Mitotic regulation of shugoshin, the “guardian spirit”, in *Cryptococcus neoformans*

A thesis submitted for the fulfilment of the degree of

Masters of Science

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By

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DECLARATION

I do hereby declare that the work described here in this thesis titled '**Mitotic regulation of shugoshin, the "guardian spirit", in *Cryptococcus neoformans***' is an original piece of work and has been carried out by myself under the guidance and supervision of **Kaustuv Sanyal**, Professor, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India.

Polisetty V S Satya Dev
Place: Bangalore
Dated:

CERTIFICATE

This is to certify that this thesis titled '**Mitotic regulation of shugoshin, the "guardian spirit", in *Cryptococcus neoformans***', submitted by **Polisetty V S Satya Dev** towards the partial fulfilment of Integrated PhD Program, as part of the project work for Master of Science, at Jawaharlal Nehru Centre for Advanced Scientific Research, is based on the studies carried out by him under my supervision and guidance.

Prof. Kaustuv Sanyal
Dated:

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Satya Dev

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Abbreviations

Abbreviations	Full form
^{o}C	degree Celsius
µg	microgram
µl	microliter
ANOVA	analysis of variance
APC/C	anaphase promoting complex/cyclosome
bp	base pair
CFU	colony forming units
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
G-418	Genticin-418
h	hour
HMM	hidden markov modelling
kb	kilo base
kDa	kilo Dalton
KT	kinetochore
M	molar
MAFFT	multiple alignment using fast fourier transform
Mb	mega base

min	minutes
ml	millilitre
mM	mill molar
MSA	multiple sequence alignment
NAT	Nourseothricin
$O.D_{600}$	optical density at 600 nm wavelength
ORF	open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rpm	revolutions per minute
SAC	spindle assembly checkpoint
SMC	structural maintenance of chromosome
TBZ	Thiabendazole

1 Introduction

1.1 Cell cycle

The regulated set of events that eventually result in the generation of two cells is referred to as cell cycle. The eukaryotic cell cycle consists of four stages G_1, S, G_2 and M phase. G_1, G_2 and S phase constitute interphase. G_1 and G_2 phases are referred to as gap phases. During this phase, the cell prepares itself for the division and it allows growth of the cell. S phase refers to synthesis phase where the genome of the cell is duplicated once. The M phase refers to mitosis phase where the chromosomes are segregated followed by cell division. It is subdivided into prophase, metaphase, anaphase, telophase and cytokinesis.

In prophase, chromatin condenses to form chromatids and the assembly of the mitotic spindle takes place. At metaphase, chromosomes align along the equator of the spindle to form the metaphase plate and the kinetochores of sister chromatids are attached to the microtubules from the opposite spindle poles. During anaphase, the chromatids are pulled apart and it is divided into two stages; anaphase A and B. In anaphase A, sister chromatids move towards opposite poles and in anaphase B the spindle poles are pushed apart from each other. In telophase, the chromatids decondense to form chromatin. During cytokinesis, the cytoplasm divides forming two daughter cells. In higher eukaryotes like metazoans, cell cycle phases have defined boundaries. However, in certain organisms such as budding yeast *Saccharomyces cerevisiae,* there is no defined G_2 phase between S and M phases and it is referred to as a G_2/M phase.

1.2 Centromere

The centromere is a specific region on a chromosome which is required for faithful segregation of chromosomes by recruiting the kinetochore complex. Centromeres are associated with a histone H3 variant called CENP-A/Cse4 which is a constitutive component of the centromere (Roy and Sanyal 2011). Centromeres are defined as either genetic or epigenetic-based on the factors that determine centromere formation (Roy and Sanyal 2011, Talbert et al. 2009). In genetically determined centromeres such as *Saccharomyces cerevisiae,* the position of the centromere is solely determined by DNA sequence. In certain organisms, the position of the centromere is not primarily dependent on DNA sequence but is influenced by epigenetic factors such as chromatin organization or DNA/histone modifications. This type of epigenetically determined centromeres are found in humans, *Schizosaccharomyces pombe* and *Candida albicans* etc. Based on the size, centromeres are

of three types: point, regional and large regional centromeres. In point centromeres, example *S. cerevisiae,* centromeres are relatively short <400bp. In regional centromeres, the size is < 40 kb such as 4-18 kb centromeres in *C. albicans*. In large regional centromeres, the size is >40kb. The common feature is that they usually contain repetitive DNA sequences and are epigenetically defined.

1.3 Kinetochore

The kinetochore is a multi-subunit protein complex composed of more than 100 proteins. It assembles onto the centromere DNA and facilitates chromosome segregation by attaching chromosomes to the mitotic spindle. The kinetochore is divided into two layers: inner and outer kinetochore (Figure 1).

1.4 Inner kinetochore

The components of the inner kinetochore complex interact directly with centromere DNA. This complex includes evolutionarily conserved proteins such as CENP-A, CENP-C and CCAN (Constitutive Centromere Associated Network) proteins. CENP-A/Cse4 is a centromere-specific histone H3 variant which is responsible for the assembly of the kinetochore components (Yamagishi et al. 2014). It contains the centromere targeting domain (CATD) and a conserved histone fold (HFD) domain (Malik and Henikoff 2003, Roy and Sanyal 2011). Scm3/ HJURP, the chaperone of CENP-A/Cse4, regulates the recruitment of CENP-A/Cse4 to centromeres (Pidoux et al. 2009, Stoler et al. 2007). The CCAN network consists of four sub-complexes CENP-LN, CENP-HIKM, CENP-OPQRU and CENP-TWSX (Amano et al. 2009, Foltz et al. 2006, Hori et al. 2008, Izuta et al. 2006, Obuse et al. 2004, Okada et al. 2006). It acts as a structural framework for the assembly of the kinetochore. CENP-C (Mif2 in *S. cerevisiae*) acts as the structural hub for the kinetochore assembly (Przewloka et al. 2011, Screpanti et al. 2011). It contains the “CENP-C-box” - a 25-amino acid sequence which is required for the kinetochore localization. It has a C-terminal globular structure known as the cupin domain which is responsible for dimerization and important for its kinetochore recruitment. Among the CCAN proteins, the CENP-TWSX complex is well characterized and it forms a nucleosome-like structure. It is a tetrameric complex which contains proteins with the histone fold domain. The CENP-T of this complex interacts with the Ndc80 of the outer kinetochore region.

1.5 Outer Kinetochore

The outer kinetochore is the region which links the centromere to the microtubules and transduces the force generated by depolymerizing microtubules to facilitate chromosome movement along the microtubules. It consists of the KMN network and the Dam1 complex (Ska complex in humans).

The KMN network forms the core of the outer kinetochore, and consists of ten subunits. It is composed of three main sub-complexes Knl1 (Spc105 in yeast), Ndc80 and Mis12. Ndc80 is a dumbbell-shaped 4-subunit complex containing Spc24, Spc25, Ndc80 and Nuf2 (Wigge and Kilmartin 2001). The N-terminal region of Nuf2 and Ndc80 contains the calponin-homology (CH) domain which facilitates binding to microtubule lattice (Valverde et al. 2016, Wei et al. 2007). The Spc24 and Spc25 form the C-terminal region that mediates kinetochore-targeting. These two proteins interact with CENP-T (Nishino et al. 2013).

The Mis12/Mtw1 complex (the MIND complex in budding yeast) is a 4-subunit complex containing Mis12/Mtw1, Dsn1/Mis13, Nsl1/Mis14 and Nnf1 and acts as an interaction hub linking Ndc80 and Knl1 complexes (Cheeseman et al. 2006). This complex connects the KMN network with inner kinetochore component CENP-C.

The Knl1 complex is composed of Knl1 (Spc105 and Spc7 in yeast) and ZWINT (Ydc532 and Kre28 in yeast). The Knl1 complex contains an array of protein docking motifs to act as a scaffold for the recruitment of spindle assembly checkpoint components (SAC) (discussed in a later section) (Musacchio and Desai 2017). The Dam1/DASH complex is a 10-subunit complex which consists of Dam1, Duo1, Dad1, Dad2, Dad3, Spc34, Ask1, Spc19 and Hsk3 (Cheeseman et al. 2001) (Janke et al. 2002) (Li J. M. et al. 2005). The Dam1 complex proteins are conserved and are specific to the fungal kingdom. Electron microscopy studies have revealed that this complex forms a ring-like structure around microtubules (Westermann et al. 2005). The Ska complex is observed in humans and it consists of three subunits Ska1, Ska2 and Ska3 is a functional counterpart of the fungal Dam1 complex (Sridhar et al. 2017). The Ska complex and the Dam1 complex do not share any sequence or structural homology. The Ska complex forms a wedge shape around microtubules and it plays a role in attaching microtubules to kinetochores (Sridhar et al. 2017).

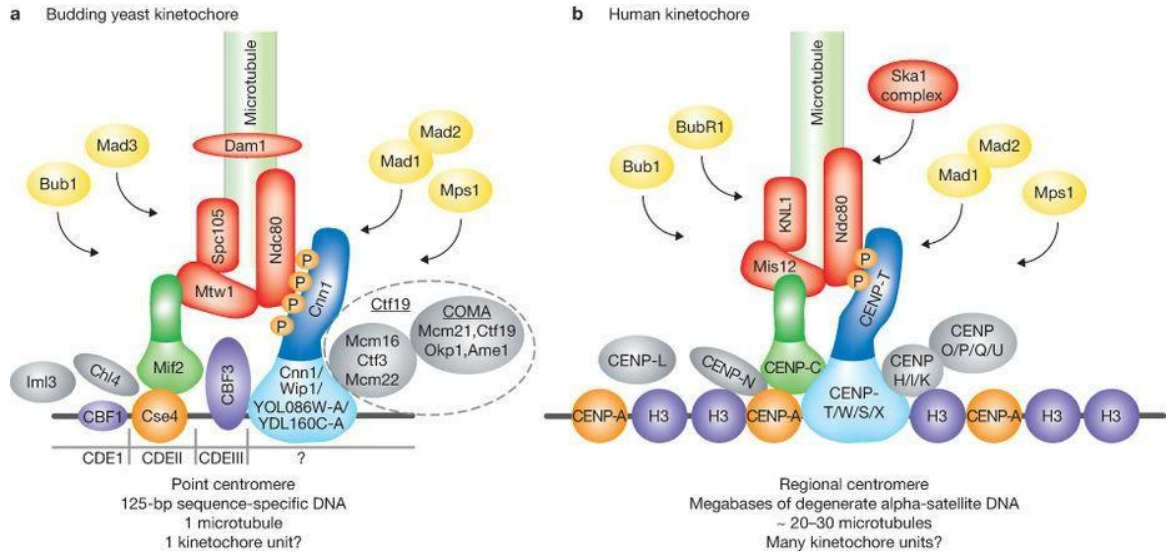

Figure 1: Organization of kinetochore structure in yeast and humans (Gascoigne and Cheeseman 2012).

1.6 Mitosis

During mitosis, the duplicated genomes are segregated equally to daughter cells. To ensure equal segregation of chromosomes, they must be prevented from premature separation by cohesins (Marston 2015) (Figure 2a). Cohesins are heterotrimeric protein complexes that hold the replicated chromosomes together by topologically embracing two sister DNA molecules (Haering et al. 2008, Ivanov and Nasmyth 2005). The core tripartite ring structure consists of Smc1, Smc3 (Structural Maintenance complex) and the α-kleisin subunit Rad21/Scc1. The Smc1 and Smc3 heterodimerize to form a V-shaped structure. The α-kleisin subunit closes the ring by connecting the Smc's (Nasmyth and Haering 2009, Peters et al. 2008). The heterotrimeric ring is associated with additional proteins (Pds5, Waplb, Sororin and Scc3/SA) which regulate the dynamics of cohesin (Mirkovic and Oliveira 2017).

In mitosis, segregation of chromosomes occurs as a result of the loss of cohesion among the sister chromatids. Loss of cohesin occurs in a stepwise manner. First, the arm cohesion is lost followed by loss of cohesion at the pericentromeric region. In mammalian cells loss of cohesion at arms and the pericentromeric region is regulated by two distinct pathways (Waizenegger et al. 2000). The prophase pathway regulates the removal of arm cohesion and the seperase-dependent cleavage of cohesion is responsible for the loss of pericentromeric cohesion. In the prophase pathway, removal of cohesin takes place by disruption of the interaction between Scc1 and Smc3 subunit by Wapl during early mitosis

(Buheitel and Stemmann 2013, Eichinger et al. 2013). Many key mitotic kinases such as Aurora B and cyclin-dependent kinase 1 (Cdk1) play a role in the removal of cohesins during the prophase pathway (Mirkovic and Oliveira 2017). These mitotic kinases phosphorylate sororin and promote dissociation of sororin from Pds5 (Dreier et al. 2011, Nishiyama et al. 2013). As a result of sororin dissociation, Wapl comes into the picture and binds to Pds5 thereby destabilizing the Smc3-Scc1 interaction (Nishiyama et al. 2013). Combined all these activities promote dissociation of cohesins from arms during the prophase pathway (Mirkovic and Oliveira 2017).

Loss of cohesion at the centromere is mediated through the activity of a cysteine protease known as seperase or Esp1 protein which cleaves the α-kleisin subunit Rad21/Scc1 (Ciosk et al. 1998, Cohen-Fix et al. 1996, Funabiki et al. 1996). Until the chromosomes are properly bioriented and all the kinetochores are attached to the microtubules, the cells cannot enter into anaphase. Seperase-dependent cleavage of cohesins is considered as a universal trigger for chromosome segregation (Marston 2015). The activity of seperase is controlled by E3 ubiquitin ligase known as the anaphase promoting complex/cyclosome (APC/C) (Irniger et al. 1995) and it is under the tight control by a cell surveillance mechanism known as spindle assembly checkpoint (SAC) which is discussed later. To prevent premature chromosome segregation, the seperase inhibitor securin or Pds1 binds to seperase preventing it from cleaving α-kleisin (Ciosk et al. 1998). Upon activation of APC/C, it polyubiquitinates securin thereby subjecting it to the 26S proteasome-mediated degradation (Ciosk et al. 1998, Cohen-Fix et al. 1996, Funabiki et al. 1996). This mechanism allows separase to access Rad21/Scc1 resulting in the release of cohesin thereby promoting chromosome segregation.

1.7 Meiosis

Meiosis is a type of cell division in which a diploid cell gives rise to four haploid cells. It involves two successive rounds of cell division which are meiosis I and meiosis II following a single round of DNA replication (Figure 2b). During meiosis I homologous chromosomes are segregated to opposite poles followed by segregation of sister chromatids in meiosis II. In meiosis I, the cohesion along the arms of the sister chromatids is lost and during meiosis II the pericentromeric cohesion is lost (Marston and Amon 2004). Similar to mitosis, in meiosis sister chromatid cohesion is established through the cohesin complex. However, they undergo meiosis specific modifications (Marston and Amon 2004). The common

modification is the replacement of Rad21/Scc1 subunit of cohesin with meiosis specific Rec8 (Klein et al. 1999, Pasierbek et al. 2001, Watanabe et al. 2001). In meiosis, loss of cohesin occurs in a stepwise manner. It is regulated by seperase-dependent cleavage of cohesins from arms and pericentromeric regions. Cleavage of Rec8 is dependent on its phosphorylation (Brar et al. 2006, Clyne et al. 2003, Ishiguro et al. 2010, Katis et al. 2010, Lee and Amon 2003). The kinases that are involved in phosphorylation of Rec8 are casein kinases (in case of budding and fission yeast), Dbf4-dependent kinase (DDK) and polo kinase (only in budding yeast) (Ishiguro et al. 2010, Katis et al. 2010, Lee and Amon 2003). During meiosis I, the cleavage of Rec8 at centromeres is prevented and the machinery involved in the prevention of Rec8 cleavage is discussed later.

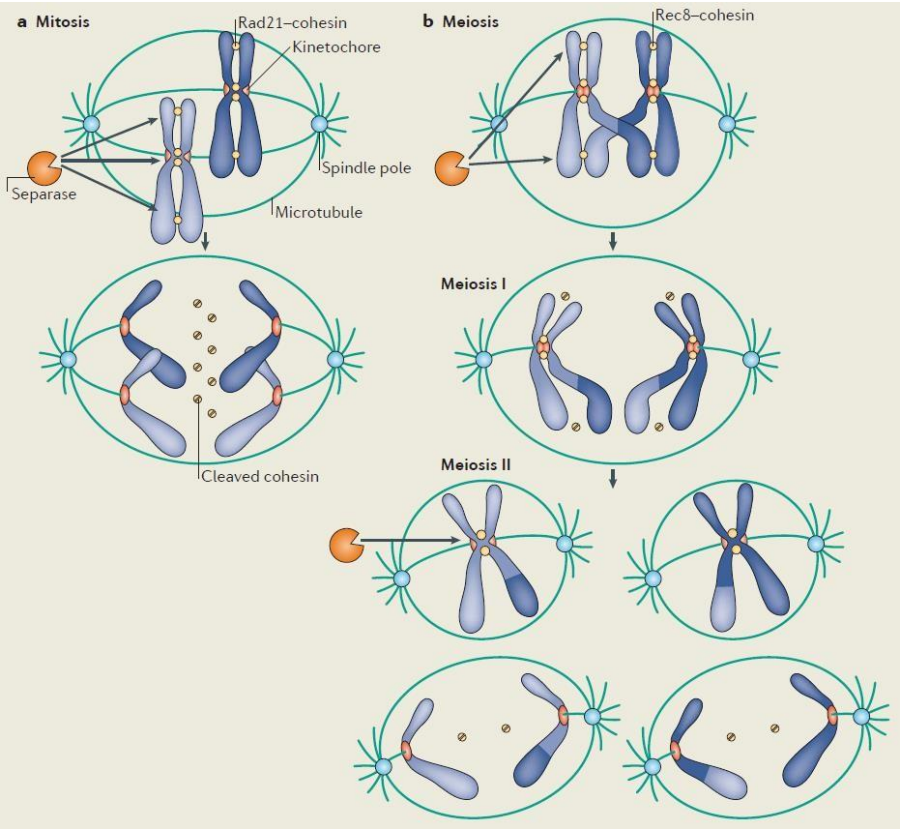

Figure 2: Chromosome segregation during mitosis and meiosis (Watanabe 2012).

1.8 Kinetochore-microtubule attachments

The segregation of chromosomes depends on the stable attachment of kinetochores with microtubules and proper orientation on the spindle. Erroneous attachments lead to improper segregation of chromosomes leading to aneuploidy. In a cell, different types of

kinetochore-microtubule attachments are possible (Figure 3). When both the kinetochores of sister chromatids are captured from the microtubules of opposite poles, it is referred to as amphitelic attachment or biorientation (Figure 3c). The amphitelic attachment generates full tension across centromeres. In cases where only one of the kinetochores is attached to microtubules originating from both the poles, it is referred to as merotelic attachment (Figure 3d). In merotelic attachments, there is no tension across the centromeres of sister chromatids. This mode of attachment is not observed in case of budding yeast since only one microtubule is associated with the kinetochore. In case of meiosis I, both the kinetochores of sister chromatids bind to microtubules originating from the same spindle pole. This attachment is referred to as syntelic attachment or mono-orientation (Figure 3b). Syntelic attachments facilitate segregation of the homologous chromosomes to opposite poles during meiosis I thereby facilitating a reduction in the ploidy. In monotelic attachments, one of the kinetochores of sister chromatids is attached to microtubule from a single spindle pole (Figure 3a).

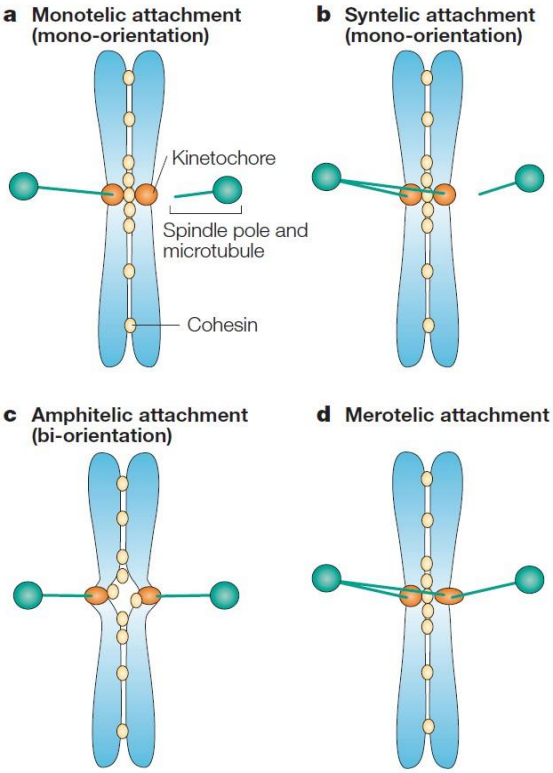

Figure 3: Types of kinetochore-microtubule attachments (Tanaka et al. 2005)**.**

1.9 Spindle assembly checkpoint

Two independent genetic screens in budding yeast, have identified several genes, when mutated they bypassed mitotic arrest in the presence of spindle poisons (Hoyt et al. 1991, Li R. and Murray 1991). The key genes identified were MAD (mitotic arrest deficient) genes *MAD1*, *MAD2* and *MAD3* (*BUBR1* in humans) and BUB genes (budding uninhibited by benzimidazole) *BUB1* and *BUB3* (Hoyt et al. 1991, Li R. and Murray 1991). These genes are involved in preventing erroneous segregation of chromosomes and it was named as spindle assembly checkpoint (SAC) (Musacchio and Salmon 2007). Though the term spindle assembly checkpoint is misleading, it does not monitor spindle assembly. The term originated from the observation that "spindle poisons which disassemble the spindle resulting in SAC activation" (LaraGonzalez et al. 2012). SAC is a surveillance system that monitors kinetochore-microtubule attachments. It essentially conveys a 'wait anaphase' signal during cell division by preventing the anaphase onset until all the erroneous and tensionless kinetochore-microtubule attachments are resolved and chromosomes are bioriented (London and Biggins 2014a) (Figure 4). The core components of SAC include Mad1, Mad2, Mad3/BubRI, Bub1, Bub3, Mps1 (Monopolar Spindle 1) and Aurora B (Kallio et al. 2002, Weiss and Winey 1996). The key target of SAC is Cdc20 which is a co-factor of APC/C and it negatively regulates the activation of APC/C (Musacchio 2015). SAC proteins Mad3/BubRI, Bub3 and Mad2 along with Cdc20 form a complex known as mitotic checkpoint complex (MCC). It is considered as the effector complex of SAC signaling and inhibits APC/C (Musacchio 2015, Sudakin et al. 2001).

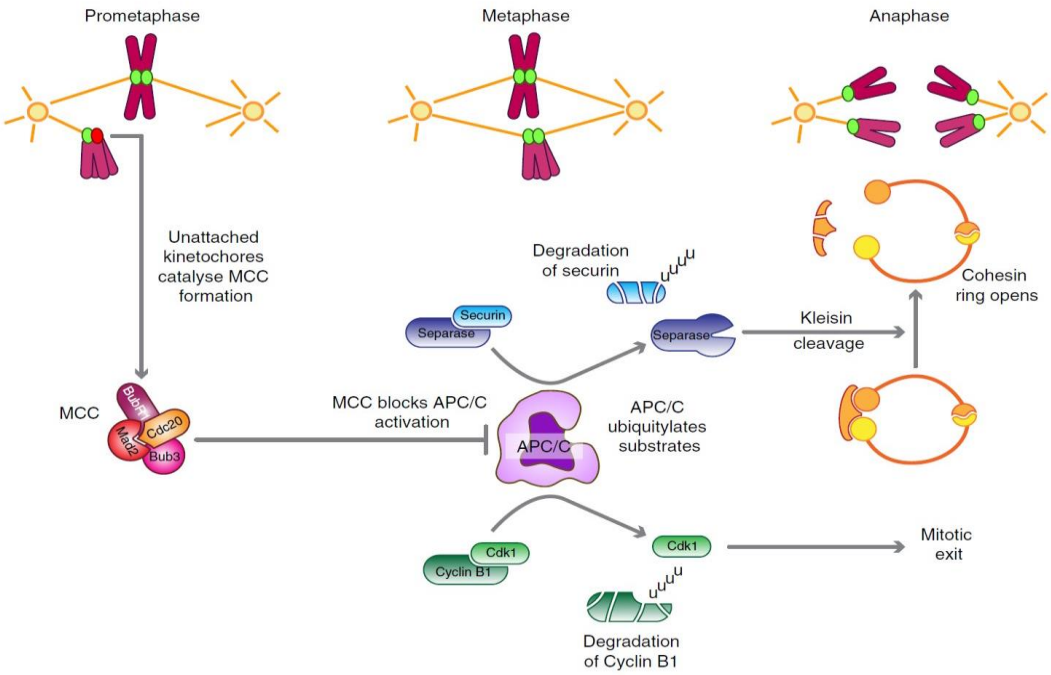

Figure 4: Schematic of spindle assembly checkpoint (SAC) function. At prometaphase stage, the kinetochores are predominantly unattached. During this state, SAC is active and components of SAC are recruited to the kinetochore. This results in the generation of the mitotic checkpoint complex which inhibits the APC/C complex. Inhibition of the APC/C complex prevents anaphase onset. Once the kinetochore-microtubule attachments are stabilized and chromosomes are bioriented the MCC generation is inhibited releasing the APC/C from inhibition and thereby promotes the onset of anaphase (Lara-Gonzalez et al. 2012).

1.10 Generating the 'on' signal

Laser ablation and cell biology studies showed that the kinetochores are the sites for generating the SAC signal in response to the unattached or tensionless situation (London and Biggins 2014a). The SAC components at the kinetochore are assembled in a stepwise fashion. The conserved cell cycle kinases Mps1 and Aurora B being the first, followed by recruitment of Bub3-Bub1 and then the Bub3-Mad3/BubRI complex. Finally, recruitment of the Mad1-Mad2 heterotetramer (London and Biggins 2014a).

1.11 Kinetochore localization of Bubs

The Knl1 of the KMN network recruits the Bubs (Bub1, Bub3 and BubRI) to the kinetochore (London et al. 2012, Yamagishi et al. 2012). Bub1 and BubRI are related proteins (paralogs) which are important for the checkpoint function. Bub1 is a kinase and BubRI is a

pseudokinase (Suijkerbuijk et al. 2012). Bub3 interacts with Bub1 and BubRI and they form a stable complex with Bub3. Bub3 is responsible for the kinetochore localization of Bub1 and BubR1 but the mechanism of recruitment is different (Overlack et al. 2015). Localization of Bub3 and Bub1 is dependent on the kinase activity of Mps1. The localization of BubRI is through a pseudo-symmetric interaction between Bub1 and BubRI which recruit BubRI directly to kinetochores (Overlack et al. 2015).

1.12 Kinetochore localization of Mad1 and Mad2

Mad1 and Mad2 are localized to the unattached kinetochores as a heterotetramer and it is the key step in checkpoint signaling (London and Biggins 2014a). Bub1 recruits Mad1 and Mad1 serves as the Mad2 receptor at the kinetochores (Musacchio 2015). In humans, in addition to Bub1-mediated localization of Mad1, another component known as Rod-Zwilich-Zw10 (RZZ) complex is also responsible for Mad1 localization. This complex is observed only in metazoans (London and Biggins 2014a).

Mad2 is a HORMA (Hop1, Rev7 and Mad2) domain containing protein and it adopts two different conformations namely, open and closed Mad2 (O-Mad2 and C-Mad2) (Luo and Yu 2008, Musacchio 2015). When Mad2 is not bound to any of its interacting partners, it adopts open conformation. Most of the soluble Mad2 is in open conformation. When Mad2 is bound to either Cdc20 or Mad1, it forms C-Mad2 (Musacchio 2015).

1.13 Generation of MCC and APC/C inhibition

Once Mad1-C-Mad2 is recruited at the unattached kinetochores by Bub1, it recruits soluble O-Mad2 at the kinetochores. The soluble O-Mad2 is converted to I-Mad2 (Intermediate) which is geared up for conversion to C-Mad2 and association with Cdc20 (Hara et al. 2015). In the process mentioned above, Mad1-C-Mad2 acts as a template for the formation of the C-Mad2-Cdc20 complex, hence it is termed as the “Mad2 template model” (De Antoni et al. 2005) (Mapelli and Musacchio 2007). Once the C-Mad2-Cdc20 complex is generated, it associates with the BubRI-Bub3 complex to form MCC (Chao et al. 2012). Once the MCC is fully assembled, it is capable of inhibiting the APC/C complex.

1.14 Silencing of SAC

Once all the kinetochores are attached to the microtubules and tension is satisfied, SAC must be inactivated to promote anaphase. This is achieved through a series of events at the kinetochores and disassembly/degradation of MCC. At kinetochores upon microtubule

attachment, the activity of Mps1 and Aurora B kinase is suppressed. This results in loss of balance in the kinase/phosphatase activity at the outer kinetochore (Corbett 2017). This results in dissociation of the Bub1-Bub3 complex and the associated SAC components (Corbett 2017, London et al. 2012). In order to progress into anaphase, the MCC component of the SAC must also be inactivated. Upon stable KT-MT attachment, the assembly of new MCC is stopped. The existing MCC's present in the cell are degraded to terminate the SAC signal (Corbett 2017).

1.15 Shugoshin

The Shugoshin (the guardian spirit in Japanese) family of proteins are involved in ensuring fidelity of chromosome segregation in both mitosis and meiosis. It associates with the pericentromeric region that surrounds the centromere of the chromosome. The primary role of shugoshin appears to serve as a pericentromeric platform or as an adaptor protein to recruit several other effector proteins that ensure precise chromosome segregation (Marston 2015). A shugoshin ortholog Mei-S332 was first identified in *Drosophila melanogaster* where mutants lacking Mei-S332 showed precocious separation of sister chromatids during anaphase I of meiosis (Kerrebrock et al. 1992). Later functional screens carried out in fission yeast *Schizosaccharomyces pombe* and budding yeast *Saccharomyces cerevisiae* identified genes required for maintaining cohesion of sister chromatids and these genes encoding a family of proteins was named as "shugoshins" (Katis et al. 2004, Marston et al. 2004, Rabitsch et al. 2004). Structural analysis suggests that the number of shugoshin paralogs encoded by an organism differs from species to species. For example, budding yeast and *Drosophila* contain only one form of shugoshin (Sgo1 and Mei-S332 respectively) whereas mammals, fission yeast and plants contain two forms of shugoshin (Sgo1 and Sgo2 in yeast and Sgol1 and Sgol2 in vertebrates) (Kitajima et al. 2004). Some of the shugoshins are found only in meiotic cells (e.g., mammals, fission yeast and plants) whereas some are found in both meiotic as well as somatic cells (e.g., budding yeast Sgo1 and Drosophila Mei-S332).

1.16 Structure of shugoshin

The orthologs of shugoshin are relatively divergent and contain a conserved basic C-terminal "SGO motif" and an N-terminal coiled-coil domain, which are the two structural features of the shugoshin family (Kitajima et al. 2004). The SGO motif is known to interact

with phosphorylated histone H2A and the N-terminal coiled-coil domain helps in dimerization of shugoshin and its association with PP2A and CPC (Chromosome Passenger Complex) (Kitajima et al. 2004, Tang T. T. et al. 1998). X-ray crystal structural studies revealed that the coiled-coil homodimer of shugoshin interacts with both catalytic subunit and the B' regulatory subunit of PP2A (Xu et al. 2009).

Apart from above mentioned conserved regions, rest of the protein shows little sequence homology and exhibits functional differences among different species (Gutierrez-Caballero et al. 2012). The other species-specific features of shugoshins include the presence of a Mad2 interacting motif, Aurora kinase phosphorylation sites, motifs responsible for degradation (KEN box and D or destruction box) of shugoshin and for association with heterochromatin protein HP1 (Marston 2015). The Mad2-interacting motif (MIM) is found in human Sgo2, Xenopus Sgo and mouse Sgo2; it is located near to the N-terminal coiled-coil domain of Sgo. It resembles with the MIMs observed in Mad1 and Cdc20 proteins. It was identified that Sgo forms a tight association with C-Mad2 (Orth et al. 2011, Rattani et al. 2013). The Aurora kinase phosphorylation sites are observed in human Sgo1, mouse Sgo2 and *Drosophila* mei-S332 (Yao and Dai 2012). The KEN box and D box motifs present close to the C terminus are observed in human Sgo1 and budding yeast Sgo1 which mediate shugoshin degradation through the anaphase promoting complex (APC) (Eshleman and Morgan 2014, Karamysheva et al. 2009). The heterochromatin protein HP1 interacting site is found in fission yeast and humans and is responsible for localization of shugoshin to the pericentromeric region (Kang et al. 2011, Yamagishi et al. 2008).

1.17 Localization of shugoshin

The localization of shugoshin to pericentromeres is regulated by different factors which include HP1, nucleosomes and cohesins. In humans and fission yeast, it is reported that HP1 (Swi6 in fission yeast) is known to localize shugoshin to the pericentromere. In fission yeast Sgo1 – HP1 interaction is essential during meiosis since in the *sgo1* mutants which are defective in interaction with HP1 are unable to protect cohesins (Yamagishi et al. 2008). In humans, it is known that HP1 recruits Sgo1 to pericentromeres during interphase but its functional relevance remains elusive (Kang et al. 2011, Perera and Taylor 2010).

Mitotic localization of shugoshin is dependent upon the Bub1 kinase and cohesins (Kitajima et al. 2004, Liu H. et al. 2013b). The Bub1 kinase is one of the components of spindle assembly checkpoint and its kinase activity is required for the localization of shugoshin to the centromere (Fernius and Hardwick 2007, Perera and Taylor 2010). In both fission yeast and humans, Bub1 kinase phosphorylates histone H2A at serine 121 (T120 in humans) which facilitates the localization of shugoshin to centromeres (Kawashima et al. 2010, Liu H. et al.
2013b).

1.18 Functions of Shugoshin

Shugoshins have diverse functions: they are known to involve in the protection of cohesins during mitosis and meiosis, promoting bi-orientation during cell division, delaying cell cycle in response to tensionless kinetochore-microtubule (KT-MT) attachments and in silencing SAC. Apart from the functions mentioned above, it is shown that the splice variant of Sgo1 prevents disengagement of centrioles in early mitosis in humans (Wang et al. 2008).

1.19 Cohesin protection during meiosis I

Shugoshin maintains the protection of cohesion at the pericentromeric region by preventing cleavage of meiosis specific Kleisin subunit Rec8. As mentioned earlier, cleavage of the Rec8 subunit is dependent upon its phosphorylation. Shugoshin prevents the cleavage of Rec8 by recruiting protein phosphatase 2A (PP2A) (Kitajima et al. 2006, Riedel et al. 2006, Xu et al. 2009) (Figure 5). PP2A is serine/threonine phosphatase which consists of three subunits: catalytic, scaffold and regulatory subunits. There are different families of regulatory subunits (B, B’ and B’’ or B’’’) that associate with the PP2A. In meiosis, shugoshin associates with specific holoenzyme PP2A-B and recruits it to the pericentromeric region. PP2A-B’ prevents phosphorylation of Rec8 thereby rendering it as a poor substrate for separase dependent cleavage (Kitajima et al. 2006, Riedel et al. 2006, Xu et al. 2009).

1.20 Cohesin protection during mitosis

Shugoshin confers protection of cohesin at the centromeres by preventing clevage through the prophase pathway in mammals. At the centromeres, shugoshin antagonizes the activity of Wapl by different ways (Marston 2015). Sgo1 prevents the phosphorylation of Sororin by mitotic kinases Aurora B and Cyclin-dependent kinase 1 (Cdk1) which prevents its

dissociation from Pds5 subunit of cohesin and also it helps in maintenance of sororin at the centromeres (Dreier et al. 2011, Liu H. et al. 2013a, Nishiyama et al. 2013). The antagonizing activity of Sgo1 is achieved by recruitment of PP2A to the pericentromeric region which prevents phosphorylation of sororin (Kitajima et al. 2006, Riedel et al. 2006, Tang Z. et al. 2006) (Figure 5). In budding and fission yeast, the prophase pathway does not seem to exist. In cells lacking shugoshin, cohesin levels and cohesion are maintained during mitosis (Indjeian et al. 2005, Kiburz et al. 2005, Verzijlbergen et al. 2014). Loss of cohesion at the arms and the pericentromeric region is mediated by a separase-dependent cleavage of cohesins in yeasts.

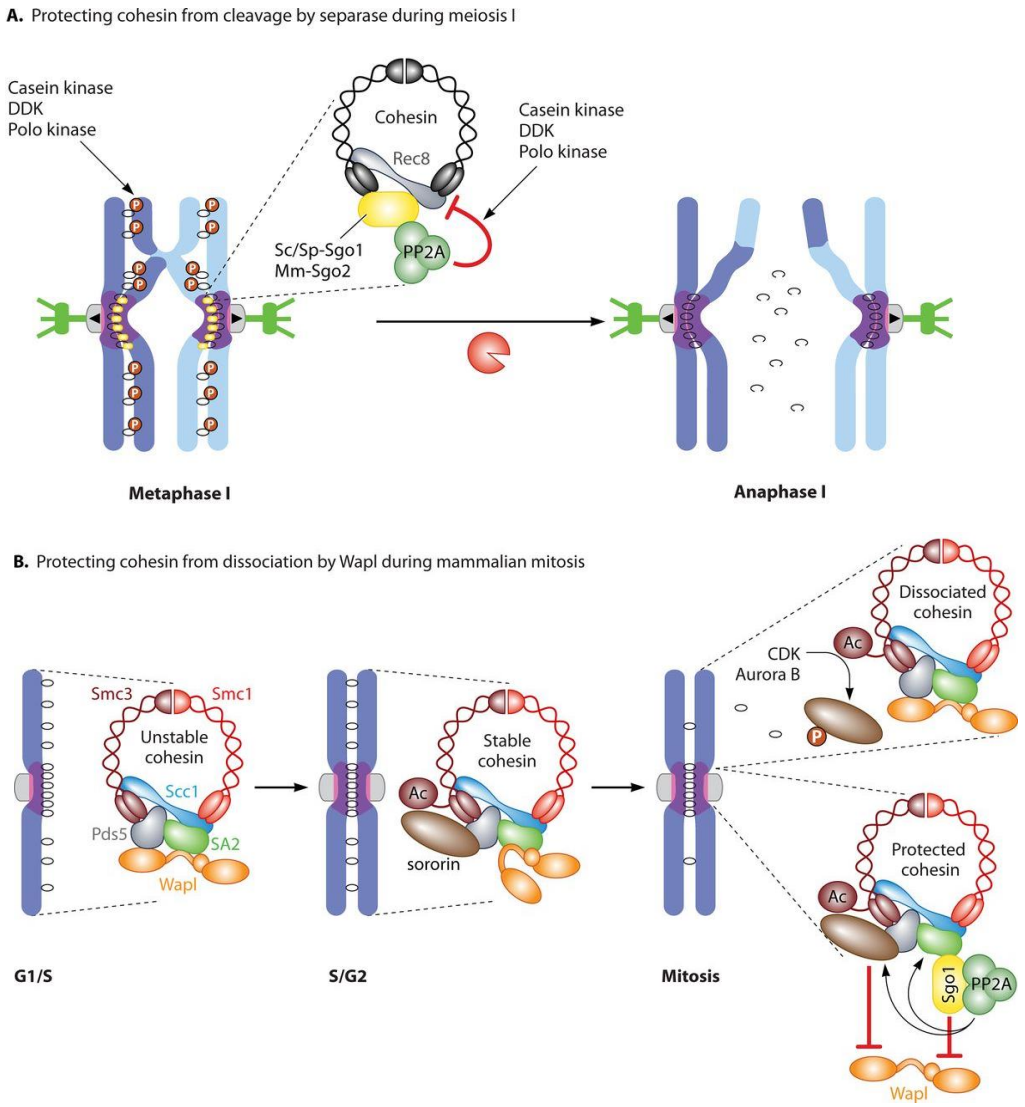

Figure 5: Protection of cohesins at centromeres by shugoshin during mitosis and meiosis. A) Mechanism of cohesin protection at the pericentromeric region in mammalian mitosis. Sgo1 recruits PP2A which antagonizes phosphorylation of sororin thereby protecting cohesion at the pericentromeres. **B)** Mechanism of protection of the pericentromeric

cohesion during meiosis I. Shugoshin recruits PP2A and antagonizes phosphorylation of Rec8 thereby protecting the pericentromeric cohesion during meiosis I (Marston 2015).

1.21 Role in chromosome bi-orientation

As mentioned earlier, to ensure proper chromosome segregation the chromosomes must be attached by the microtubules originating from the opposite poles of the cell. This mode of attachment is referred to as bi-orientation and it is observed during mitosis as well as in meiosis II. Shugoshin along with other effector proteins such as CPC (Chromosome Passenger Complex), the chromosome-shaping complex, condensin, PP2A and SAC component-Mad2 play roles in promoting bi-orientation of chromosomes (Eshleman and Morgan 2014, Huang et al. 2007, Indjeian and Murray 2007, Kawashima et al. 2007, Kiburz et al. 2008, Peplowska et al. 2014, Rattani et al. 2013, Storchova et al. 2011, Vanoosthuyse et al. 2007, Verzijlbergen et al. 2014). Bi-orientation is achieved through two mechanisms: one through the error correction process and the other by means of kinetochore geometry (Marston 2015).

1.22 Bi-orientation through error correction

The machinery that facilitates error correction during cell division is referred to as the chromosome passenger complex (CPC). CPC consists of INCENP, Survivin, Borealin and Aurora B (Carmena et al. 2012). The function of CPC is to regulate the kinetochore-microtubule attachments. One of the CPC components Aurora B is known to destabilize the KT–MT attachments which are not under tension by phosphorylating KMN network of kinetochore proteins. This process results in the generation of unattached kinetochores and subsequently results in activation of SAC (Tanaka 2010). From the perspective of bi-orientation, it has been shown that the major role of shugoshins is to serve as an adaptor for the localization of CPC to the pericentromeric region.

1.23 Bi-orientation through kinetochore geometry

During mitosis, the kinetochores of sister chromatids are in back-to-back orientation this enables kinetochores to attach microtubules originating from the opposite spindle poles (Peplowska et al. 2014, Verzijlbergen et al. 2014). It is known that condensin plays a role in altering the structural properties of the centromeres thereby affecting the dynamics of chromosomes such as resisting spindle forces (Stephens et al. 2011). In budding yeast, condensin is one of the effectors of Sgo1. It is shown that condensins are highly enriched at

pericentromeric regions and depletion of Sgo1 causes reduction in levels of condensin at the pericentromeres. Moreover, it was shown that in budding yeast condensins bias sister kinetochores to adopt a back-to-back orientation thereby promoting bi-orientation. The exact mechanistic details of how bi-orientation is achieved through condensins remain elusive (Verzijlbergen et al. 2014).

1.24 Delaying cell cycle

In budding yeast, it is shown that Sgo1 in response to the tensionless KT-MT interactions it may delay cell cycle progression independent of its function in localizing CPC. In response to lack of tension, shugoshin with the help of PP2A-B'/Rts1 or with PP2A-B/Cdc55 may delay cell cycle (Clift et al. 2009, Peplowska et al. 2014, Xu et al. 2009). PP2A-Cdc55 acts downstream of shugoshin and it is reported that it inhibits seperase activity directly independent of SAC components and securin (Clift et al. 2009).

1.25 Shugoshin 's role in spindle assembly checkpoint

It is shown that Xenopus Sgo1, human Sgo2 and mouse Sgo2 interact directly with one of the SAC components Mad2 (Orth et al. 2011). In humans, Sgo2 is dispensable during mitosis (Huang et al. 2007, Orth et al. 2011, Tanno et al. 2010). In mouse oocytes, it is shown that interaction of Sgo2 with Mad2 silences SAC instead of activating it. It is also shown that Sgo2 knockout spermatocytes undergo partial metaphase II arrest while nearly half of the cells escaped the arrest leading to aneuploid spermatozoa in mouse (Rattani et al. 2013). Recent reports show that in budding yeast Sgo1 also promotes SAC silencing but the underlying mechanism is not known (Jin and Wang 2013).

1.26 Cryptococcus neoformans

C. neoformans is a basidiomycete fungus. It is a haploid organism with 19 Mb of genome distributed among 14 chromosomes. It primarily exists in two morphological forms (dimorphic): unicellular yeast and filamentous form which includes true hyphae and pseudohyphae. The unicellular yeast and pseudohyphal forms are observed during the vegetative growth phase. True hyphae are formed as a result of mating, which constitutes the sexual growth phase. It is an opportunistic pathogen which affects immunocompromised as well as immunocompetent patients (Hoang et al. 2004).

1.27 The life cycle of *C. neoformans*

C. neoformans exhibits a bipolar mating system: mating types **a** (*MAT***a**) and α (*MATα*). It exhibits both opposite and same-sex matings. Mating results in the generation of hyphal cells containing dikaryon (heterokaryon) where the nuclei at this stage divide by mitosis (Kwon-Chung 1975, McClelland et al. 2004). Special structures called clamp cells arise from each hyphal cell and connects neighboring cell (Alspaugh et al. 2000). The terminal hyphal cell differentiates to form a round flask-shaped structure known as the basidium. Fusion of the two parental nuclei occurs in the basidium which results in a diploid nucleus. This event is followed by meiosis and gives rise to four haploid nuclei (Figure 6). These nuclei bud out to form the infectious spores known as basidiospores which are the infectious propagules and they cause disease (Jin and Wang 2013). Infection to human hosts is acquired by inhalation of the spores and they colonize the host pulmonary system without any symptoms of the disease, where it remains dormant. When host immune system is compromised, dormant form becomes active and enters the bloodstream to cause systemic infection. In more severe cases, it crosses the blood-brain barrier and enters the central nervous system (CNS) which results in meningoencephalitis.

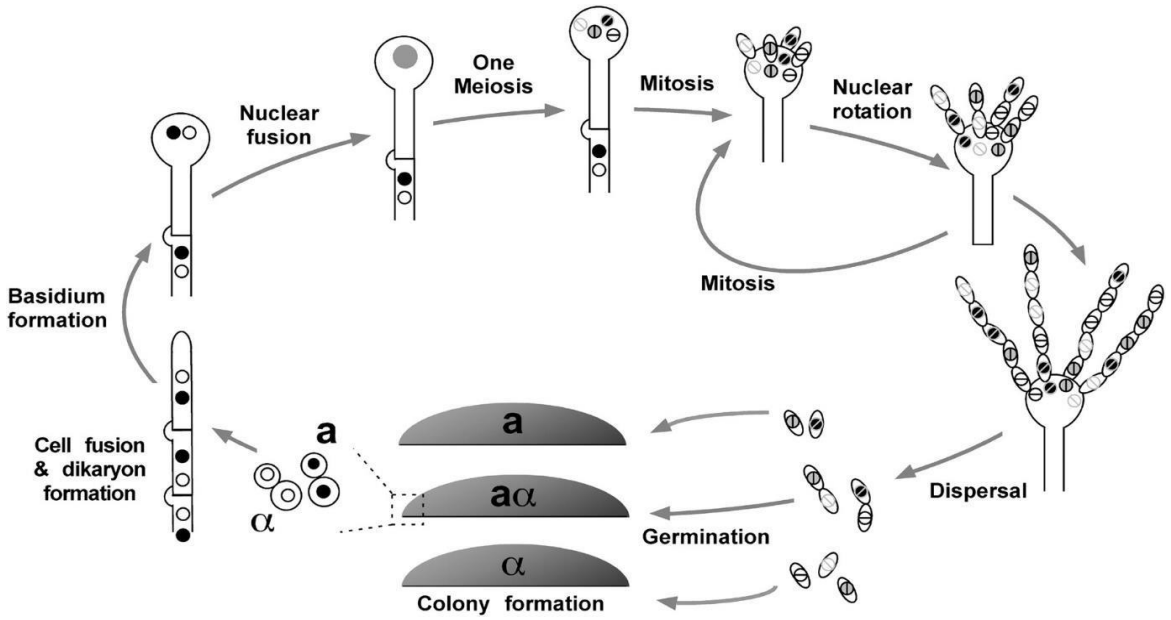

Figure 6: The life cycle of *C. neoformans.* Schematic showing both sexual and asexual life cycle of *C. neoformans*. The sexual life cycle includes mating of opposite mating types **a** and α which results in formation dikaryotic hyphae that ultimately give rise to basidiospores. The spores germinate to form yeast cells. In asexual stages, cells undergo mitosis by budding (Idnurm 2010).

1.28 Mitosis in *C. neoformans*

The vegetative form of *C. neoformans* divides by budding. The mitotic events such as the nuclear and the kinetochore dynamics in *C. neoformans* resemble more closely to metazoan mitosis (Kozubowski et al. 2013). Budding yeasts, *S. cerevisiae* and *Candida albicans*, have completely assembled kinetochores throughout the cell cycle (Goshima and Yanagida 2000, Meluh et al. 1998, Sanyal and Carbon 2002). In *C. neoformans*, the kinetochore assembly occurs in a stage-specific manner. Live cell microscopy experiments showed that the inner kinetochore components CENP-A and CENP-C form the constitutive network and are observed throughout the cell cycle. Whereas the outer kinetochore components such as Mtw1, Dad1 and Dad2 were shown to localize only during mitotic stages (Kozubowski et al. 2013). This mode of kinetochore assembly in *C. neoformans* more closely resembles metazoans; where the inner kinetochore components are present throughout the cell cycle but the outer kinetochore components assemble during mitosis (Foltz et al. 2006, Liu S. T. et al. 2006, Przewloka et al. 2007).

During interphase, the kinetochores/centromeres are declustered and they gradually cluster during mitosis. These multiple foci of the centromeres/kinetochores are shown to associate with the nuclear envelope. As the centromeres/kinetochores cluster during mitosis, they remain associated with nuclear envelope but they also co-localize with spindle pole bodies (Kozubowski et al. 2013).

Mitosis in *C. neoformans* is referred to as semi-open mitosis. During mitosis, the nuclear envelope partially breaks down at the SPBs when the spindle begins to appear (Kozubowski et al. 2013). This mode of nuclear envelope break down is similar to what was observed in another basidiomycete *Ustilago maydis* (Theisen et al. 2008).

Live cell microscopy using GFP tagged histone H4 (used as chromatin marker) shows that during mitosis, the nucleus migrates into the daughter cell first and then undergoes nuclear division. This mode of nuclear division was neither observed in budding yeast *S. cerevisiae* and *C. albicans* nor in the fission yeast *S. pombe*. It more closely resembles *U. maydis* where nucleus similarly migrates into the daughter cell (Straube et al. 2005).

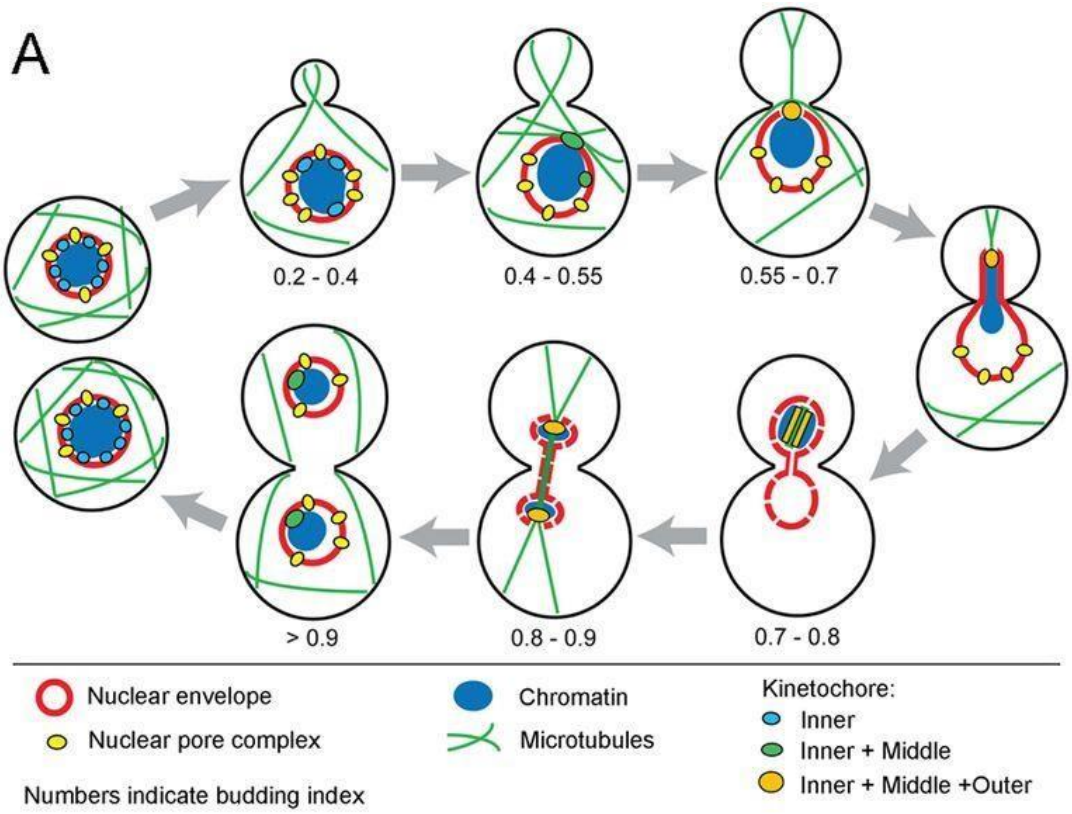

Figure 7: Schematic of mitotic events in *C. neoformans*. A cartoon representing the dynamics of Kinetochore and nucleus during various stages of cell cycle. The numbers represent budding index which is the ratio of daughter bud length and mother bud length (Kozubowski et al. 2013).

1.29 Rationale of the current study

Canonical role of shugoshin includes protection of cohesion at the pericentromeric regions during mammalian mitosis and meiosis I (Gutierrez-Caballero et al. 2012). Mechanistically, shugoshin recruits PP2A to the pericentromeric region and prevents phosphorylation of cohesin subunits such as Rec8 in meiosis I and sororin in case of mammalian mitosis. Apart from the canonical function, shugoshins have evolved species-specific functions that play a role in ensuring proper fidelity of chromosomes in both mitosis and meiosis (Gutierrez-Caballero et al. 2012). In *C. neoformans* the role shugoshin remains unknown in both mitosis and meiosis. Moreover, studies on spatial and temporal dynamics of kinetochore proteins during mitosis have indicated that the mitotic events in *C. neoformans* are more metazoan like. The divergent nature of shugoshin family of proteins and metazoan like mitosis in *C. neoformans* has prompted us to investigate the mitotic function of shugoshin in *C. neoformans.*

2 Results

In order to probe for the function of the shugoshin gene in *C. neoformans*, first we checked its localization and the consequences of its loss of function. The results chapter is divided into two sections. Section A contains results of the initial characterization of the shugoshin protein and Section B deals with the mitotic function of shugoshin in *C. neoformans*.

Section A

2.1 Identification and characterization of the shugoshin gene

2.2 Bioinformatic identification of the shugoshin gene in *C. neoformans*

The shugoshin gene (ORF CNAG_05516) in *C. neoformans* was identified by employing the Hidden Markov Modelling (HMM) method. This ORF of length 2535 bp encodes a 71 kDa protein containing 645 amino acids. Sequence analysis using the MAFFT multiple sequence alignment (MSA) identified the presence of both an N-terminal coiled-coil motif and a C-terminal SGO motif (Figure 8B and 8C). The protein sequence identified was submitted to the Pfam database and the database showed the presence of the C- terminal SGO motif (E-value: 1.2e-07). The residues from 37 – 87 amino acids represent the N-terminal coiled-coil motif and the residues from 362 – 386 amino acids represent the C-terminal SGO motif.

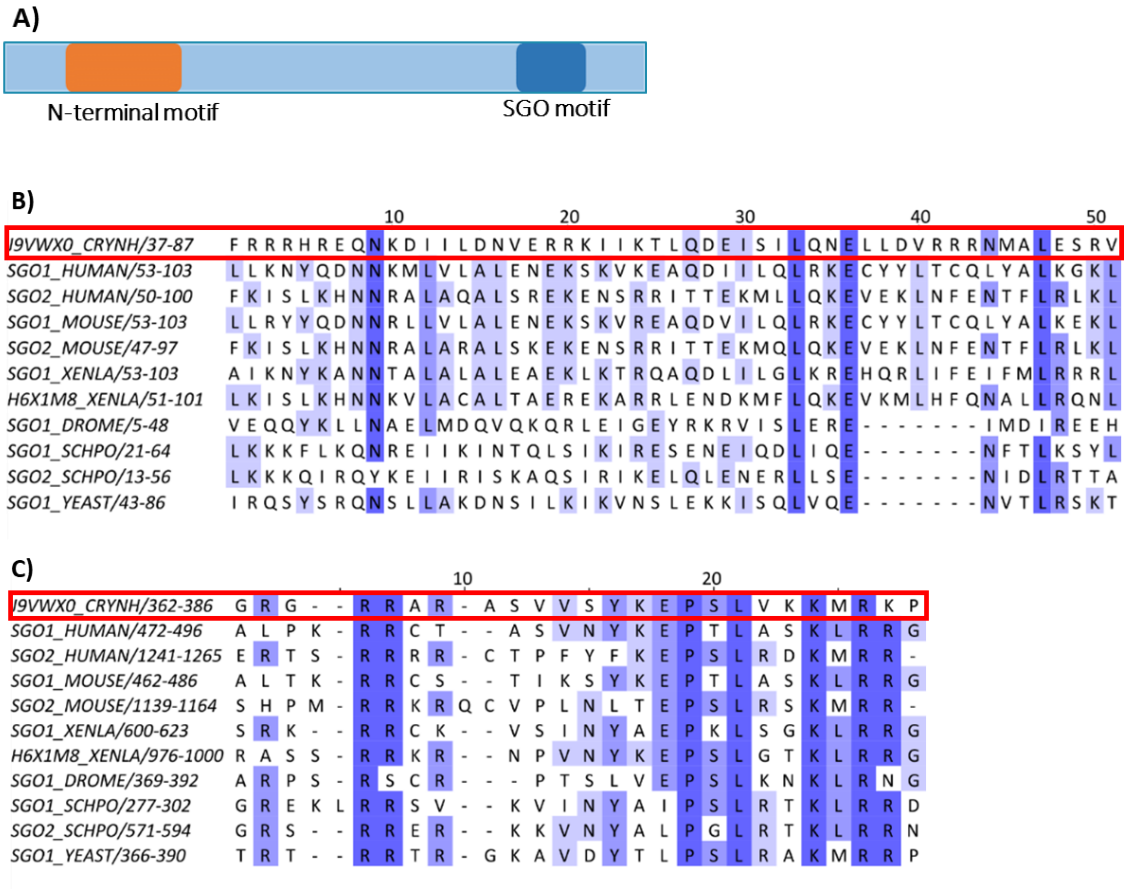

Figure 8: Identification of the *SGO1* gene *in C. neoformans*. A) Schematic of the Sgo1 protein in *C. neoformans* containing an N-terminal coiled-coil motif and a C-terminal SGO motif. **B)** MSA of the N-terminal coiled-coil motif. **C)** MSA of the C-terminal SGO motif. The red box represents the SGO motif sequence in *C. neoformans* and the blue shades in the MSA represent identical amino acids.

2.3 Shugoshin localizes to kinetochores

To check the intra-cellular localization of shugoshin, the protein was expressed with a C-terminally tagged GFP from its native locus generated by overlap PCR. This cassette was transformed into the H99 strain where a kinetochore protein Cse4/CENP-A was tagged with mCherry. To check for the intra-cellular localization of shugoshin, the cells were synchronized first by growing in YPG media until $O.D_{600}$ reached 5.5 (approximately 15 h). At this $O.D_{600}$, 98% cells were in the un-budded stage. The cells were then shifted to YPD media containing thiabendazole (10 µg/ml) and incubated for around 180 min. Sgo1-GFP co-localized as a bright dot along with mCherry-Cse4 in the daughter bud (Figure 9C). The dot-like signal was observed when the cells attained a budding index of 0.7-0.8. In un-budded and small-budded cells co-localization of Sgo1-GFP with mCherry-Cse4 was not observed (Figure 9A and 9B). In untreated conditions, we could not detect any Sgo1-GFP signals Co-localizing with both clustered and unclustered mCherry-Cse4 signals (Figure 9D).

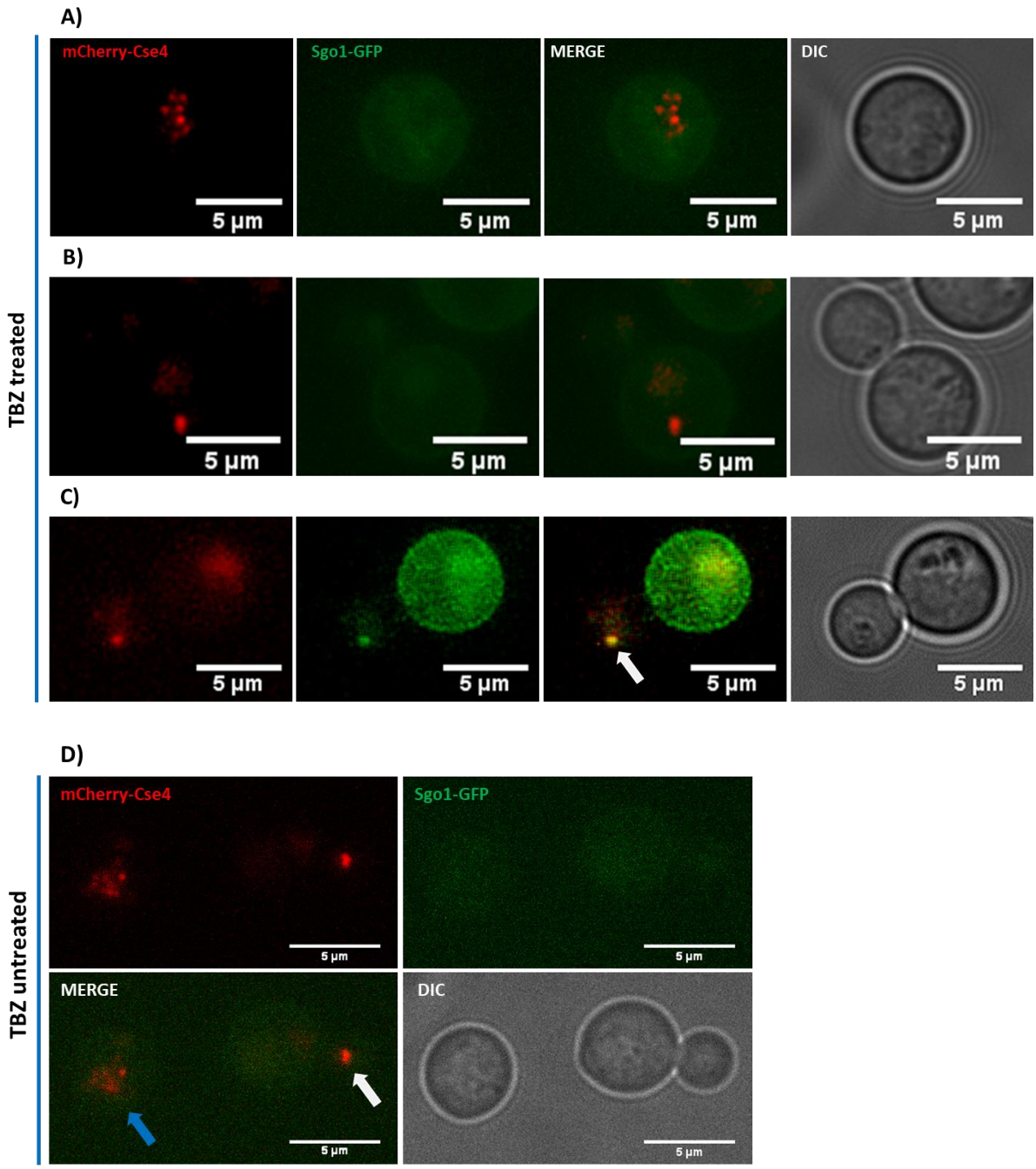

Figure 9: Fluorescence microscopy images of cells expressing Sgo1-GFP and mCherry-Cse4. A - C) Panels represent microscopy images of cells treated with thiabendazole. In un-budded and small-budded (panels A and B) Sgo1-GFP signal was not observed. When budding index of the cells was around 0.7-0.8, a dot-like co-localization of Sgo1-GFP with mCherry-Cse4 was observed (indicated by a white arrow). **D)** Represents microscopy images of cells that were not treated with the drug. Blue and white arrows represent unclustered and clustered signals of mCherry-Cse4 respectively. No signal of Sgo1-GFP was observed in an untreated condition indicating that Sgo1-GFP localizes to unattached kinetochores.

2.4 *sgo1Δ* mutant does not show any defect in growth and viability

To characterize the *SGO1* gene, a growth curve was constructed to determine the growth rate of the mutant in comparison to the wild type. For this analysis, two individual null mutants of shugoshin and the H99 (WT) strains in triplicates were used. The overnight culture of the strains were inoculated at 0.1 $O.D_{600}$ and $O.D_{600}$ was measured at every 90 min time intervals until 18 h. Samples were collected at two additional time points after 27 and 54 h of growth. The $O.D_{600}$ values obtained were plotted against time and a growth curve was generated. As it can be observed from the graph, growth of both *sgo1Δ* mutants is similar to that of wild type (Figure 10A).

To determine the viability of the s*go1Δ* mutant strain, two individual *sgo1Δ* mutants and wild type strains in triplicates were used. A total of hundred cells were plated in each case and the plates were incubated at 30^{o}C for 2 days. The number of colony forming units (CFU) were calculated and was plotted. Compared to the wild type the mutants do not show any significant loss in the viability as it is evident from the graph (Figure 10B).

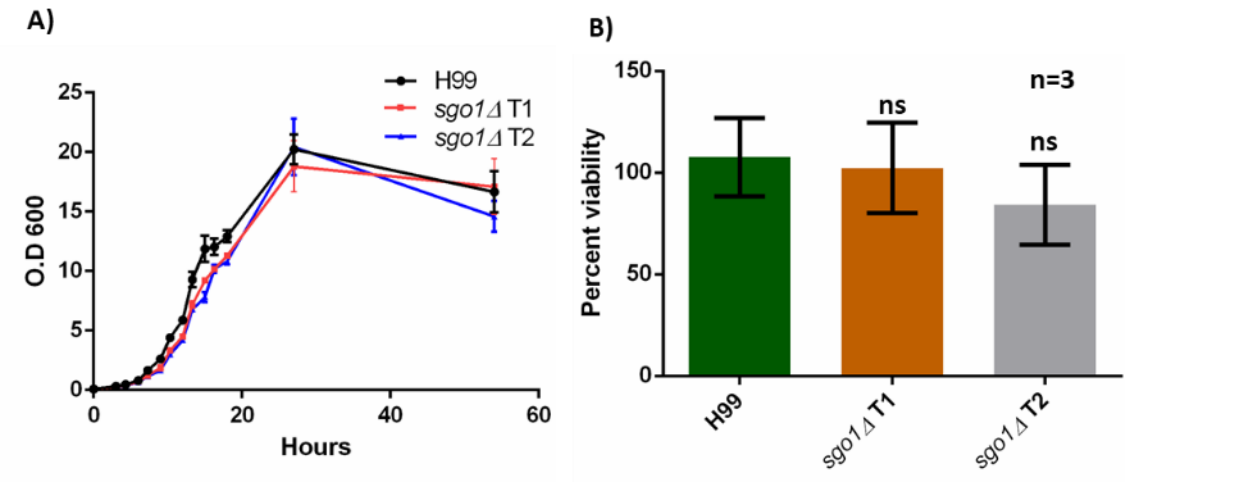

Figure 10: Growth curve and viability of the *sgo1Δ* mutant. A) The Graph represents the growth curve of the s*go1Δ* mutants and the H99 strains. From the graph, the growth of the s*go1Δ* mutants is comparable to that of the wild type. **B)** The Graph represents the viability of the s*go1Δ* mutant and the wild type. The number of colonies obtained on the plate were counted and were plotted as percent viability. One-way ANOVA was performed using 3 independent replicates and it was observed that the difference in viability between the wild type and the mutants is non-significant. Error bars represent standard error of the mean (SEM) calculated from three replicates. T1 and T2 indicate individual transformants of shugoshin null mutants. ns means non-significant.

2.5 *sgo1Δ* mutant is sensitive to microtubule depolymerizing drugs

From the growth curve experiment described in previous section, it was observed that *SGO1* is non-essential for the cell growth in *C. neoformans*. Shugoshin as mentioned earlier is relatively divergent and it also evolved species-specific functions. This prompted us to check whether Sgo1 has any role in cellular processes such as DNA repair, DNA replication and spindle assembly checkpoint function. If the mutant creates any problem in the cellular functions, then treating with known inhibitors that negatively affect the cellular process would exacerbate the growth of the cell. Creating these conditions elevates the phenotype which can be mild or undetected. To test this, we used drugs that perturb DNA repair, DNA replication or spindle checkpoint function.

To probe for the drug susceptibility, the *sgo1Δ* mutants and the H99 strains were spotted on respective drug plates. The *sgo1Δ* mutants did not show any susceptibility to DNA damage inducing drug methyl methane sulphonate (MMS). The *sgo1Δ* mutants also did not show any sensitivity to DNA replication inhibiting drug hydroxyurea (HU). The above results indicate that Sgo1 is not involved in DNA damage repair pathway and DNA replication process. To check whether is there any chromosome loss or aneuploidy, cells were treated with ergosterol synthesis inhibiting drug called fluconazole and the strain did not show any resistance to fluconazole (Figure 11A). It was observed that the *sgo1Δ* mutants are sensitive to microtubule depolymerizing drugs benomyl and thiabendazole (2.5 µg/ml concentration) (Figure11A and11B). These results indicate that the *sgo1Δ* mutants show defects in kinetochore-microtubule interactions. The sensitivity observed in the *sgo1Δ* mutants was similar to the phenotype observed for one of the spindle checkpoint mutant *mad2* (Figure 11B).

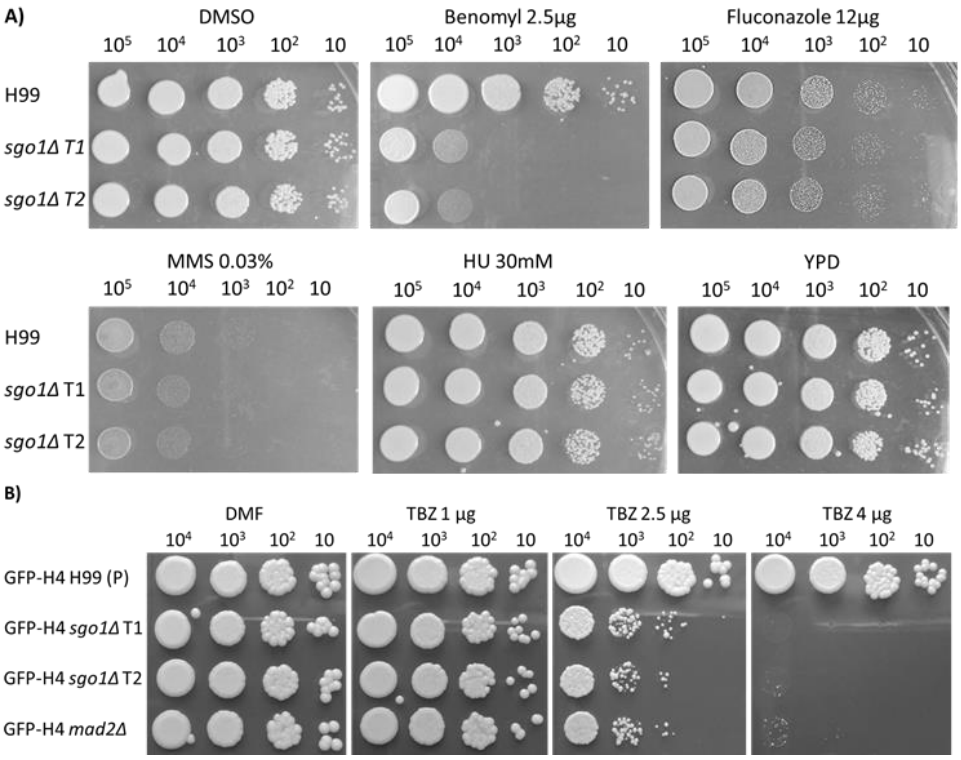

Figure 11: Drug sensitivity assay. A) The sensitivity of the *sgo1Δ* mutants was assayed by spotting various dilutions of cells on different drug plates. The *sgo1Δ* mutants show sensitivity to benomyl at 2.5 µg/ml concentration as compared to H99 (WT). **B)** GFP-H4 tagged strains of H99 (WT), *sgo1Δ* and *mad2Δ* were spotted on different concentrations of thiabendazole containing YPD plates. DMF (Dimethylformamide) represents no drug control. The *sgo1Δ* mutants show sensitivity to thiabendazole at 2.5 µg/ml concentration similar to the *mad2Δ* mutant. `

Section B

2.6 Mitotic function of shugoshin in *C. neoformans*.

2.7 *sgo1Δ* mutants bypass spindle assembly checkpoint when treated with thiabendazole

As mentioned in the previous section that the *sgo1Δ* mutants showed sensitivity to thiabendazole this prompted us to look into the cell cycle defects in these mutants under thiabendazole treated conditions. In a wild type cell, when the cells were subjected to treatment with microtubule depolymerizing drugs such as thiabendazole it creates unattached kinetochores. This situation in a cell activates SAC thereby arresting the cells. In *C. neoformans*, the above mentioned situation results in a large-budded arrest where the nucleus is either present in the daughter bud or mother bud. In situations where one of the SAC components such as *MAD2* is deleted, the cells are unable to sense unattached kinetochores and fail to arrest the cells at any specific stage of the cell cycle.

In this experiment, H99 (WT), *sgo1* and *mad2* null mutants were treated with thiabendazole at 10 µg/ml concentration and cells were harvested for microscopy analysis. Various cell cycle stages such as un-budded, small-budded, large-budded and multi-budded were counted and the percentage values were plotted. For the shugoshin null mutant, two independent transformants were considered and each experiment was performed with three individual technical replicates. For the *mad2Δ* mutant, only two technical replicates were considered for the analysis.

With the increase in time (150 min), the H99 strain showed accumulation of cells in large-budded stage (Figure 12B). Whereas in the *mad2Δ* mutant, cycling population was observed without any accumulation of large-budded cells, which is the expected phenotype (Figure 12C). In the *sgo1Δ* mutants, the phenotype observed was similar to that of the *mad2Δ* mutant with only a slight increase in the number of large-budded cells at 150 min time point. Also, the percentage of un-budded cells in the s*go1Δ* mutants was less than the *mad2Δ* mutant (Figure 12D and 12E). One interesting result observed is that as the time increased there was an increase in the accumulation of multi-budded cells in the s*go1Δ* mutants compared to the H99 and the *mad2Δ* mutant (Figure 12D and 12E). These results indicate that in the absence of Sgo1, the thiabendazole treated cells bypass spindle

assembly checkpoint function similar to the *mad2Δ* mutant. This indicates that shugoshin might play a role in spindle assembly checkpoint.

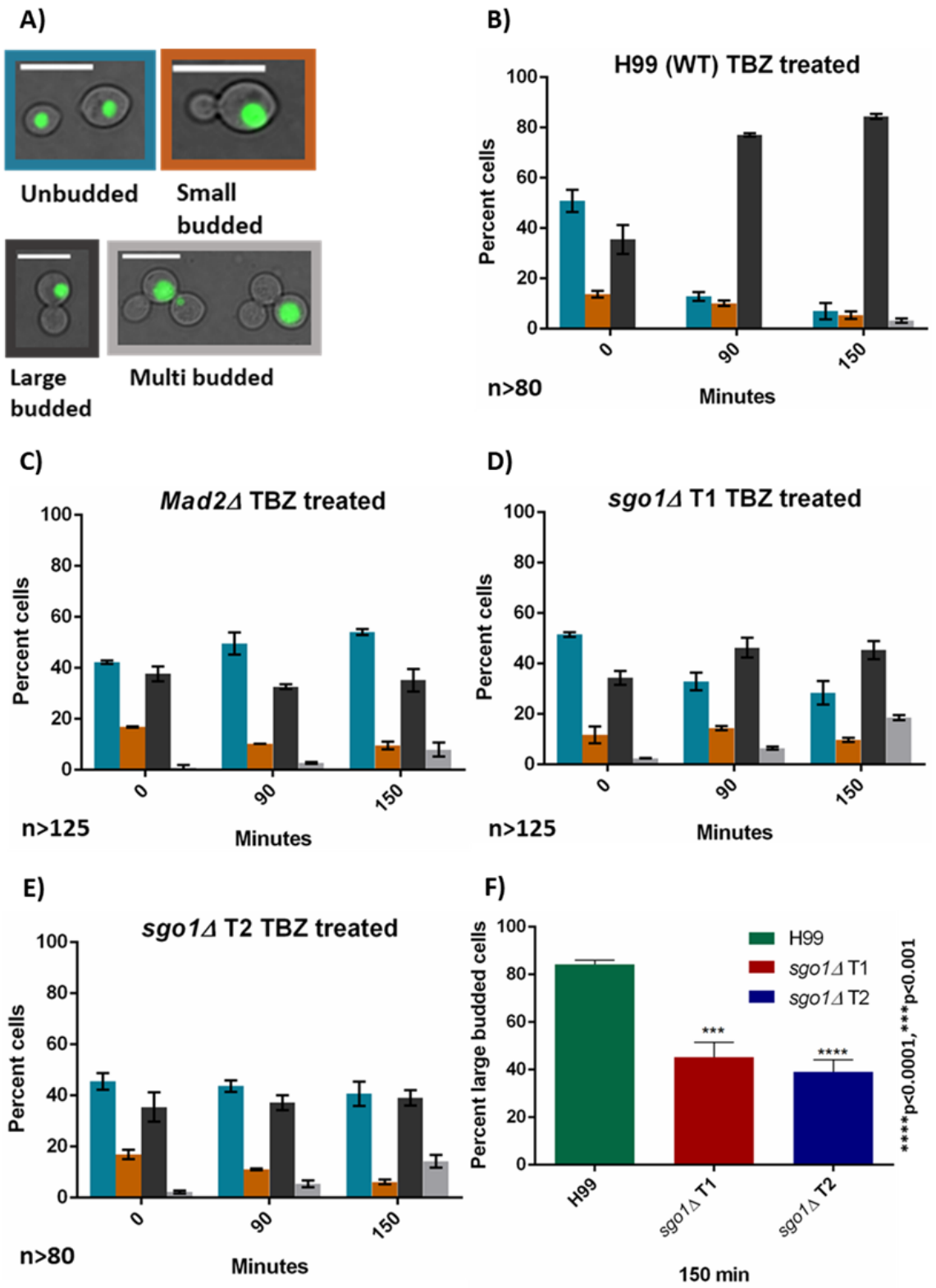

Figure 12: *sgo1Δ* mutants bypass SAC checkpoint. A) Microscopy images represent the various cell cycle stages used to score for the cell cycle defects. The green fluorescence

represents nucleus where nuclear marker H4 was tagged with GFP. **B - E)** panels represent plots of the percentage of cells in various cell cycle stages. 0 min represents untreated condition (DMF control). Cells treated with thiabendazole for various time durations were indicated. The percentage of large-budded cells observed in both the transformants of the *sgo1Δ* mutant is comparable to the *mad2Δ* mutant. This indicates that the *sgo1Δ* mutant bypasses SAC when treated with thiabendazole. Error bars represent SEM. **F)** The graph represents the percent large-budded cells from 150 min time point. One-way ANOVA analysis was performed with the wild type, *sgo1Δ* mutants and the analysis indicated that the decrease in percentage of large-budded cells was significant between wild type and the mutant ($p<0.001$ for *sgo1Δ* T1 and $p<0.0001$ for *sgo1Δ* T2). T1 and T2 represents different transformants of *sgo1Δ.*

2.8 Thiabendazole treated *sgo1Δ* strain show an increase in genome content

To study cell cycle defects in thiabendazole treated *sgo1Δ* mutants, flow cytometry was carried out. This experiment was performed with drug treated and untreated controls. Upon treatment with thiabendazole, the H99 strain showed arrest at the G_2/M stage (Figure 13A). As expected the *mad2Δ* mutants showed both G_1 and G_2/M peaks indicating the occurrence of cycling population of cells (Figure 13C). In case of the *sgo1Δ* mutant two peaks were observed; one indicating the G_2/M stage (or 2C genome content) and the other peak was observed beyond the G_2/M peak (Figure 13B) indicating a higher genomic content of the cell. Thus treatment with thiabendazole results in an increase in genome content of the cell in *sgo1Δ* mutants. The above mentioned situation might arise because of improper nuclear division or having more than one round of DNA replication.

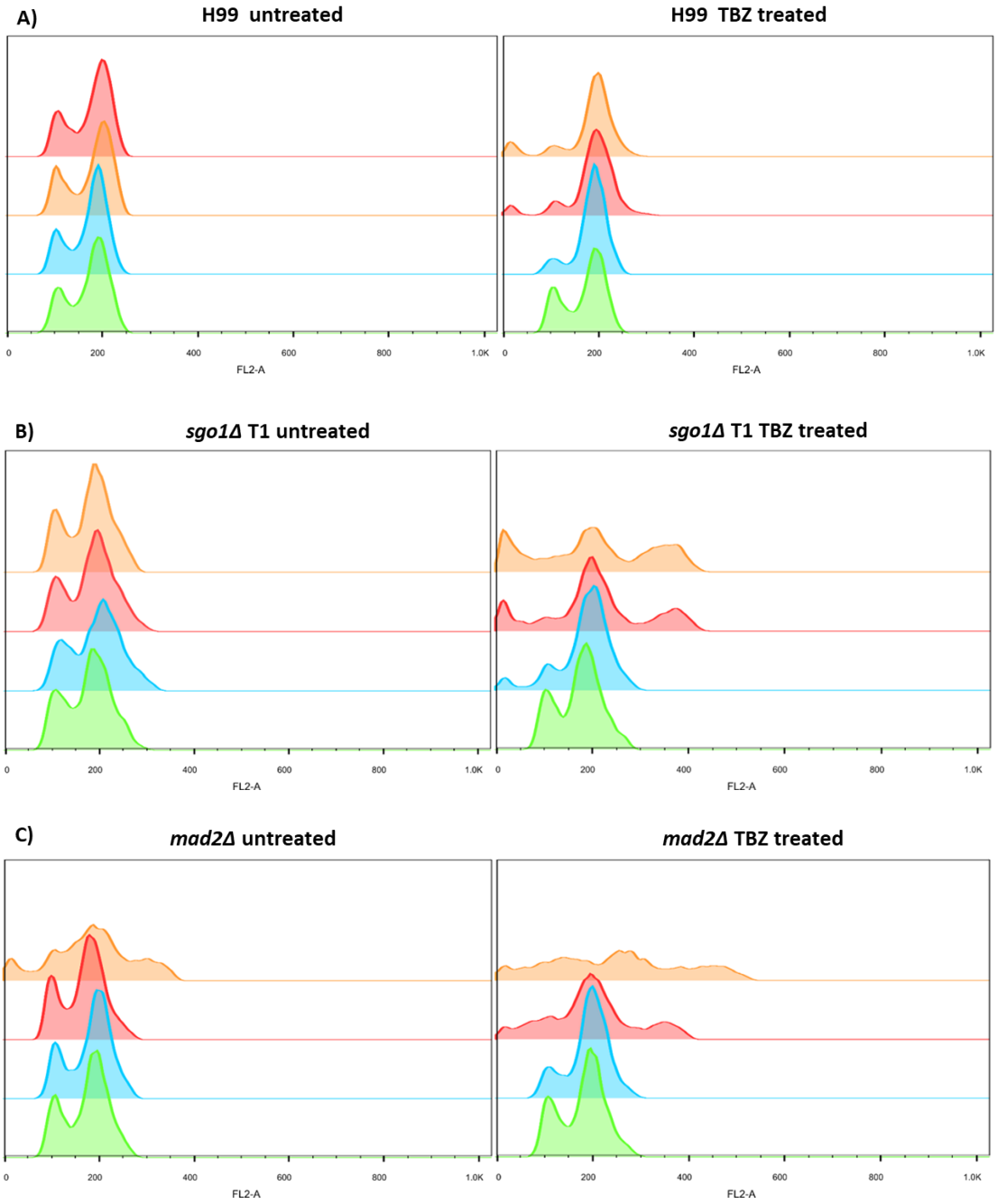

Figure 13: Thiabendazole treated *sgo1Δ* strains show an increase in genome content. Panels **A, B** and **C** represent flow cytometry profile of thiabendazole treated and untreated conditions wild type H99, *mad2Δ* and *sgo1Δ* mutant strains. The X-axis represents fluorescence intensity FL2-A and Y-axis represents counts or events. Colour code represents time points.

2.9 *sgo1Δ* mutants show partial bypass of spindle assembly checkpoint upon depletion of outer kinetochore component

Microscopy analysis of the *sgo1Δ* mutants treated with thiabendazole showed bypass of spindle assembly checkpoint which was similar to what was observed in the *mad2Δ*

mutants. The above mentioned experiment was repeated using a different approach. In this method, the *SGO1* gene was deleted in a strain where outer kinetochore protein Dad1 is placed under the controllable promoter of the *GAL7* gene. The expression of the *DAD1* gene is repressed in the presence of glucose/dextrose and induced in the presence of galactose. We used the H99 (parent) strain and the *mad2Δ* strain containing *GAL7-DAD1* construct as controls. For microscopy analysis of the cell cycle defects, the strains were grown in the repressive condition (YPD) for 6 h and samples were collected every 1 h until 8 h.

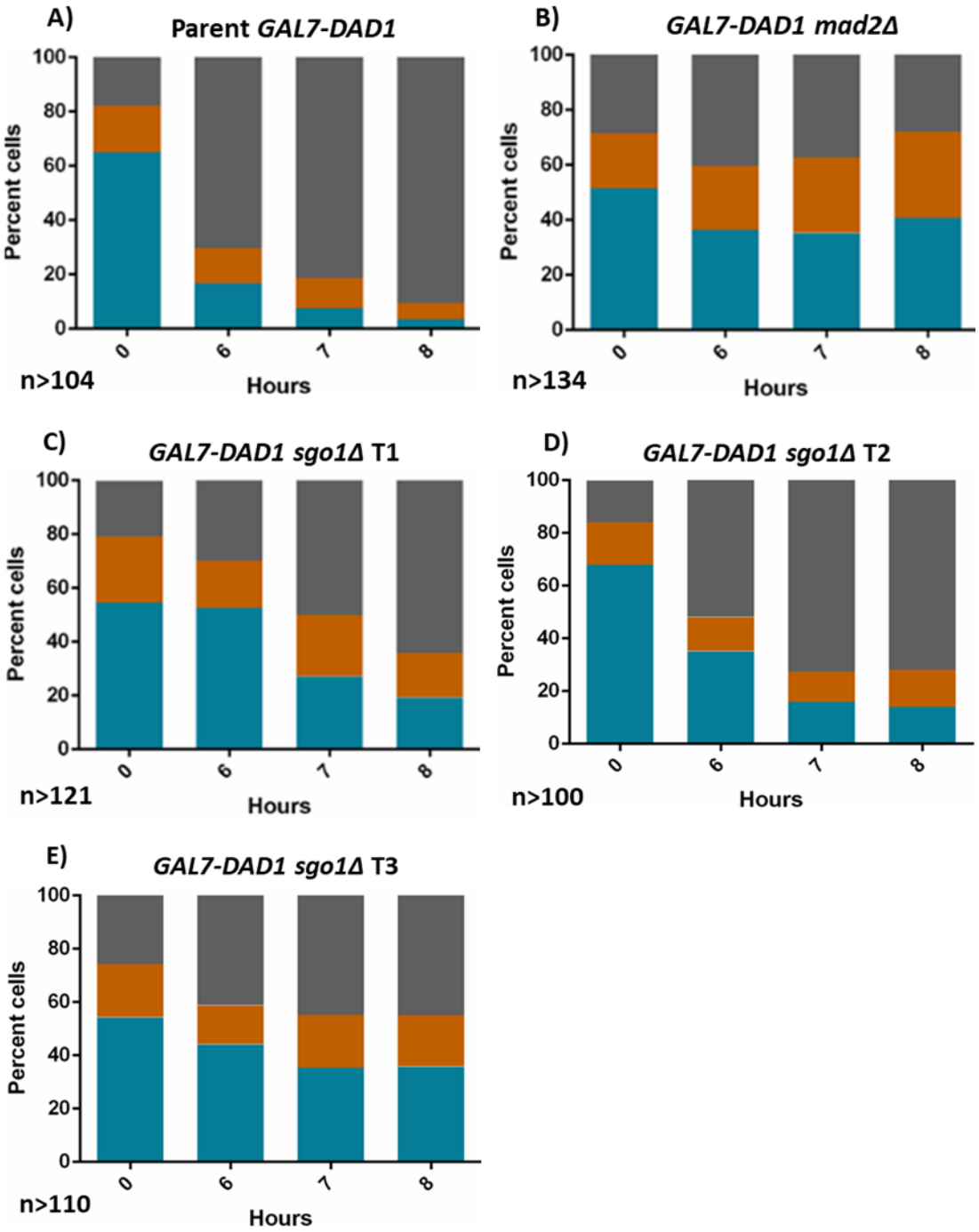

Figure 14: *GAL7-DAD1 sgo1Δ* strains show partial bypass of SAC. A - E) Panels represent plots of percent cells in various cell cycle stages grown under repressive conditions (YPD). n represents number of cells counted. The s*go1Δ* mutants show partial bypass of the checkpoint when compared to the *mad2Δ* mutant. T1, T2 and T3 represents different transformants of *sgo1Δ.*

At 8 h time-point, 90% of the cells were arrested at large-budded stage in parent strain (Figure 14A). As expected in the *mad2Δ* strain, cycling population of cells were observed (Figure 14B). Surprisingly in the *sgo1Δ* mutants, the percent of large-budded cells observed was intermediate between parent and the *mad2Δ* strain (Figure 14C,14D and 14E). The experiment was performed with three individual *sgo1Δ* transformants and they showed a range of 45 to 70% cells arrested in large-budded stage.

3 Discussion

Previous studies in yeasts and higher eukaryotes have highlighted diverse functions of shugoshin. Canonical function of shugoshin includes protection of cohesins at the pericentromeres during meiosis I and mammalian mitosis. Apart from canonical function other species-specific functions such as bi-orientation, SAC silencing and cell cycle delay were also identified. Till date, there were no reports on either mitotic or meiotic functions of shugoshin in basidiomycetes. In this study, we explored the mitotic function of shugoshin in *C. neoformans* by looking into its localization and the consequences of loss of function mutation.

3.1 Shugoshin null mutants do not show any growth defect and are sensitive to microtubule depolymerizing drugs

It has been reported in *S. cerevisiae*, that the *Scsgo1Δ* cells are viable but grow slowly compared to wild type cells. The possible reason for slow growth phenotype in *S. cerevisiae* was not addressed (Indjeian et al. 2005). In *C. neoformans*, we identified that the s*go1Δ* mutants are viable and the growth rate was similar to that of wild type cells. This observation implies that the *SGO1* gene in *C. neoformans* is non-essential for its viability when grown in rich media.

Shugoshin null mutants in *S. cerevisiae* and *S. pombe* show sensitivity to microtubule depolymerizing drugs such as benomyl (Indjeian et al. 2005) (Vanoosthuyse et al. 2007). In *C. neoformans,* the *sgo1Δ* mutants are sensitive to microtubule depolymerizing drugs similar to that of shugoshin null mutants in *S. cerevisiae* and *S. pombe*. We observed that the sensitivity to microtubule depolymerizing drug thiabendazole was similar to that of canonical SAC mutant *mad2Δ* in *C. neoformans.* These results indicate that the phenotype observed might be a result of impaired kinetochore-microtubule interactions in the s*go1Δ* mutant.

3.2 Shugoshin localizes to kinetochores/centromeres in response to unattached kinetochores

In *S. cerevisiae*, shugoshin localizes to the pericentromeric region as a dot-like signal and diffuses into the nucleus during the onset of anaphase. Its localization to the pericentromeric region is stabilized upon treatment with microtubule depolymerizing drugs (Clift et al. 2009). The temporal dynamics of the mitotic Sgo2 in *S. pombe* is similar to *S.*

cerevisiae Sgo1, but the spatial dynamics differ. In *S. pombe*, during interphase Sgo2 localizes to telomeres and its localization shifts to centromeres during early anaphase. During late anaphase, Sgo2 signal appears diffused in the nucleus (Vanoosthuyse et al. 2007).

In *C. neoformans*, we could not detect any signal of Sgo1-GFP during untreated conditions and it is absent during all stages of cell cycle. During drug treated conditions, a dot-like diffused signal of Sgo1-GFP was observed that co-localized with the mCherry-Cse4 signal. This signal was observed only in the daughter bud of the budded cell and was not present during any other stages of the cell cycle. This behavior might indicate that shugoshin in *C. neoformans* localizes to kinetochores/centromeres only during drug-treated condition. This can be either in response to tensionless KT-MT attachments or because of unattached kinetochores. This behavior is different from what has been reported in *S. cerevisiae* and *S. pombe*. In these organisms, the localization of shugoshin is observed even without the treatment of microtubule depolymerizing drugs. The localization of shugoshin to kinetochores only in response to unattached kinetochore might indicate a possible spindle checkpoint function in *C. neoformans*.

3.3 *sgo1Δ* mutants show partial bypass of the spindle assembly checkpoint

It is reported that the *sgo1Δ* mutants of S. cerevisiae arrest normally in the presence of nocodazole (Indjeian et al. 2005). Similarly, in *S. pombe*, when spindle integrity is perturbed the cells get arrested at metaphase stage in the *sgo2Δ* mutants (Vanoosthuyse et al. 2007). These results indicate that shugoshin is not required to activate spindle assembly checkpoint in the presence of unattached kinetochores. However, in both the yeasts, Sgo is required to maintain spindle checkpoint function in the absence of tension among the sister chromatids.

In *C. neoformans* the behavior of the *sgo1Δ* mutants is different from other yeasts. From the localization pattern of Sgo1 in *C. neoformans*, we speculated that there might be a possible role of Sgo1 in SAC function. We identified that when KT-MT attachments in the *sgo1Δ* mutant were severed, the phenotype was intermediate between the wild type and the *mad2Δ* mutant. It was not a complete arrest or a complete bypass of the checkpoint. This partial bypass in checkpoint can be as a result of a reduction in the levels of Mad2 in

the absence of shugoshin. It is also possible that it might be due to a reduction in the levels of PP2A as it is the direct binding partner of shugoshin.

One attractive hypothesis can be that shugoshin dependent control of the anaphase onset during mitosis. Recently it is reported that in *S. pombe*, Sgo2 might play a role in controlling the onset of anaphase which is independent of canonical Mad2 mediated pathway, i.e., through the formation of MCC (Meadows et al. 2017). It might be possible that in *C. neoformans,* Sgo1 might play a partial role in detecting unattached kinetochores. It is also possible that it might regulate the Mad3-Cdc20 complex to inhibit APC/C in response to unattached kinetochores. The above mentioned hypothesis has to be further validated. We also observed that when spindle integrity was perturbed using microtubule poisons, we observed a bypass of checkpoint and increase in ploidy content. The FACS analysis revealed that the *sgo1Δ* mutants show an increase in the ploidy content treated with thiabendazole. This phenotype can be attributed to the endoreduplication of DNA. Further experiments have to be done to validate the above hypothesis. In conclusion, these results shed light on the new undiscovered role of shugoshin in SAC, especially in basidiomycete yeasts. Further experiments have to be performed to decode the mechanistic details of the shugoshin action in modulating SAC.

4 Materials and Methods

4.1 Strains and Media

Strains used in this study are listed in the table2. *C. neoformans* cultures were grown and maintained on YPD (1% yeast extract, 2% peptone and 2% dextrose) and YPG (1% yeast extract, 2% peptone and 2% galactose) media at 30^{o}C unless specified. For transformation YPD+1M sorbitol or YPG + 1M sorbitol plates were used. For selection of transformants nourseothricin (NAT) (100 µg/ml final concentration) and neomycin (G418) (200 µg/ml final concentration) were used.

4.2 Biolistic transformation

The biolistic transformation was done based on a method previously described by (Davidson et al. 2000). 5 ml of *C. neoformans* culture was grown overnight, centrifuged at 4,000 rpm for 5 min and resuspended in 300 µl sterile dH_2O. Approximately 300 µl of cell suspension was spread on YPD+1M sorbitol plate and was allowed to dry. Gold micro-carrier beads (0.6µm) coated with 2-5 µg of DNA to be transformed were prepared and 10 µl of 2.5M $CaCl_2$, 2 µl of 1M spermidine free base (Sigma-Aldrich) were added. The mixture was vortexed for 10-20 s and incubated for 10 min at room temperature, centrifuged, washed with 500 µl of 100% ethanol and then resuspended in 10 µl of 100% ethanol. The solution was placed on micro-carrier disk and allowed to dry. Dried disks were placed in Bio-Rad biolistic transformation apparatus (Biolistic® PDS-1000/He Particle Delivery System). Helium gas pressure of approximately 1300 psi under a vacuum of minimum 25 inches of Hg was generated to bombard the cells with micro-carrier beads. The cells were incubated for 5-6 hours on YPD+1M sorbitol medium at 30^{o}C. Cells were then plated on YPD + NAT/NEO selection plate and incubated at 30℃ for 3-4 days.

4.3 Flow cytometer analysis

5ml of *C. neoformans* culture was grown overnight, and 2 $O.D_{600}$ cells were taken and washed with sterile dH_2O and fixed in 1 ml of 70% ethanol overnight at 4℃. Fixed cells were centrifuged at 4000 rpm and washed with 1 ml NS buffer(10 mM Tris-HCl pH 7.5, 250 mM sucrose, 1 mM EDTA (pH 8.0), 1 mM MgCl2, 0.1 mM CaCl2, 0.1 mM ZnCl2, 0.4 mM phenyl methyl sulphonyl fluoride and 7 mM β-mercaptoethanol) and resuspended in 200 µl of NS buffer added with 2 µg of RNase and incubated at 37℃ for 3-4 h. To this mixture Propidium Iodide (final concentration 12 µg/ml) was added and incubated for 30 min at room temperature in the dark. 50 µl of the sample was suspended in 2 ml of 1X PBS (Phosphate

buffered saline) solution, vortexed, sonicated for 10 s at 30% Amp. 30,000 cells were analyzed by flow cytometry using FL2-A channel on a Becton–Dickinson FACS Calibur.

4.4 Fluorescence microscopy

Cell grown in YPD or YPG were pelleted at 4000 rpm. Cells were washed once with distilled water and suspended in distilled water. The cell suspension was placed on a slide and imaged at 100X using either Olympus BX51 or Zeiss Axio Observer. Images obtained were processed using ImageJ or Zen Blue software.

4.5 Drug sensitivity Assay

For drug sensitivity assays overnight cultures of wild type, *sgo1Δ* mutants and *mad2Δ* mutants were inoculated to the secondary culture at 0.2 $O.D_{600}$ /ml and grown until 0.8 $O.D_{600}$ /ml. The number of cells per ml were calculated and were serially diluted. The serially diluted cultures were spotted on plates containing various inhibitors such as hydroxyurea, methyl methanesulphonate, Thiabendazole and fluconazole. As controls DMF, DMSO and YPD plates without drugs were used. The drug plates were incubated for 72 h and photographed.

4.6 Thiabendazole treatment of the *sgo1Δ* mutants

For thiabendazole treatment cells were first grown in YPD and incubated overnight. The overnight culture was inoculated into secondary YPD culture at 0.2 $O.D_{600}$ /ml and grown until 0.8 $O.D_{600}$ /ml. The secondary culture was treated with 10 µg/ml concentration of thiabendazole and samples were collected.

4.7 Microscopy analysis of *GAL7-DAD1 sgo1Δ* mutants

For depleting the Dad1 protein, the cells were grown in YPG overnight. The cells were washed with distilled water twice and are transferred to YPD media at 0.2 $O.D_{600}$ /ml and grown for 6 h. From 6 h onwards cells were collected at every 1 h interval until 8 h time point.

4.8 Construction of Sgo1-GFP tagging cassette

The fluorescent fusion protein of shugoshin was generated by overlap PCR method. GFP was tagged to the C-terminal region of *SGO1* gene. Primers used for overlap cassette construction were listed in the table1. The 1 kb 3’ region upstream of stop codon (without

including stop codon) and 1 kb 3' UTR region after the stop codon was amplified from the *SGO1* gene using H99 genomic DNA. The GFP sequence with antibiotic resistance marker NAT was amplified form the pCN19 plasmid using primers SDP12 and 13. The individual PCR fragments were gel purified. Using individual PCR fragments final overlap cassette of 4.5 kb was constructed using primers SDP10 and 16. The final cassette was transformed. The transformants obtained were confirmed using PCR.

4.9 Construction of *sgo1Δ* overlap PCR cassette

sgo1Δ deletion cassette was constructed using overlap PCR method. Primers used for overlap cassette construction were listed in the table1. The 1 kb upstream region to ATG start codon and 1 kb downstream region of stop codon was amplified from the *SGO1* gene using H99 genomic DNA. The NEO resistance marker fragment was amplified from pLK25 plasmid using primers SDP4 and SDP5. The individual PCR fragments were gel purified. Using individual fragments final overlap cassette of 3.8 kb was constructed using primers SDP2 and SDP8. The final cassette was transformed. The transformants obtained were confirmed using PCR.

4.10 Bioinformatic identification of *C. neoformans SGO1* gene

Sgo1 homolog of *C. neoformans* was identified using raw HMM file obtained from Pfam database (http://pfam.xfam.org/family/PF07557#tabview=tab6). The HMM file was submitted to jackhmmer search tool in HMMER web server (https://www.ebi.ac.uk/Tools/hmmer/search/jackhmmer). The sequence identified from jackhmmer was submitted to Pfam database to check for the presence of motifs. The putative N-terminal and C-terminal sequences were also identified using MAFFT Multiple Sequence Alignment tool. For MSA Jalview 2.0 software (Waterhouse et al. 2009) was used. An identity threshold of 30 % was set as a cut-off for representing the sequence identity in the MSA.

4.11 Growth curve assay

For growth curve assay respective strains were grown in YPD for overnight and inoculated in secondary YPD culture at 0.1 $O.D_{600}$ /ml. The experiment was performed with three independent technical replicates. Samples were collected every 90 min and $O.D_{600}$ was measured and plotted using Graphpad Prism 6 software.

4.12 Viability assay

For viability assay cultures were first inoculated and grown for overnight and $O.D_{600}$ was measured. The number cells per ml were calculated from the $O.D_{600}$ value obtained (1 $O.D_{600}$ = 2 X 10^7 cells). The cells were diluted accordingly and 100 cells were plotted per plate. This experiment was performed using 3 independent technical replicates for each strain. The plates were incubated for 48 h and the number of colonies obtained were counted and plotted. One-Way ANOVA was performed to check for statistical significance between the viability of the wild type and the mutant.

4.13 Localization of Sgo1-GFP signal

To localize Sgo1-GFP signal, the cells were first grown in YPD. The cells were inoculated At 0.2 $O.D_{600}$ /ml in YPG and grown for around 15 h ($O.D_{600}$ 5 – 5.5). The Cells arrested at the un-budded stage were harvested by centrifugation, washed and inoculated in YPD media containing thiabendazole (10 µg/ml concentration). The cells were collected at 180 min time point and were imaged. For imaging, both untreated and treated controls were used.

Table 1: Primers used in this study

Primer name	Alias	Sequence (5'-3')	Purpose
SDP1	SGO1_DEL_P1	CCAGTTCCTCAAAGAGCTTGAC	
SDP2	SGO1_DEL_P1_FULL	CCGATGAGCGTACACCACTTAC	
SDP3	SGO1_DEL_P2	CCAGCTCACATCCTCGCAGCTGCTACG AGATTGAGACGAATG	
SDP4	SGO1_DEL_P3	CATTCGTCTCAATCTCGTAGCAGCTGCG AGGATGTGAGCTGG	
SDP5	SGO1_DEL_P4	CTCCCAACTTCCCCGCTTTTGGTTTATCT GTATTAACACGGAAGAG	
SDP6	SGO1_DEL_P5	CTCTTCCGTGTTAATACAGATAAACCAA AAGCGGGGAAGTTGGGAG	
SDP7	SGO1_DEL_P6	ATCAGAACGCACGTCGCAC	

SDP8	SGO1_DEL_P6_FULL	CAGGCAGAGGATTGGGAAGTC	*SGO1* deletion construct
SDP9	SGO1_F_P1	CTGCTGATTACTTGGTAGCCGAGT	Sgo1-GFP tagging
SDP10	SGO1_F_P1_FULL	CACGTGCCAGTGTCGTCAG	
SDP11	SGO1_F_P2	CTCGCCCTTGCTCACCATCTGCAAGACT CCAACGGCCCTC	
SDP12	SGO1_F_P3	GAGGGCCGTTGGAGTCTTGCAGATGG TGAGCAAGGGCGAG	
SDP13	SGO1_F_P4	CTAACTAGCATCATTATCGTCGCAGCGC GCGTAATACGACTC	
SDP14	SGO1_F_P5	GAGTCGTATTACGCGCGCTGCGACGAT AATGATGCTAGTTAG	
SDP15	SGO1_F_P6	GCAGAGGATTGGGAAGTCATC	
SDP16	SGO1_F_P6_FULL	GGTCTGACGTCGATAAGCCAC	

Table 2: List of strains used in this study

Strain ID	**Background**	**Source**
H99	α	Perfect *et al.* 1993
CNVY108	α H99*::GFP-H4: NAT*	Kozubowski *et al.* 2013
CNSD110	α *SGO1::sgo1-NEO*	This study
CNSD113	**a** KN99*::mCherry-CENP-A: NEO*, *SGO1-GFP-NAT*	This study
CNSD117	α H99*::GFP-H4: NAT*, *SGO1::sgo1-NEO*	This study
CNSD120	α H99*::GAL7p-GFP-DAD1:HYG, SGO1::sgo1-NEO*	This study

SHR710	α H99*::GAL7p-GFP-DAD1:HYG*	Shreyas thesis 2014
SHR733	α *MAD2::mad2-NEO, GAL7p-GFP-DAD1:HYG*	Shreyas thesis 2014
SHR741	α H99*::GFP-H4-NAT, MAD2::mad2-NEO*	Shreyas thesis 2014

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