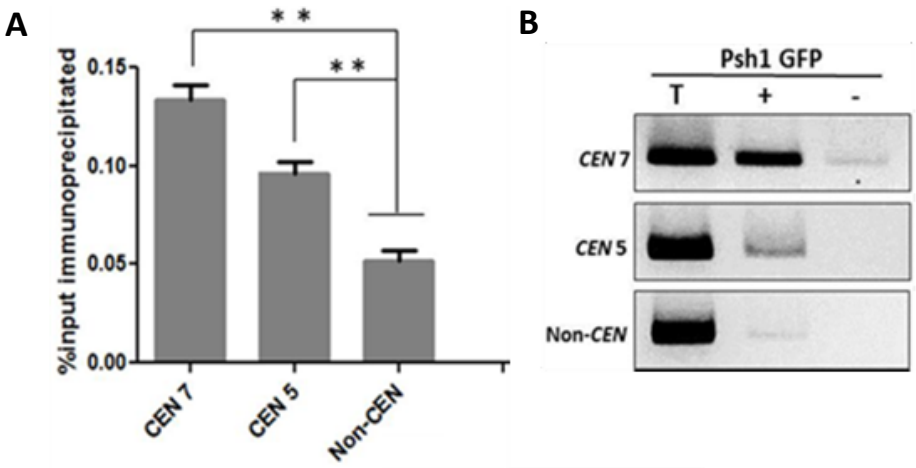
2.2.2 Chromatin immunoprecipitation (ChIP) assays with Psh1-GFP

Association of Psh1 with chromatin was analysed by performing ChIP assays. Briefly, cells were cross-linked for 30 min, chromatin was fragmented to an average size of 300-500 bp by sonication, and immuno-precipitated with or without anti-GFP antibodies. The binding of the recovered DNA fragments to chromatin was analysed using the primer pairs mentioned in Table2. One of the regions where Psh1 was shown to bind in *S. cerevisiae* was centromere (HEWAWASAM *et al.* 2010). We sought to determine if ChIP recovered DNA fragments include any centromere DNA sequences. We analysed binding of Psh1 at two centromeres, *CEN*5 and *CEN*7, and found that Psh1 is enriched at those sites by both semi-quantitative and qPCR (Figure 9A-B). Notably, we also observed a marginal enrichment of Psh1 binding at a far non-*CEN* region.

Psh1 binding at the core centromere regions made us curious to further analyse its enrichment in and around the centromere. For this, we scanned the whole 3kb centromere of chromosome7 and ~ 2kb region flanking to it, for Psh1 binding and then compared it with the CENP-A enrichment profile, by semi-quantitative PCR. In *C. albicans*, CENP-A binds to a 3kb region which specifies the centromere location. The binding pattern within the 3kb region is bell shaped wherein it is highest in the middle and then starts decreasing at the ends (SANYAL *et al.* 2004). We found Psh1 binds in the middle of the centromere. Strikingly, the enrichment starts decreasing at the end of the centromere and then it again increases after the 3 kb region (Figure 9C).

Psh1 is responsible for degrading CENP-A from the ectopic loci in other organisms. Thus, Psh1 is expected to bind throughout the genome. Binding to Psh1 across many chromosomal sites has been observed in *S. cerevisiae* (Sue Biggins, personal communication). Thus it is difficult to choose a region as a negative control for

Psh1 ChIP-PCR assays. This could also explain why we observed a marginal Psh1 enrichment at the far non-*CEN* regions. Regardless of the region we chose, we always found a higher enrichment of Psh1 at the core centromere. This is consistent with *Sc*Psh1 binding which was shown to be present at the centromeres, promoters of different genes and intergenic loci (Sue Biggins, personal communication). The reason behind localisation of Psh1 at the centromere is not known. Functionally, we do not expect Psh1 to be at the centromere as *CEN* associated CENP-A is not degraded (COLLINS *et al.* 2004). This raises an important question regarding the association of Psh1 with centromere chromatin, whether it is direct or indirect. Within the centromeric region, we observed a drop in Psh1 binding and then a sudden increase in the enrichment after the 3 kb region. We do not know the significance of this at present. However, we also need to validate these results by qPCR. Together, we conclude, Psh1 associates with centromeric and pericentric chromatin.



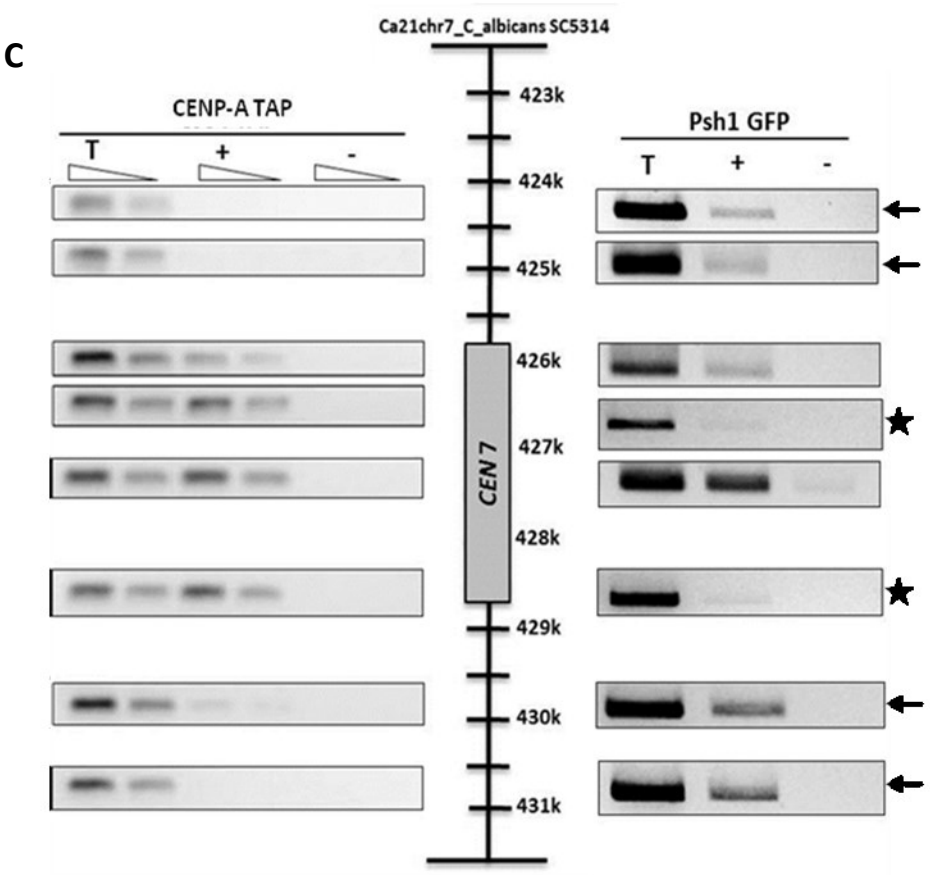


Figure 9. Psh1 associates with chromatin. (A-B) Psh1 GFP ChIP showing enrichment at centromere 5 and 7 by qPCR and semi-quantitative PCR, respectively. **(C)** Comparison of Psh1 binding with CENP-A binding at CEN7 by ChIP assay. Left and right panels show enrichment of CENP-A (SANYAL *et al.* 2004) and Psh1, respectively by semi-quantitative PCR. Stars show the sites within the 3kb centromere region where Psh1 binding drops. Arrow heads show regions flanking the centromere where Psh1 binding increases. T, total input DNA; +, immuno-precipitated DNA with anti-GFP antibody; -, beads only control without antibody, n=3.

2.3 *PSH1* deletion does not affect nuclear segregation and cell cycle progression

Enrichment of Psh1 at the centromere led us to examine its possible role in the process of chromosome segregation. For this, we first analysed the nuclear morphology in *psh1/psh1* null mutants (PJ100 and PJ102) after DAPI staining followed by confocal microscopy. We did not find any significant difference in nuclear morphology between the wild type and *psh1/psh1* null mutants. We also examined the spindle morphology of

null mutants by immuno-staining using anti-tubulin antibodies. We did not find any significant spindle defects in the null mutants, suggesting Psh1 deletion does not directly affect the chromosome segregation process (Figure 10A). To further elucidate the function of Psh1, we examined if it has any role in cell cycle progression. For this we quantified cells in a particular cell cycle stage in both wild type (J127) and *psh1/psh1* null mutant (PJ100) by flow cytometry after staining the cells with propidium iodide (Figure 10B). We did not find any cell cycle arrest in the *psh1/psh1* null mutants, except for the one transformant (T1) which showed slightly more number of cells in G2/M stage. Together, *PSH1* deletion does not affect the process of nuclear segregation or cell cycle progression.

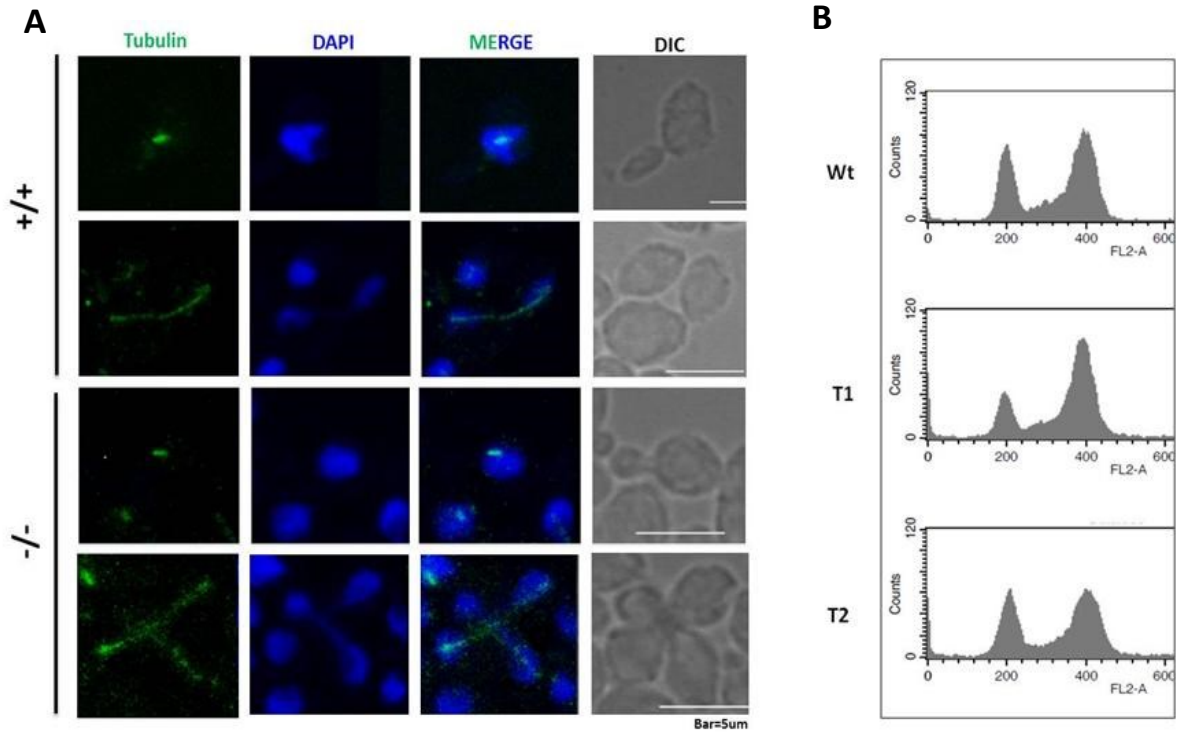


Figure 10. *PSH1* deletion does not affect nuclear segregation and cell cycle progression. (A) Wild type (+/+) and *psh1/psh1* null mutants (-/-) were fixed and stained with DAPI and anti-tubulin antibody followed by confocal microscopy, n=5 in each case, bar (white line), 5µm. **(B)** Flow cytometry analysis of wildtype (wt) and *psh1/psh1* null mutants (T1 and T2 are two transformants), n=1.

2.4 Absence of Psh1 does not prevent CENP-A de-localisation from the centromere upon kinetochore depletion.

In *C. albicans*, assembly of the KT is a highly coordinated and interdependent process wherein depletion of any of the proteins from the inner, middle or outer KT results in disassembly of the entire KT (THAKUR and SANYAL 2012). Depletion of any of the essential KT proteins in *C. albicans* also results in CENP-A de-localisation and degradation (THAKUR and SANYAL 2012) (Figure 11A). However, a non-degradable form of CENP-A in which all the lysines were mutated to arginines was still de-localised from the centromere, but not degraded, upon KT depletion (THAKUR and SANYAL 2012), suggesting CENP-A de-localisation and degradation are two separate events. We speculated Psh1 is involved in this process of CENP-A de-localisation and degradation when a KT is absent. Hence, we wanted to analyse CENP-A localisation in absence of Psh1 upon KT depletion.

For this we deleted both the copies of Psh1 in J127 strain where CENP-A is GFP tagged and an essential outer kinetochore protein Dam1 is under a repressible *MET3* promoter, to give the resultant strain PJ100. The *MET3* promoter is repressed in presence of cysteine (Cys) and methionine (Met) (CARE *et al.* 1999). The cells were grown under permissive (+Cys +Met) or non-permissive conditions (-Met -Cys) of the *MET3* promoter for 8 h and examined for GFP signals by confocal microscopy. Under permissive conditions, we found CENP-A GFP signals in both wild type and *psh1/psh1* null mutants. But, under repressive conditions, CENP-A was still de-localised from the centromere, both in wild type and *psh1/psh1* null mutant (Figure 11B).

Absence of Psh1 could not prevent CENP-A from getting de-localised upon KT depletion. This suggests that there might be some other proteins involved in taking out

CENP-A from chromatin upon KT depletion and exposing it to the degradation machinery. In support of this, a recent study has shown that in budding yeast, the FACT (facilitates chromatin transcription/transactions) complex is required for Psh1-mediated CENP-A degradation (DEYTER and BIGGINS 2014). The FACT complex takes out CENP-A from chromatin and exposes the CATD domain of CENP-A to Psh1 for ubiquitination. The similar mechanism could be present in *C. albicans* as well. Furthermore, since, Psh1 deletion mimics the situation where CENP-A was non-degradable, it is possible that CENP-A is de-localised but not degraded in absence of Psh1. Hence, it will be interesting to determine CENP-A levels by western blot analysis in absence of Psh1 upon KT depletion. The required strain is under construction.

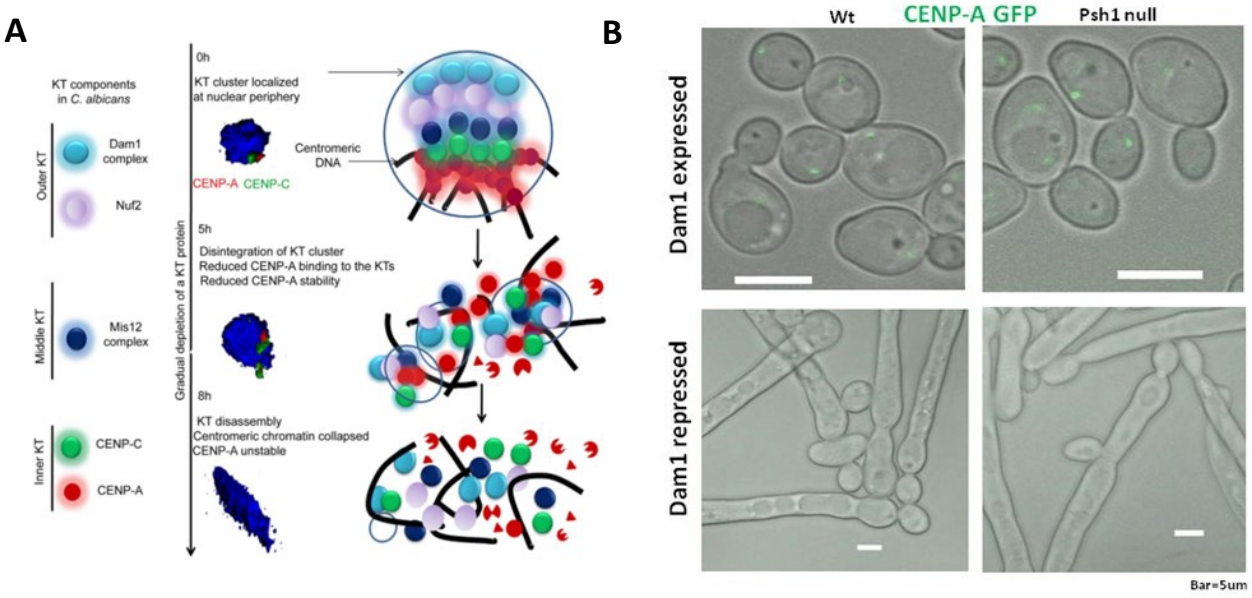


Figure 11. Psh1 does not de-localise CENP-A upon KT depletion. (A) Schematic showing dynamics of KT disassembly in *C. albicans*. Depletion of any of the essential KT proteins in *C. albicans* also results in CENP-A de-localisation and degradation (THAKUR and SANYAL 2012). **(B)** Wild type (J127) and *psh1/psh1* null mutants (PJ100) were grown in permissive (-Met -Cys) or repressive (+Met +Cys) conditions for 8 h to express or repress Dam1, respectively. Bar (white line), 5µm.

2.5 Effect of *PSH1* deletion on CENP-A levels.

Psh1 has been shown to ubiquitinate CENP-A for proteasomal degradation. We hypothesized that absence of Psh1 prevents CENP-A from degradation and leads to its increased levels inside the cell. Hence, we wanted to analyse CENP-A levels in absence of Psh1.

For this, we made a *psh1/psh1* null mutant in a strain where CENP-A is TAP tagged (CAKS102) to get the resultant strain PJ102. We first determined CENP-A levels in this strain by western blot analysis. We found in absence of Psh1, CENP-A levels were higher than the wild type (Figure 12A). Over-expression of CENP-A leads to its increased binding at the centromere in *C. albicans* (BURRACK et al. 2011). We speculated Psh1 deletion may mimic the CENP-A over-expression condition and may result in its increased binding at the centromere. Hence, we examined CENP-A levels at the centromere in PJ101 by ChIP assay. The preliminary results showed a higher enrichment of CENP-A at *CEN*7 than wild type (Figure 12B). To further confirm that absence of Psh1 leads to higher CENP-A levels, we measured the intensity of CENP-A-GFP signals in PJ100 under permissive condition (-Met -Cys). For measurement, G1 or anaphase cells were considered, as cells in other stages (such as S phase) may have variable CENP-A intensities due to chromosome duplication. To our surprise, we found one of the transformants (T1) had unusually high CENP-A GFP intensities, while the other two had the same intensity as that of wild type (T2 and T3) (Figure 12C).

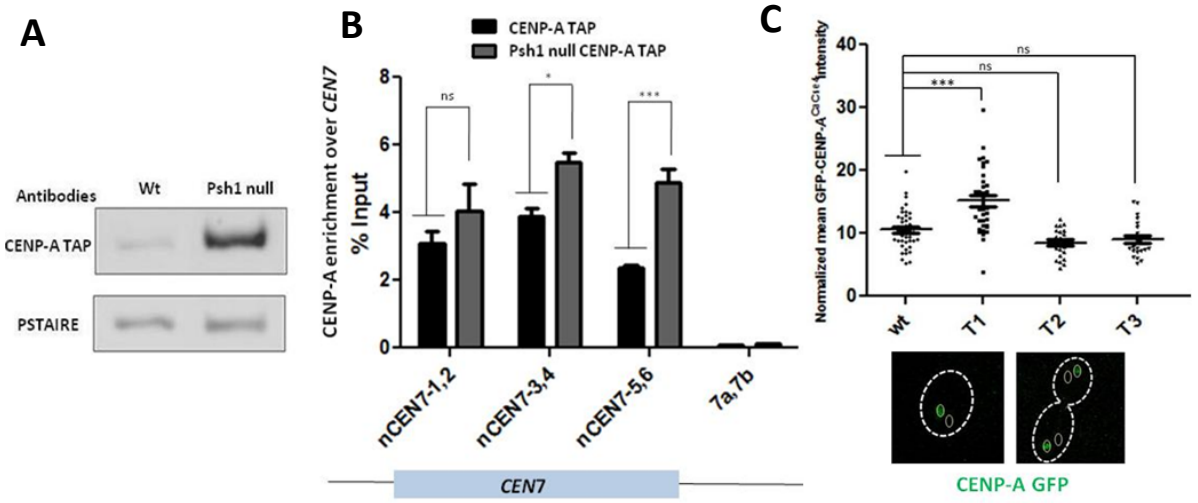


Figure 12. CENP-A levels in absence of Psh1. (A) Western blot showing CENP-A levels in wild type (CAKS102) and *psh1/psh1* null mutant (PJ101), n=1. **(B)** ChIP assay showing CENP-A enrichment at CEN7 in wild type (CAKS102) and *psh1/psh1* null mutant (PJ101) by qPCR, n=1. X-axis shows different primer pairs used for the analysis, nCEN7-1 to 6 are primer pairs across the 3 kb CEN7, 7a and 7b is a primer pair for the far-CEN region. **(C)** Intensity of the GFP-CENP-A signals was measured by the ImageJ software for wild-type (J127, n=80) and *psh1/psh1* null mutants, T1-T3 (PJ100, n=30 for T1, n=80 for T2&T3) for the G1 and anaphase stage under permissive conditions (-Met -Cys). Lower panel shows the measurement technique for calculating GFP intensity.

In *S. cerevisiae*, Psh1 deletion only leads to a marginal increase in CENP-A levels with a huge heterogeneity between the cells (HERRERO and THORPE 2016). We too observed the similar heterogeneity in CENP-A levels in *C. albicans*, but we need to screen more transformants in order to conclude that CENP-A levels are high in absence of Psh1.

Conclusions

In this study, we have tried to understand the function of a putative CENP-A E3 ubiquitin ligase Psh1, in *C. albicans*. E3 ligases confer specificity to ubiquitination reaction by recognizing and ubiquitinating the target substrate. Psh1 in budding yeast (*Sc*Psh1) targets specifically CENP-A for proteasomal degradation when mis-incorporated, hence ensuring the specificity of CENP-A at the centromere. With the aim of determining a putative homolog of CENP-A E3 ligase in *C. albicans*, we began our study by identifying Psh1 in *C. albicans* using *Sc*Psh1 sequence as a query. The identified homolog in *C. albicans* (*Ca*Psh1) contained the similar domains as that of *Sc*Psh1. We also identified Psh1 homologs in other ascomycetes, suggesting it may be a conserved protein in fungi. In order to determine its function, we generated a psh1/psh1 null mutant. We found, like *Sc*Psh1, *Ca*Psh1 is also non-essential for viability suggesting the possibility of existence of additional pathways for regulating CENP-A in *C. albicans.* To further investigate its role as an E3 ligase of CENP-A (which is a DNA binding protein), we examined if Psh1 associates with chromatin. We found *Ca*Psh1 binds to chromatin, including the centromere by ChIP. However, we observed a different binding profile of Psh1 than that of CENP-A, the significance remains unknown at present. Though *Ca*Psh1 is localised at the centromere, it does not play any obvious role in chromosome segregation or cell cycle progression. We also showed that Psh1 does not play any role in de-localising CENP-A from the centromere upon depletion of an essential outer kinetochore protein Dam1. Analysis of CENP-A levels in *psh1/psh1*Psh1 null mutants showed heterogeneity among the transformants. We observed high CENP-A levels in some but not in all transformants. We need to screen more transformants to conclude whether absence of Psh1 stabilises CENP-A in *C. albicans*.

Materials and Methods

4.1 Strains, plasmids and primers

The strains and plasmids used in this study are listed in Table 1. The primers used for PCR are listed in Table 2.

4.2 Media and growth conditions

C. albicans strains were grown in YPDU (1% Yeast extract, 2% peptone, 2% dextrose and 100 µg/ml uridine) at 30°C. For recycling of the *SAT1* flipper cassette, strains were grown in YPMU (1% Yeast extract, 2% peptone, 2% Maltose and 100 µg/ml uridine). NAT^R transformants were selected on YPDU with 100 µg/ml nourseothricin. Psh1 GFP tagged transformants were selected on complete medium (CM) (2% dextrose, 1% YNB and auxotrophic supplements). Conditional mutant strains J127 and PJ100, where *DAM1* is under the regulation of the *MET3* promoter, were grown in YPDU as permissive media and YPDU with 10 mM Cysteine and 10 mM Methionine as non-permissive media.

4.3 Lithium acetate transformation of *C. albicans*

C. albicans cells were transformed by standard techniques (SANYAL and CARBON 2002). Briefly, Cells were grown till an OD_{600} of 0.8, harvested, washed and resuspended in lithium acetate and 10X TE mix (Tris, EDTA). Resuspended cells were aliquoted as 100µl and ~ 6µg DNA was added along with the carrier DNA (Herring salmon sperm DNA). Cells were incubated with PEG 3350 overnight at 30°C and spreaded onto the selection plates.

4.4 Southern hybridization

psh1/psh1 null mutants were confirmed using standard Southern hybridization technique as described in (SOUTHERN 1975). Genomic DNA was isolated by glass beads (Sigma G8772), digested with enzymes *Eco*RV. DNA was transferred using capillary transfer, blotted on the Zeta probe membrane (BioRad 162-0153). The blots were hybridized with specific probes (Table 2). The blots were then washed, dried and exposed on a X-ray film.

4.5 ChIP assay and PCR analysis

The ChIP assay was done as described previously (SANYAL *et al.* 2004). Briefly, asynchronous cultures of *C. albicans* strains were grown in YPDU till OD_{600} of 1.0. They were cross-linked with 37% formaldehyde for 30 min (for Psh1 GFP) and 15 min (for Cse4 TAP) and quenched by adding 2.5 M glycine to a final concentration of 125 mM for 5 min. Subsequently, sonication was performed with Biorupter (Diagenode) to get sheared chromatin fragments of an average size of 300–500 bp. The fragments were immuno-precipitated with anti-GFP antibodies (Roche) and anti-Protein A (Sigma) at a final concentration of 4 ug/ml and 20µg/ml of immunoprecipitate (IP), respectively. Protein A Sepharose beads (50% slurry in TE) was then added to the IP and incubated at 4°C overnight. After washing the beads, IP DNA was eluted and precipitated in 100% ethanol overnight at -20°C. The isolated DNA was then dried and resuspended in TE. The recovered IP DNA was assayed by PCR using specific set of primers as mentioned in Table 2. Realtime PCR analysis was carried out in i- Cycler (BIO-RAD) with IQ Sybr Green Supermix (Bio-Rad). Cycling parameters were as follows: 94°C/30s, 55°C/30s, 72°C/45s, repeated 40×.

4.6 Indirect immunofluorescence

C. albicans cells (CAKS102, PJ100, J127 and PJ101) were grown in YPDU till OD_{600} of 1 and were fixed by 37% formaldehyde at room temperature for 1 h. Fixed cells were washed and resuspended in 0.1 M Phosphate buffer (pH 6.4). Approximately 80-90% spheroplasting was achieved using lyticase (Sigma) and β-mercaptoethanol. Spheroplasts were pelleted down and washed with PBS (Phosphate Buffer Saline buffer). About 15 µl spheroplasted cells were placed onto each well of polylysine (1 mg/ml) treated teflon coated slide. Cells attached to the slide were fixed in ice cold methanol (-20˚C) for 6 min and in ice cold acetone (-20˚C) for 30s. Blocking was done with 2% skim milk for 30 min. Subsequently cells were incubated in primary antibodies for 1 h and washed with PBS for 8-10 times. Cells were treated with secondary antibodies for 1 h in dark humid chamber followed by washing with PBS 8-10 times. DAPI was added at a final concentration of 100 ng/ml in 70% glycerol for mounting. Antibodies were diluted as described, 1:200 for rat anti-tubulin antibody (abcam), 1:500 for Alexa Fluor 488 Goat anti-rat IgG (Invitrogen). Cells were examined at 100× magnification on a confocal laser scanning microscope (LSM 510 META, Carl Zeiss).

4.7 FACS analysis.

C. albicans cells (J127 and PJ100) were grown till OD_{600} of 1 under permissive conditions (-Met -Cys). Cells equivalent to OD_{600} 1.5 were pelleted and washed in PBS buffer and fixed in 70% ethanol overnight at 4 °C. Fixed cells were washed in 500 µl of RNAse buffer (0.2M Tris-HCl pH 7.5, 20mMEDTA, pH 8.0) and resuspended in 100ul of RNAse buffer with 10µl of RNAse (10mg/ml) followed by incubation at 37˚C for 4 h. The cells were then washed with PBS and stained with propidium iodide after resuspension

in 900 µl of PBS and 100ul of PI (0.005µg/ml) followed by half an hour incubation in dark at room temperature. Stained cells were diluted into PBS and sonicated. Flow cytometry was performed on 10,000 cells and analysed using the FL2 channel on a Becton–Dickinson FACS calibur.

4.8 Fluorescence microscopy

The *C. albicans* strains (J127 and PJ100) were grown overnight in YPDU. These cells were transferred into permissible (-Met -Cys) and repressible media (+Met +Cys) with a starting $O.D._{600}$ of 0.2 and grown for 8 h. Cells were pelleted and washed once with distilled water and finally resuspended in distilled water. Cell suspension (0.5-1 µl) was placed on a slide containing a thin 2% agarose patch and covered with a coverslip. Images were captured at 100X using either Zeiss LSM Meta 510 confocal microscope or the Delta vision system (Olympus IX-71 base) equipped with a Cool snap HQ2 high-resolution CCD camera. Image processing was done using either Zeiss image processing software LSM, Image J, Image Pro-plus or Photoshop (Adobe Systems, San Jose, CA).

4.9 Protein preparation and western blot analysis

Strains were grown overnight in YPDU. Overnight cultures were then pelleted down and washed with water twice to remove any residual medium. 3 OD cells were taken, washed with water once and resuspended in 12.5% TCA (tri-chloro acetic acid) and incubated at -20˚C overnight, for precipitation. The cells were pelleted down and washed twice with ice cold 80% acetone. The pellet was then allowed to air dry and then finally resuspended in lysis buffer (0.1N NaOH and 1% SDS and 5X protein loading dye).

For western blot assays, protein samples were boiled at 95˚C for 10 min and run in 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to a nitrocellulose membrane and blocked in 5% non-fat milk in PBS. Membranes were incubated with a 1:5000 dilution of anti-Protein A (Sigma) or anti-PSTAIRE (Sigma) in 2.5% non-fat milk in PBS-Tween. Membranes were washed 3 times in PBS-Tween and then exposed to a 1:10,000 dilution of either anti-mouse- or anti-rabbit -horseradish peroxidase antibody (Pierce) in 2.5% nonfat milk in PBS-Tween. Membranes were washed 3 times in PBS-Tween, incubated with Super Signal West Dura Extended Duration Substrate (Pierce) and exposed to X-ray films.

Table1: List of *C. albicans* strains used in this study

Strain	Genotype	Reference
SN148	*Δura3*::*imm434/Δura3*::*imm434 Δhis1::hisG/Δhis1::hisG Δarg4::hisG/Δarg4::hisGΔleu2::hisG/Δleu2::hisG*	(NOBLE and JOHNSON 2005)
J127	*ura3Δ-iro1Δ::imm434/ ura3Δ-iro1Δ::imm434 his1Δ/his1Δ arg4Δ/arg4Δ MET3prDAM1(CdARG4)/dam1::HIS1 cse4::dpl200-URA3/CSE4:GFP:CSE4*	(THAKUR and SANYAL 2012)
CAKS102	*Δura3*::*imm434/Δura3*::*imm434 Δhis1::hisG/Δhis1::hisG Δarg4::hisG/Δarg4::hisGΔleu2::hisG/Δleu2::hisGCSE4/CSE4-TAP(URA3)*	(MITRA et al. 2014)
PJ10	*Δura3*::*imm434/Δura3*::*imm434 Δhis1::hisG/Δhis1::hisG Δarg4::hisG/Δarg4::hisGΔleu2::hisG/Δleu2::hisGPSH1/PSH1-GFP(HIS1)*	This study
PJ99	*ura3Δ-iro1Δ::imm434/ ura3Δ-iro1Δ::imm434 his1Δ/his1Δ arg4Δ/arg4Δ MET3prDAM1(CdARG4)/dam1::HIS1 cse4::dpl200-URA3/CSE4:GFP:CSE4 psh1::FRT/PSH1*	This study
PJ100	*ura3Δ-iro1Δ::imm434/ ura3Δ-iro1Δ::imm434 his1Δ/his1Δ arg4Δ/arg4Δ MET3prDAM1(CdARG4)/dam1::HIS1 cse4::dpl200-URA3/CSE4:GFP:CSE4 psh1::FRT/psh1::NATflp*	This study
PJ101	*Δura3*::*imm434/Δura3*::*imm434,Δhis1::hisG/Δhis1::hisG Δarg4::hisG/Δarg4::hisGΔleu2::hisG/Δleu2::hisGCSE4/CSE4-*	This study

	TAP(URA3)psh1::FRT/PSH1	
PJ102	*Δura3*::*imm434/Δura3*::*imm434,Δhis1::hisG/Δhis1::hisG Δarg4::hisG/Δarg4::hisG Δleu2::hisG/Δleu2::hisGCSE4/CSE4-TAP(URA3) psh1::FRT/psh1::NATflp*	This study
Plasmid	**Description**	**Reference**
pPJ1	*pSFS2a + 5'CaPSH1+ 3'CaPSH1*	This study
pPsh1 GFP	*pGFP-His+CaPSH1C-term*	This study

Table 2: primers used in this study

Oligo Name	Sequence	Description
Primers used for Psh1 deletion		
PJ4	agtaggtaccggtagtactgcttaccattgag	Psh1 US forward primer
PJ5	agatctcgaggaattatggacacatacaagtgtgg	Psh1 US reverseprimer
PJ6	gataccgcgggatgatgatgatggttggtgg	Psh1 DS forward primer
P7J	atgagagctcctaaaaatgccgattgtctacctc	Psh1 DS reverse primer
Primers used for probing the Southern Blot		
PJ20	gattagtccaattacatctcatattg	Forward primer
PJ21	cttgatcagttcgacttatcc	Reverse primer
Primers used for tagging Psh1 with GFP		
PJ14	Gtcaccgcgggtgacgacattaattgattgttctgatggag ttgcaag	Psh1 C-term forward primer
PJ15	Cagtactagtccaccaaccatcatcatcatcatcatcatta tcattatctac	Psh1 C-term reverse primer
PJ10	gataccgcggataatacaattgagtggagatg	Tagging verification forward primer
GFP Internal RP	ccatacgcgaaagtagtg	Tagging verification reverse primer
Primers used for Psh1 GFP ChIP, Cse4 TAP ChIP		
2498-17	gcttggccctcagtataactggat	Semi-quantitative ChIP PCR primers forCEN7
2498-18	cttcaggacaagctccatatctcttc	Semi-quantitative ChIP PCR primers forCEN7
2498-19	gccatacggtagtcaaactcctgg	Semi-quantitative ChIP PCR primers for CEN7
2498-20	cctgaaccactactgcagaaacgt	Semi-quantitative ChIP PCR primers for CEN7
2498-5	gcgtaacgggcctagtttcgataagag	Semi-quantitative ChIP PCR primers for CEN7

2498-6	catgcacaggctcttatagcaagt	Semi-quantitative ChIP PCR primers for CEN7
2498-7	gcctgtagcgatgtaagtatatggag	Semi-quantitative ChIP PCR primers for CEN7
2498-8	ccacctctgcactaatctacaatgc	Semi-quantitative ChIP PCR primers for CEN7
2498-21	ctagtgcaagaccctcatagaagc	Semi-quantitative ChIP PCR primers for CEN7
2498-22	cctgacactgtcgtttcccatagc	Semi-quantitative ChIP PCR primers for CEN7
2498-9	gaaacgatccttcctgtacaccac	Semi-quantitative ChIP PCR primers for CEN7
2498-10	cttgatagcgatcagtgggttcag	Semi-quantitative ChIP PCR primers for CEN7
2498-23	gtatgacctaaagctgtgagctgc	Semi-quantitative ChIP PCR primers for CEN7
2498-24	cagagcaatggcccttgtgattgt	Semi-quantitative ChIP PCR primers for CEN7
2498-15	caagctgccttgtcaggcaaagcatc	Semi-quantitative ChIP PCR primers for CEN7
2498-16	ccatctccaacccgccatgccagc	Semi-quantitative ChIP PCR primers for CEN7
Cen 5e	tgttctgacatactgggtagacttt	Semi-quantitative ChIP PCR primers for CEN5
Cen 5f	cgaagcattttgtataacagccc	Semi-quantitative ChIP PCR primers for CEN5
CaCH5F1	ttcatggaagaggggtttca	qPCR primers for CEN5
CaCh5R1	cccgcaaataagcaaacact	qPCR primers for CEN5
NCEN7-1	cacctctgcactaatctacaatg	qPCR primers for CEN7
NCEN7-2	tgttgagatgtcttattgattaggt	qPCR primers for CEN7
NCEN7-3	gcatacctgacactgtcgtt	qPCR primers for CEN7
NCEN7-4	aacggtgctacgttttttta	qPCR primers for CEN7
NCEN7-5	tcaattatcgcttgatagcg	qPCR primers for CEN7
NCEN7-6	ctatcatcatgccagcctag	qPCR primers for CEN7
Ctrl 7 a	actcgccttcccctcctttaaatag	qPCR and semi-quantitative ChIP PCR primers for non centromeric region
Ctrl 7 b	ccactactacgactgtggattcact	qPCR and semi-quantitative ChIP PCR primers for non centromeric region

References

AMATO, A., T. SCHILLACI, L. LENTINI and A. DI LEONARDO, 2009 CENPA overexpression promotes genome instability in pRb-depleted human cells. Mol Cancer **8:** 119.

ARDLEY, H. C., and P. A. ROBINSON, 2005 E3 ubiquitin ligases. Essays Biochem **41:** 15-30.

BLACK, B. E., L. E. JANSEN, P. S. MADDOX, D. R. FOLTZ, A. B. DESAI *et al.*, 2007 Centromere identity maintained by nucleosomes assembled with histone H3 containing the CENP-A targeting domain. Mol Cell **25:** 309-322.

BORDEN, K. L., and P. S. FREEMONT, 1996 The RING finger domain: a recent example of a sequence-structure family. Curr Opin Struct Biol **6:** 395-401.

BURRACK, L. S., S. E. APPLEN and J. BERMAN, 2011 The requirement for the Dam1 complex is dependent upon the number of kinetochore proteins and microtubules. Curr Biol **21:** 889-896.

CAMAHORT, R., B. LI, L. FLORENS, S. K. SWANSON, M. P. WASHBURN *et al.*, 2007 Scm3 is essential to recruit the histone h3 variant cse4 to centromeres and to maintain a functional kinetochore. Mol Cell **26:** 853-865.

CARE, R. S., J. TREVETHICK, K. M. BINLEY and P. E. SUDBERY, 1999 The MET3 promoter: a new tool for Candida albicans molecular genetics. Mol Microbiol **34:** 792-798.

CHATTERJEE, G., S. R. SANKARANARAYANAN, K. GUIN, Y. THATTIKOTA, S. PADMANABHAN *et al.*, 2016 Repeat-Associated Fission Yeast-Like Regional Centromeres in the Ascomycetous Budding Yeast Candida tropicalis. PLoS Genet **12:** e1005839.

COLLINS, K. A., S. FURUYAMA and S. BIGGINS, 2004 Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. Curr Biol **14:** 1968-1972.

DEYTER, G. M., and S. BIGGINS, 2014 The FACT complex interacts with the E3 ubiquitin ligase Psh1 to prevent ectopic localization of CENP-A. Genes Dev **28:** 1815-1826.

FOLCO, H. D., A. L. PIDOUX, T. URANO and R. C. ALLSHIRE, 2008 Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres. Science **319:** 94-97.

FOLTZ, D. R., L. E. JANSEN, A. O. BAILEY, J. R. YATES, 3RD, E. A. BASSETT *et al.*, 2009 Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. Cell **137:** 472-484.

GIETZ, R. D., and R. A. WOODS, 2002 Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol **350:** 87-96.

GKIKOPOULOS, T., V. SINGH, K. TSUI, S. AWAD, M. J. RENSHAW *et al.*, 2011 The SWI/SNF complex acts to constrain distribution of the centromeric histone variant Cse4. EMBO J **30:** 1919-1927.

HAYASHI, T., Y. FUJITA, O. IWASAKI, Y. ADACHI, K. TAKAHASHI *et al.*, 2004 Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. Cell **118:** 715-729.

HERRERO, E., and P. H. THORPE, 2016 Synergistic Control of Kinetochore Protein Levels by Psh1 and Ubr2. PLoS Genet **12:** e1005855.

HEUN, P., S. ERHARDT, M. D. BLOWER, S. WEISS, A. D. SKORA *et al.*, 2006 Mislocalization of the Drosophila centromere-specific histone CID promotes formation of functional ectopic kinetochores. Dev Cell **10:** 303-315.

HEWAWASAM, G., M. SHIVARAJU, M. MATTINGLY, S. VENKATESH, S. MARTIN-BROWN *et al.*, 2010 Psh1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. Mol Cell **40:** 444-454.

HU, Z., G. HUANG, A. SADANANDAM, S. GU, M. E. LENBURG *et al.*, 2010 The expression level of HJURP has an independent prognostic impact and predicts the sensitivity to radiotherapy in breast cancer. Breast Cancer Res **12:** R18.

KOREN, A., H. J. TSAI, I. TIROSH, L. S. BURRACK, N. BARKAI *et al.*, Epigenetically-inherited centromere and neocentromere DNA replicates earliest in S-phase. PLoS Genet **6:** e1001068.

LI, Y., Z. ZHU, S. ZHANG, D. YU, H. YU *et al.*, 2011 ShRNA-targeted centromere protein A inhibits hepatocellular carcinoma growth. PLoS One **6:** e17794.

LOMONTE, P., K. F. SULLIVAN and R. D. EVERETT, 2001 Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. J Biol Chem **276:** 5829-5835.

MAEHARA, K., K. TAKAHASHI and S. SAITOH, 2010 CENP-A reduction induces a p53-dependent cellular senescence response to protect cells from executing defective mitoses. Mol Cell Biol **30:** 2090-2104.

MAYER, F. L., D. WILSON and B. HUBE, 2013 Candida albicans pathogenicity mechanisms. Virulence **4:** 119-128.

METZGER, M. B., V. A. HRISTOVA and A. M. WEISSMAN, 2012 HECT and RING finger families of E3 ubiquitin ligases at a glance. J Cell Sci **125:** 531-537.

MITRA, S., J. GOMEZ-RAJA, G. LARRIBA, D. D. DUBEY and K. SANYAL, 2014 Rad51-Rad52 mediated maintenance of centromeric chromatin in Candida albicans. PLoS Genet **10:** e1004344.

MORENO-MORENO, O., S. MEDINA-GIRO, M. TORRAS-LLORT and F. AZORIN, 2011 The F box protein partner of paired regulates stability of Drosophila centromeric histone H3, CenH3(CID). Curr Biol **21:** 1488-1493.

MORENO-MORENO, O., M. TORRAS-LLORT and F. AZORIN, 2006 Proteolysis restricts localization of CID, the centromere-specific histone H3 variant of Drosophila, to centromeres. Nucleic Acids Res **34:** 6247-6255.

NOBLE, S. M., and A. D. JOHNSON, 2005 Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen Candida albicans. Eukaryot Cell **4:** 298-309.

RANJITKAR, P., M. O. PRESS, X. YI, R. BAKER, M. J. MACCOSS *et al.*, 2010 An E3 ubiquitin ligase prevents ectopic localization of the centromeric histone H3 variant via the centromere targeting domain. Mol Cell **40:** 455-464.

REUSS, O., A. VIK, R. KOLTER and J. MORSCHHAUSER, 2004 The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene **341:** 119-127.

ROSIC, S., F. KOHLER and S. ERHARDT, 2014 Repetitive centromeric satellite RNA is essential for kinetochore formation and cell division. J Cell Biol **207:** 335-349.

SANYAL, K., M. BAUM and J. CARBON, 2004 Centromeric DNA sequences in the pathogenic yeast Candida albicans are all different and unique. Proc Natl Acad Sci U S A **101:** 11374-11379.

SANYAL, K., and J. CARBON, 2002 The CENP-A homolog CaCse4p in the pathogenic yeast Candida albicans is a centromere protein essential for chromosome transmission. Proc Natl Acad Sci U S A **99:** 12969-12974.

SELMECKI, A., A. FORCHE and J. BERMAN, 2006 Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science **313:** 367-370.

SHIVARAJU, M., R. CAMAHORT, M. MATTINGLY and J. L. GERTON, 2011 Scm3 is a centromeric nucleosome assembly factor. J Biol Chem **286:** 12016-12023.

SOUTHERN, E. M., 1975 Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol **98:** 503-517.

STELLFOX, M. E., A. O. BAILEY and D. R. FOLTZ, 2013 Putting CENP-A in its place. Cell Mol Life Sci **70:** 387-406.

THAKUR, J., and K. SANYAL, 2012 A coordinated interdependent protein circuitry stabilizes the kinetochore ensemble to protect CENP-A in the human pathogenic yeast Candida albicans. PLoS Genet **8:** e1002661.

THAKUR, J., and K. SANYAL, 2013 Efficient neocentromere formation is suppressed by gene conversion to maintain centromere function at native physical chromosomal loci in Candida albicans. Genome Res **23:** 638-652.

TOMONAGA, T., K. MATSUSHITA, S. YAMAGUCHI, T. OOHASHI, H. SHIMADA *et al.*, 2003 Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. Cancer Res **63:** 3511-3516.

VAN HOOSER, A. A., OUSPENSKI, II, H. C. GREGSON, D. A. STARR, T. J. YEN *et al.*, 2001 Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. J Cell Sci **114:** 3529-3542.

VERDAASDONK, J. S., and K. BLOOM, 2011 Centromeres: unique chromatin structures that drive chromosome segregation. Nat Rev Mol Cell Biol **12:** 320-332.

WILLIAMS, J. S., T. HAYASHI, M. YANAGIDA and P. RUSSELL, 2009 Fission yeast Scm3 mediates stable assembly of Cnp1/CENP-A into centromeric chromatin. Mol Cell **33:** 287-298.

XIAO, H., G. MIZUGUCHI, J. WISNIEWSKI, Y. HUANG, D. WEI *et al.*, 2011 Nonhistone Scm3 binds to AT-rich DNA to organize atypical centromeric nucleosome of budding yeast. Mol Cell **43:** 369-380.

YAMAGISHI, Y., T. SAKUNO, Y. GOTO and Y. WATANABE, 2014 Kinetochore composition and its function: lessons from yeasts. FEMS Microbiol Rev **38:** 185-200.

ZEITLIN, S. G., N. M. BAKER, B. R. CHAPADOS, E. SOUTOGLOU, J. Y. WANG *et al.*, 2009 Double-strand DNA breaks recruit the centromeric histone CENP-A. Proc Natl Acad Sci U S A **106:** 15762-15767.