Perturbing the *Cryptococcus neoformans* kinetochore: insights into kinetochore architecture, chromosome segregation and the spindle assembly checkpoint

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Master of Science

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By

Shreyas Sridhar

Molecular Biology and Genetics Unit,

Jawaharlal Nehru Centre for Advanced Scientific Research,

Jakkur, Bangalore- 560064, India

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Stay Hungry.
Stay foolish.

- Stewart Brand
Final edition “The Whole Earth Catalogue”.

DECLARATION

I hereby declare that this thesis entitled **"Perturbing the *Cryptococcus neoformans* kinetochore : insights into kinetochore architecture, chromosome segregation and the spindle assembly checkpoint"** is an authentic record of research work carried out by me towards my Master of Science under the supervision of **Dr. Kaustuv Sanyal** at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India and that this work has not been submitted elsewhere for the award of any other degree.

In keeping with the norm of reporting scientific observations, due acknowledgements have been made whenever the work described was based on the findings of other investigators. Any omission, which might have occurred by oversight or misjudgment, is regretted.

Shreyas Sridhar

Place: Bangalore

Date:

Certificate

This is to certify that the work described in this thesis entitled **"Perturbing the *Cryptococcus neoformans* kinetochore: insights into kinetochore architecture, chromosome segregation and the spindle assembly checkpoint"** is the result of the investigations carried out by **Mr. Shreyas Sridhar** towards his Master of Science as part of the Integrated-PhD program at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under my guidance, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Kaustuv Sanyal

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Bangalore 560064

Place: Bangalore

Date:

Acknowledgement

It's been a rather short duration spent at JNCASR in comparison to the amount I have been able to learn. Ironically, I knew negligible to non-existent amounts of yeast biology, more so on the topics of kinetochore, centromere, chromosome segregation and autophagy; towards which I have worked and hopefully had an impact on over the past few years. This contribution and understanding of the topics would not have been without the constant encouragement, correction, freedom and time provided by my mentors Dr. G. R. Ramesh, Prof. Kaustuv Sanyal and Dr. Ravi Manjithaya.

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ABBREVATIONS

A	Adenosine
bp	base pair
C-terminus	Carboxy/COOH terminus
C	Cyotsine
DNA	Deoxyribonucleic Acid
DIC	Differential interference contrast (microscopy)
GFP	Green fluorescent protein
G	Guanosine
h	hour
Kb	Kilo base pair
µg	Microgram
µl	Microlitre
ml	Millilitre
min	minutes
N-terminus	Amino/NH_2 terminus
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolution per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
Sec	Second
T	Thymidine
UV	ultraviolet

Setting the scene

Abstract

High fidelity chromosome segregation ensures proper inheritance of the genetic material, one of the most fundamental cellular processes. Chromosome segregation requires the linking of the chromosomes to a dynamic pulling and pushing force that enables equal division of chromosomes between the mother and daughter cells. The kinetochore is the chromosomal attachment site of this segregation machinery, the spindle, comprised of microtubules. Any errors occurring in this critical attachment are monitored with by the spindle assembly checkpoint (SAC). Several proteins, more than 80 known in yeast, assemble in a regulated manner to form the kinetochore on centromeric chromatin linking a chromosome to the dynamic plus ends of microtubules. Intriguingly, although the centromere DNA sequence in various eukaryotes is highly divergent, the basic architecture and function of the kinetochore remains conserved. However, the process of kinetochore assembly including localization patterns and localization dependency of various proteins at the kinetochore varies significantly. To understand the underlying principles involving the generation of this diversity, we studied the kinetochore assembly dynamics of the human fungal pathogen and an evolutionarily distinct basidiomycetous yeast *Cryptococcus neoformans var. grubii*. Utilizing a stringent controllable promoter of the *GAL7* gene, we expressed fluorescently tagged kinetochore proteins and sought to address the essentiality, localization dependency and regulation of assembly among proteins that belong to different layers of the proposed tri-laminar kinetochore network. Our results suggest a putative kinetochore architecture wherein depleting the protein pools of an outer layer (farther from centromere DNA but nearer to microtubule ends) does not appreciably effect the localization of underlying layers, while disrupting the inner layers (nearer to centromere DNA) perturbs the whole multi-subunit assembly of the kinetochore. Yet, to our surprise we observed that centromeric localization of a middle kinetochore protein complex (Ndc80) is Cse4 (the inner kinetochore) independent, hinting at the existence of a parallel /bi-partite assembly via an unidentified protein. In addition to this finding it was observed that depletion of kinetochore proteins other than the fungal specific Dam1 complex did not result in the cell cycle arrest mediated by SAC. Thereby these cells depleted of specific kinetochore proteins proceeded through the cell cycle with defective segregation machinery, resulting in cellular destruction due to faulty chromosome

segregation. We demonstrate that cell cycle arrest at the G2/M stage in those kinetochore mutants that exhibited the arrest phenotype is via the activation of the Mad2-mediated SAC. Several inner and middle kinetochore mutants did not show any cell cycle arrest but lost viability due to segregation defects. It would be interesting to see whether recruitment of some of these kinetochore proteins is required for SAC activation. Overall, this study provided us a window to look through the precise assembly and architecture of the kinetochore, the process of chromosome segregation and the functioning of the spindle assembly checkpoint in this organism. Though more work is needed in the future for a definitive understanding of the kinetochore and the mitotic checkpoint, we have gained significant leads towards understanding the possibility for the existence of a master regulator required for kinetochore assembly. However several questions are to be answered such as timing, requirement and regulation of assembly, and how this complex structure is propagated and maintained in this organism.

Introduction

1.1 Historical perspective on the cell cycle

The Nobel prize in physiology or medicine was awarded to Leland H Hartwell, Paul Nurse and Tim Hunt for their contributions towards understanding cell cycle regulation (Nobelprize.org). This dissection of the mitotic process began in the late 1960s with budding yeast mutants that blocked cell cycle progression and later in the 1970s, isolation of fission yeast mutants that sped up the cell cycle (Gautier, Norbury et al. 1988). These studies were further supplemented by the work on sea urchin with the identification of proteins in these extracts expression of which varied through the progression of the cell cycle, consequently naming them as "cyclins" (Evans, Rosenthal et al. 1983). The combined work of these three scientists and a multitude of others led to the understanding of additional classes of cell cycle dependent kinases (CDKs), cyclins, checkpoints proteins, inhibitors and the process of coupling cell morphology to the cell cycle.

1.2 Cell cycle

The cell cycle can be defined as the time period between the mother cell division and its subsequent daughter cell division. Cell division ensures and enables the propagation of heritable genetic material to be transmitted from mother to daughter cells. For most cells, environmental cues determine the state and progression of the cell cycle. Cells undergo two types of cell division, mitosis and meiosis. The former is involved in growth and repair while the latter is required for sexual reproduction. The mitotic cell cycle can be broadly defined into two stages a) interphase which involves genome duplication and growth and b) mitosis phase where the duplicated genome of the mother is divided equally between its daughters. These stages are not exclusive, as S phase of genome duplication can overlap significantly with the mitotic phase of the cell cycle in yeasts. Interphase can be further sub-divided into G1, S and G2 stages **(Figure 1A)**. G1 and G2 phases (Gap phases) primarily involve the growth of cells in preparation for synthesis (S) phase and mitotic (M) phase respectively (Mitchison 1971).

It is in the S phase that the genome duplication occurs by DNA replication **(Figure 1B)**. Upon completion of genome duplication and preparation for division, cells enter the M phase. It is noteworthy, that while in most organisms cell division occurs by fission; in the budding yeast it takes place via a process of budding. This unique feature of budding yeast results in the changes in the cell morphology that is in synchrony with the cell cycle **(Figure 1A)**.

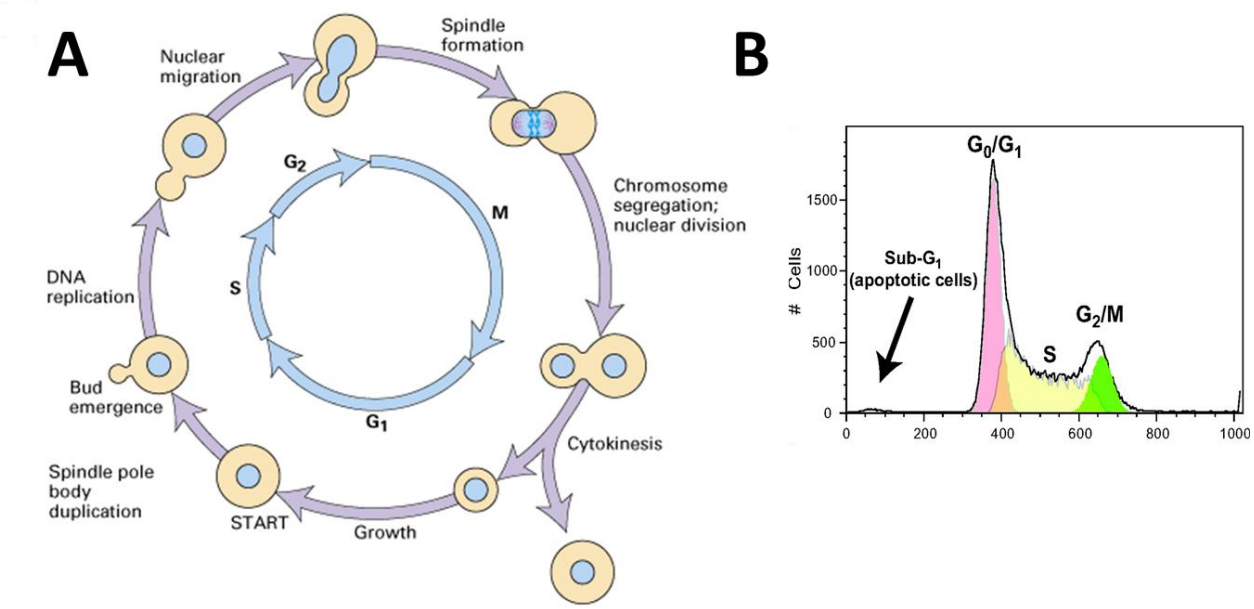

Figure 1. Budding yeast cell cycle. A) Schematic representing major events during the budding yeast cell cycle. B) Flow cytrometric analysis of how DNA content varies through cell cycle. (Trina A. Schroer, 2005)

Based on distinctive events, mitosis can be sub-divided into: a) prophase, where chromatin condenses to give rise to sister chromatids in parallel to initiating the duplication of centrosomes/spindle pole bodies (SPBs), in mammals and yeast respectively; b) metaphase, involves the invasion and capturing of the kinetochore by forming spindle, leading to alignment of chromosomes at the equatorial plane. In yeasts, although anaphase A has been observed no metaphase plate is reported (Straight, Marshall et al. 1997). At the end of metaphase, bi-orientation of the chromosomes is established resulting in tension between sisters chromatids. This prepares a cell for the next stage, c) anaphase, which involves two simultaneous processes of spindle fiber shortening and the pole-ward movement of the centrosomes/SPBs, leading to the movement of the segregated genomes to their destinations. Mitosis is completed in d) telophase which in addition to un-coiling of

the highly condensed chromatin, leads to the breakdown of the spindle. In metazoans, telophase also involves nuclear membrane and nucleoli re-formation. Completion of the cell division is brought about by cytokinesis where a contractile ring divides the cell into two identical daughter cells **(Figure 1),** (Glotzer 2005).

The time spent at various stages of the cell cycle varies among the organisms and the developmental stage, yet most cells spend ~90% of their time in interphase, while G_0 or resting/quiescent phase could last indefinitely.

As the need of the cell cycle is to ensure faithful inheritance of the genomic content, a number of processes aid in this precise segregation of chromosomes. Checkpoint mechanisms, segregation machinery and chromatin maintenance are some of those processes that involve more than 10% of the genes in a eukaryote.

Defects in the cycle and chromosome segregation are barely tolerated by the cell, with most being lethal during development, while others lead to developmental disorders and non-communicable diseases including cancers.

1.3 Basics of chromosome segregation in budding yeast

The decision to divide and transmit heritable DNA by chromosome segregation is made rather early in the cell cycle. A key point in the cell cycle is the "start" (Nasmyth 1993) signal that is satisfied in G1, which irreversibly commits cells to undergo at least one round of the cell division. The start signal initiates processes like genome duplication, cell budding and spindle pole body duplication in S phase. The start signal also initiates cell growth (Nasmyth 1993). Environmental signals such as rich nutrient conditions satisfy the start signal, while nutrient starvation and mating block this process. The requirement of the cell to satisfy certain criteria that are monitored by cellular components can be defined as a checkpoint. As the process of cell division is most important to cells, several such checkpoints exist throughout the cell cycle. Some of the key checkpoints are the G1-S, G2-M and spindle assembly checkpoint (Hartwell and Weinert 1989).

These checkpoint pre-requisites are monitored by the presence and activity of cell cycle kinases known as cyclin dependent kinases (CDKs) in concert with their activating partners – the "cyclins" (Nurse 2000). The expression of cyclins is transcriptionally controlled and their levels are monitored throughout cell cycle by proteolysis. These cyclins behave as specific substrates and activate CDKs, which in turn promote a specific cell cycle event or deter transition into the subsequent cell cycle phase **(Figure 2)**. At least 11 known cyclins exist in yeast. Cln3, G1 cyclin, is a putative cell size sensor that regulates the level of other G1 cyclins. For the start signal the activity of the Cdk1p has to be induced, which is brought about by G1 cyclins. Dependent on the cyclin substrate, Cdk1p (yeast homologue) is important for S phase as well as transition into M phase **(Figure 2)** (Ghiara, Richardson et al. 1991).

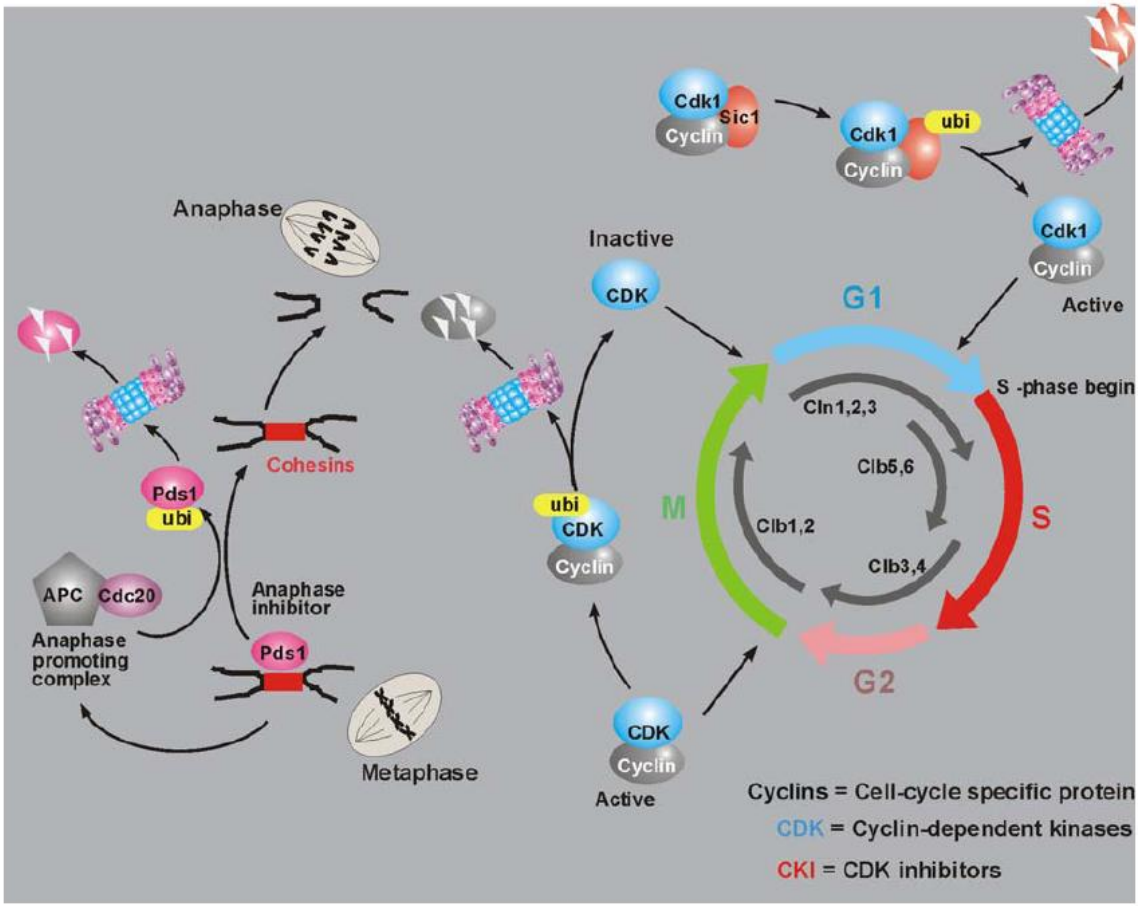

Figure 2. Regulatory network of the yeast cell cycle. Transitions between various stages of cell cycle occur upon satisfying "checkpoints" that are determined by the presence/absence of "cyclins" and their ability to activate CDKs.

Upon initiation of "start", yeast progress through cell cycle, encountering their first checkpoint, the G1-S checkpoint that delays the onset of S phase if DNA damage is detected. Upon genome replication in S phase, a cell encounters the G2/M checkpoint that in addition to detecting DNA damage also checks for the complete duplication of the yeast genome (Sancar, Lindsey-Boltz et al. 2004). On satisfying the G2/M checkpoint, cells progress through the mitotic phase where chromosomes are segregated to daughter cells. This delicate and accurate process of chromosome segregation requires the proper attachment of chromosomes to the push-pull forces of the spindle that facilitates migration of chromosomes to their destination (daughter cells). Hence the attachment of the spindle to the kinetochore, a supra-molecular bridge, is monitored and cells with improper attachments are prevented from transitioning from metaphase to anaphase. This checkpoint is watched by the anaphase promoting complex which is an E3 ubiquitin ligase that promotes the degradation of mitotic cyclin (Clb1/2) and securing upon activation **(Figure 2)** (Reddy, Rape et al. 2007).

Cohesins are the glue holding sister chromatids and are required to be degraded for sister chromatid separation, prior to anaphase separation. Securin, the anaphase inhibitor, is an antagonist of the cleavage enzyme separase, that needs to be activated for the breakdown of cohesins from the chromosomes (Michaelis, Ciosk et al. 1997).

When cells are unable to satisfy the checkpoints, they get arrested at various stages of the cell cycle exhibiting distinct cell morphologies, as cell cycle is coupled with cell morphology in budding yeast. These were the findings that led Lee Hartwell to identify cell division cycle (CDC) mutants **(Figure 2)** (Hartwell, Mortimer et al. 1973, Hartwell 1974, Weinert 1989).

Chromosome segregation requires the connection of the chromosome to the mitotic forces of the spindle that provides the force required to move these oddly shaped structures through the milieu of the cell. The connection occurs through a special protein supra-complex called kinetochores, which form at special locations on the chromosome called the centromere.

1.4 The centromere

The centromere is one of the few cellular structures that have yet an unknown mode of initiation, origin and maintenance in most systems; hence various other parameters are used to define it. Cytologically, the centromere is the primary constriction, the pinched waist-line area that holds the sister chromatids together on the metaphase chromosomes. At the molecular level, it is the chromosomal locus onto which the kinetochore assembles. The epigenetic definition would be, the chromosomal loci which is bound by the centromeric histone CENP-A. Genetically, a centromere can be defined as the region of reduced recombination, while biochemists define the centromere as a transcriptionally inactive, gene poor and heterochromatic region. This unique, chromosomal locus is the basic foundation upon which the kinetochores assemble, sister chromatin cohesion is established and chromosomal movements occur.

The relationship between centromere function and the underlying DNA sequence is one of few mysteries waiting to be solved in this modern day. While centromeres are indispensable for cell cycle, no two species exhibit any centromere DNA sequence conservation. The cloning and characterization of the centromere was first performed in *Saccharomyces cerevisiae* (Clarke and Carbon 1980). Plasmids that contain these centromeric sequences are stably maintained through mitosis and meiosis in *S. cerevisiae*.

1.4.1 Types of centromeres

Centromeres can be broadly classified based on DNA length, sequence, the presence of repetitive DNA elements and whether the kinetochore assembly is centromere DNA sequence dependent or independent. Upon these parameters centromeres can be grouped into point, regional, intermediate and diffused centromeres **(Figure 3b)**.

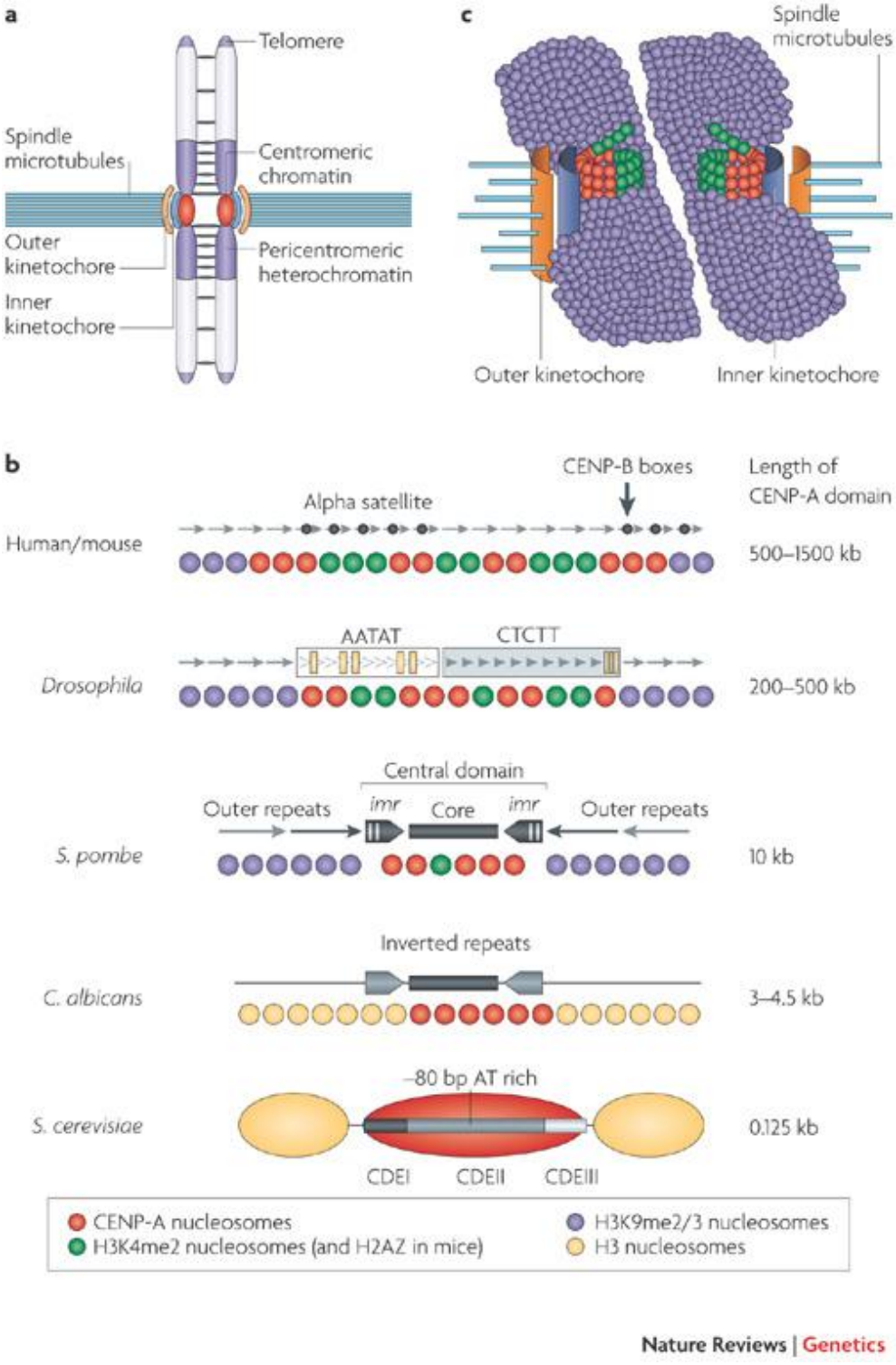

Figure 3. Organization of the centromere. A) The centromere is the DNA foundation onto which the kinetochore assembles. B) Schematic representation of the organization and histone binding at the centromere in various organisms. C) A predicted three-dimensional organization of the centromere (Allshire and Karpen 2008).

1.4.1.1 Point centromere

This class of centromeres, as the name suggests, are short in size and bound by kinetochore proteins in a sequences dependent manner. The centromere of the aforementioned budding yeast was the first described point centromere. This point centromere was found to have three distinct DNA sequence motifs, of which two act as sequence specific binding sites for kinetochore proteins (Clarke and Carbon 1980, Fitzgerald-Hayes, Clarke et al. 1982, Clarke 1990). The 125 bp centromere contains three consensus centromere DNA elements (CDEs). The non-conserved central element is AT-rich (>86%) with a sequence length of 78-86 bp (Fitzgerald-Hayes, Clarke et al. 1982, Hieter, Pridmore et al. 1985). The role of the spacer element is player by CDEII which is flanked by two conserved motifs, an 8 bp CDEI and a 25 bp CDEIII (Clarke and Carbon 1983, Cumberledge and Carbon 1987). The CDEIII sequence is an imperfect palindrome. Subsequently, several other budding yeasts including *Candida maltose*, *Camdida glabrata, Yarrowia lipolytica* and *Kluyveromyces lactis* have been shown to have centromere structures analogous to that of *S. cerevisiae* **(Figure 3b)** (Fournier, Abbas et al. 1993, Heus, Zonneveld et al. 1993, Heus, Zonneveld et al. 1994, Ohkuma, Kobayashi et al. 1995, Kitada, Yamaguchi et al. 1997, Vernis, Abbas et al. 1997, Vernis, Poljak et al. 2001).

Interestingly in *Y. lipolytica* it was found that the functions of centromers and autonomous replicating sequences (ARSs) exist together and are inseparable (Fournier, Abbas et al. 1993). The point centromere seems to be restricted to *S. cerevisiae* and closely related specific. Over the years identification of centromeres in other organisms suggests that sequence specificity in determining centromere identity may not be the only determinant, instead it appeared that in many eukaryotes centromere function is regulated epigenetically.

1.4.1.2 Regional centromeres

Centromeres that are longer in DNA sequence than point centromeres and to which bind kinetochore proteins in a sequences independent manner are described as regional centromeres. In addition all regional centromeres discovered so far have repetitive DNA sequences that comprise of the centromeric sequence. The highly repetitive nature of these regional centromeres has made them difficult to analyze. The regional centromere of the fission yeast *Schizosaccharomyces pombe* is 40-110 kb in length, and is well studied. It is

organized in a non-repetitive 4-7 kb central core region which is flanked by pericentric repeats on either side. The pericentric repeats can be further divided into centromere specific innermost (*imr*), perfect inverted repeats which are surrounded by a tandem array of outer repeats (*otr*) that vary in size and orientation (Fishel, Amstutz et al. 1988). The *otr* repeats form heterochromatin and are involved in sister chromatid cohesion, while the central core and *imr* repeats constitute the CENP-A binding region **(Figure 3b)**.

Similar to the repetitive regional centromeres of *S. pombe* several higher fungi also carry highly repetitive regional centromeres; such is the case with *Aspergillus nidulans, Neurospora crassa* and *Cryptococcus neoformans.* The centromeres of *A. nidulans and N. crassa* contain repeats that are highly divergent due to the operation of repeat induced point mutations (RIP) (Centola and Carbon 1994, Cambareri, Aisner et al. 1998).

In the well-studied invertebrate *Drosophila melanogaster*, the regional centromere consists of tandem repeats of AATAT and TTCTC sequences which are organized in a uniform "head-to-tail" orientation with few exceptions of "head-to-head" and "tail-to-tail" **(Figure 3b)** (Sun, Wahlstrom et al. 1997).

Amongst primate and human regional centromeres, higher order structure of nucleosome length long, 171 bp alpha satellite DNA units are arranged in tandem with a "head-to-tail" orientation, with each chromosome containing a specific number of repeats **(Figure3b)** (Willard 1985, Waye, Durfy et al. 1987, Willard 1990, Lee, Wevrick et al. 1997). It was revealed by immunocytochemistry that among these large centromeres only a small part of it was occupied by CENP-A.

Intermediate centromeres

This third type of centromeres is found to be present in the human fungal pathogen *Candida albicans*. The classification is based on the fact that they lack both sequence motifs which are characteristic of point centromeres, and repetitive elements which is the hallmark of regional centromeres. In *C. albicans* the centromere is composed of 3-5 kb long unique centromeric chromatin on each of its eight centromeres **(Figure 3b)** (Sanyal, Baum et al. 2004). While naked centromeric DNA was found to be sufficient to confer centromere activity in vivo, it was shown to be unable to assemble functional centromeric chromatin

and kinetochores *de novo,* when introduced into cells, implying at a possible epigenetic propagation of centromeres in *C. albicans* (Baum, Sanyal et al. 2006).

1.4.1.3 Diffused centromeres

Unlike the cytological definition of centromere, it was found that the centromeres in nematodes including *Ceanorhabditis elegans* are holocentric, without a clear primary constriction. The kinetochore proteins and spindle microtubules was found to bind along the entire length of the chromosome (Oegema, Desai et al. 2001, Meraldi, McAinsh et al. 2006) . With these findings it is speculated that all other centromeres were derived from holocentric centromeres with gradual inactivation of the so called non-centromeric regions in other organisms.

1.4.2 Centromeric determinants

1.4.2.1 Genetic determinants

1.4.2.1a DNA sequence motifs

The identity of point centromeres to a large extent is determined by genetic factors. The 125 bp long centromere of *S. cerevisiae* contains 3 functional elements CDEI, CDEII and CDEIII. The AT-rich central region is flanked by two palindromic sequence elements CDEI and CDEIII (McAinsh, Tytell et al. 2003). CDEIII serves as the binding site for CBF3, a sequence dependent kinetochore protein, thereby playing a predominant role in centromere identity. A complete disruption of centromere function is observed on even a single substitution in the CCG of CDEIII. The binding of CBF3 in turn recruits CENP-A and hence the sequence is self-sufficient in maintaining the centromere identity in *S. cerevisiae*. A similar mechanism for genetic determination of the centromere is observed in *C. glabrata, Y. lipolytica*, *C. maltose* and *K. lactis,* which contain point centromeres.

Interestingly sequence-dependent binding of kinetochore proteins onto the centromere is also observed in some epigenetically determined centromeres. CENP-B binding boxes present in human, ferrets, giant panda, tree shrews, gerbils and mouse centromers exhibits a sequence-specific binding of CENP-B. Formation of the centromere was suppressed when alpha satellite DNA was integrated into the chromosomal site; however *de novo* centromere assembly on alpha satellite DNA is dependent on CENP-B. Thus it has been proposed that

CENP-B plays a role during *de novo* centromere formation and also in the prevention of excess centromere formation on chromosomes (Ohzeki, Nakano et al. 2002, Okada, Ohzeki et al. 2007).

1.4.2.1b Centromere replication

Contrary to the belief that gene-poor, heterochromatic regions replicate late in S phase; heterochromatic centromeres have been found to replicate early in S phase such as the point centromere of *S. cerevisiae.* This finding negates the hypothesis that a delay in replication of centromeres until mitosis is required for sister chromatid adherence and proper chromosome segregation at anaphase. This observation has been found to hold true not only in the point centromeres of the *Saccharomyces* family but also in the sequence independent, intermediate centromeres of *C. albicans,* including its neocentromeres, which have been shown to replicate early in S phase (Koren, Tsai et al. 2010).

The regional centromeres of *S. pombe* have also been shown to replicate early in S phase, with active chromosomal origins of replication being identified within centromeric regions (Kim, Dubey et al. 2003). The complex centromeres of *D. melanogaster* have also been shown to replicate early in S phase (Ahmad and Henikoff 2002). To complement these findings, recent studies have shown that late replication is not a pre-requirement for heterochromatic regions. Thus, the early replicating nature of centromeres is independent of the type of centromere, although amongst higher eukaryotes the centromere has been found to replicate in mid or late S phase.

1.4.2.1c Recombination at centromeres

Recombination at the centromere is an enigma. Centromeres are known to be recombination deficient. On the contrary, centromeres are a rapidly evolving loci and recombination is necessary to bring about changes that drive evolution (Lambie and Roeder 1986). It has been proposed that crossovers close to centromeres disrupt pericentric sister chromatid cohesion resulting in the premature separation of sister chromatids leading to random segregation. The pressure to reduce crossing over near the centromeres seems to be strongly selected for (Rockmill, Voelkel-Meiman et al. 2006). Heterochromatin was previously implicated towards recombination-deficient nature of centromeres. But this is no longer the notion as heterochromatic regions were shown to

undergo recombination with frequencies that depend upon the distance from centromere (Lamb, Sherman et al. 2005). Yet this is a debated topic with varying reports emerging from several model systems. Studies analyzing meiotic events suggest that centromeres were recombinant-deficient, however analysis of mitotic recombination later revealed that recombination tract can penetrate through centromeric regions during mitosis. Mitotic recombination was found to be taking place at a very high rate in mammalian centromeres (Talbert and Henikoff 2010).

1.4.2.1d Gene conversion

The act of nonreciprocal exchange of a stretch of DNA from one homologue to another is known as gene conversion. The predominant function of gene conversion is in aiding the repair of double stranded breaks by recombination followed by copying of information from the homologues chromosome **(Figure 4)**.

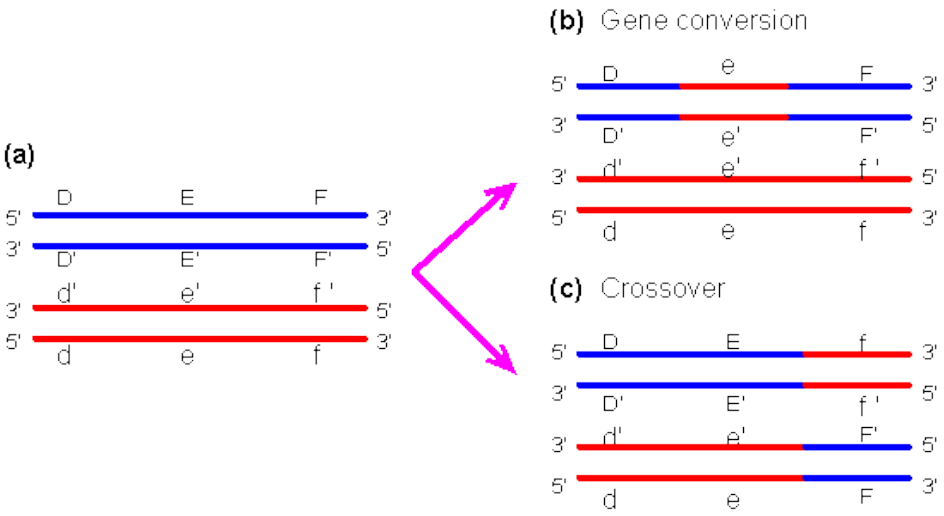

Figure 4. Schematic representation of gene conversion and crossover.

Replication fork stalling creates double strand breaks; and so if they occur at centromeres the resulting breaks could be repaired by gene conversion. This phenomenon has been implicated to occur within centromeres. The rate of this gene conversion at centromeres was found to be similar to those at other chromosomal loci.

It has now been hypothesized that gene conversion is a general feature of all higher eukaryotes. In addition, it has been proposed that unequal exchange between sites is the

cause of large expansion and contraction of alpha satellite repeat arrays, with gene conversion also being responsible for periodic homogenization of these satellite repeats (Talbert and Henikoff 2010).

1.4.2.1 Epigenetic determinants

1.4.2.2a RNA interference

The well-studied regional centromere of *S. pombe* relies upon the proper functioning of the RNAi machinery. It was found that inactivation of genes involved in the RNAi pathway led to defects in chromosome segregation. RNAi established heterochromatin at the pericentromeric regions by the recruitment of histone modifying enzymes (Martienssen, Zaratiegui et al. 2005, Kloc and Martienssen 2008, Grewal 2010). It was found that RNA-dependent RNA polymerase complex (RDRC) transcribes the pericentric repeats generating dsRNAs. The irony in this was the finding that transcription takes place from supposedly heterochromatic regions, which are thought to be transcriptionally inactive. It was later explained by findings that showed that transcription takes place during a short period in S phase when heterochromatin is displaced from the pericentric regions (Chen, Zhang et al. 2008, Kloc, Zaratiegui et al. 2008). Subsequent analysis revealed that Dicer (Dcr1) converts dsRNA into siRNA which are then incorporated into Argonaute (Ago1) containing the RNA induced silencing complex (RISC) (Motamedi, Verdel et al. 2004, Colmenares, Buker et al. 2007). Subsequently, Ago1 and siRNA become part of the RNA-induced transcriptional silencing complex (RITS) (Motamedi, Verdel et al. 2004, Verdel, Jia et al. 2004). RITS is then involved in targeting the siRNA that are made at the pericentric loci towards establishing a self-circulatory loop by again recruiting RDRC to amplifying the RNAi response (Sugiyama, Cam et al. 2005). The RITS also recruits Clr4 containing CLRC complex that catalyzes H3K9 methylation. Followed by Swi6 that binds to H3K9me2 resulting in heterochromatin formation. The spreading of the heterochromatin occurs through recruitment of Snf2/HDAC-containing repressor complex (SHREC) by Swi6 **(Figure 5)** (Sugiyama, Cam et al. 2007).

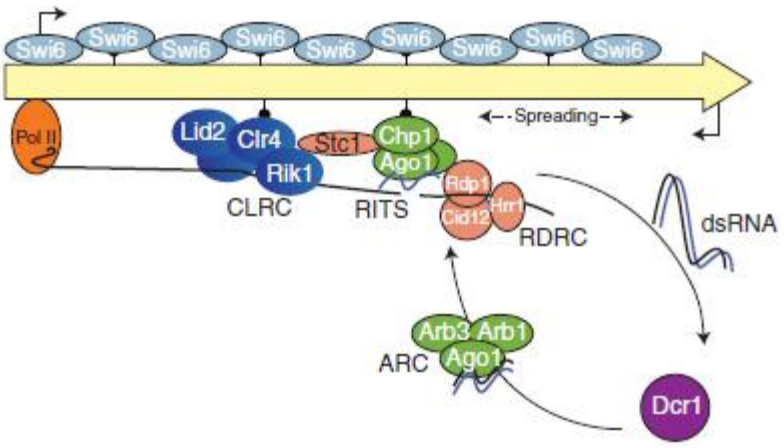

Figure 5. Molecular mechanism of heterochromatin maintenance, assembly and spreading at centromeric outer repeats in *S. pombe* (Volpe and Martienssen 2011)**.**

Although this RNAi machinery is absent in *S. cerevisiae*, other homologues and the existence of small RNAs have been observed in *S. castelli, Kluyveromyces polysporus, C. albicans* and *C. neoformans* (Drinnenberg, Weinberg et al. 2009, Skowyra and Doering 2012, Wang, Darwiche et al. 2013).

1.4.2.2b Chromatin organization

The centromere, through extensive analysis has been shown to have a unique chromatin organization onto which the kinetochore organizes. In *S. cerevisiae* the centromeric region is found to bind only one CENP-A nucleosome, which has a distinct organization and forms a ~200 bp nuclear-resistant core flanked by DNAse I hypersensitivity sites containing an array of positioned nucleosomes (Bloom and Carbon 1982). CENP-A assembles onto the central core in addition to canonical H3 nucleosomes in *S. pombe*. Upon treatment of the central core with micrococcal nuclease, it yields a smeary pattern rather than a characteristic ladder, suggesting at the lack of a regular periodic nucleosome array at the centromere (Polizzi and Clarke 1991). An increasing amount of evidence is pointing towards the altered micrococcal nuclease digestion pattern having a crucial role in CENP-A incorporation (Takahashi, Chen et al. 2000).

1.4.2.2d. Chromatin modifications

Unlike the point centromeres of *S. cerevisiae,* that have been found to contain only one CENP-A nucleosome, most other regional centromeres contain CENP-A nucleosomes inter-spread with blocks of canonical H3 containing nucleosomes. It has been known that

canonical H3 has a distinct pattern of post-translational modifications that provide a unique mark to the centromeric chromatin. Centromeric chromatin is generally hypoacetylated by the activity of histone deacetylases. Incidentally it was found that treatment with trichostatin A, a histone deacetylase inhibitor, causes an increase in acetylation of centromere chromatin, affecting chromosome segregation (Ekwall 2007).

The general heterochromatin modifications of H3K9me2/H2K9me3 are absent from centromeric chromatin. However it was found from studies in *S. pombe, D. melanogaster* and human that the H3 present at the centromeres are di-methylated at lysine 4, H3K4me2, a modification that is generally identified with open but transcriptionally inactive chromatin **(Figure 6)**. These findings could be explained by the need of transcription at the centromere in order to generate RNA that serves both a structural and silencing roles. This also implies that, centromere chromatin exists with an intermediate nature, between that of heterochromatin and euchromatin (Sullivan and Karpen 2004).

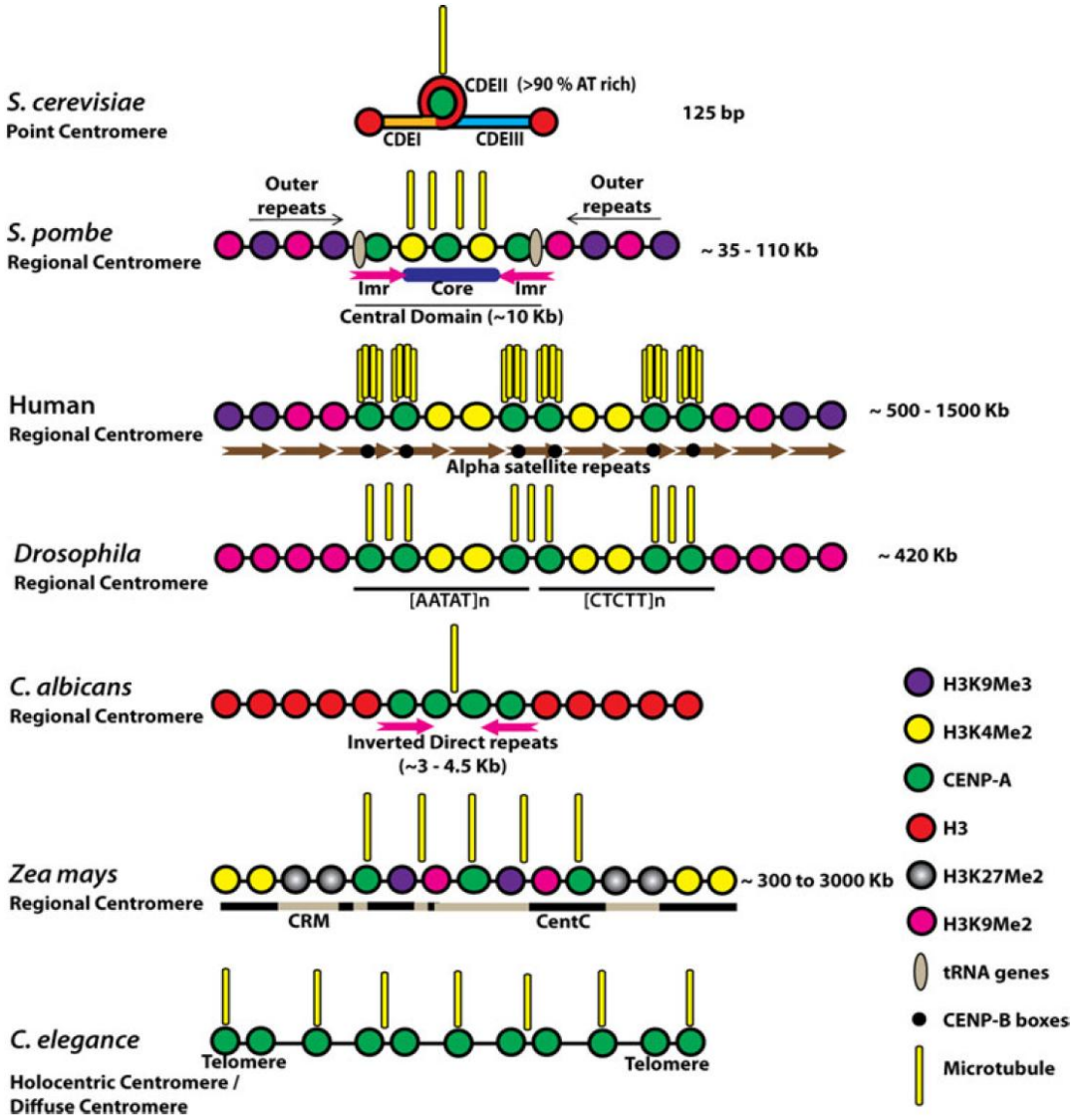

Figure 6. Centromeric and pericentromeric histone modification present across various organisms (Mehta et al, 2010)**.**

1.4.2.2e CENP-A: the ubiquitous marker of centromeric chromatin.

Most eukaryotes, in addition to the canonical histones (H2A, H2B, H3 and H4) contain histone variants (H2AZ, H2AX, H2AV and H3.3 amongst others). Towards carrying out special operations on chromatin, histone variants replace canonical histones at the nucleosome. Likewise, a centromere in all known eukaryotes is associated with its very own histone H3 variant, known as Centromere Protein-A (CENP-A) (Stoler, Keith et al. 1995). CENP-A has a constitutive presence at the centromere throughout cell cycle. It is

intriguing how the centromere, an entity that is so variable across many parameters such as length, sequences and organization share one common feature: CENP-A containing chromatin. CENP-A is known as Cse4 is *S. cerevisiae*, Cnp1 in *S. pombe*, CID in *D. melanogaster* and CENP-A in humans and vertebrates. Most kinetochore proteins, in various systems, have been shown to require CENP-A for their recruitment and localization at the centromere, yet the presence of CENP-A is most often insufficient for maintaining, forming and propagating the centromere. In addition, observations have shown that while KT proteins require CENP-A for its localization, depletion of KT proteins do not affect the localization of CENP-A. On the other hand, over-expression of CENP-A results in mis-localization of KT proteins. Recent studies have found CENP-A to depend on other proteins for its localization at the centromere, suggesting CENP-A may not be alone at the top of the hierarchy.

1.4.2.2e.1 CENP-A structure and nucleosome organization.

Like all histones, CENP-A contains the globular histone fold domain (HFD), comprised of three alpha helices separated by loops (Morey, Barnes et al. 2004). Yet unlike all other histones CENP-A sequences are highly divergent across species. The N-terminal domain has been found to be hyper variable, hinting at its requirement to keep up with the rapid evolution of the underlying centromeric DNA (Malik and Henikoff 2001). CENP-A has also been found to have a CENP-A targeting domain (CATD), that helps with the loading of CENP-A onto the centromere, by directing the interaction of CENP-A with Scm3, a CENP-A chaperone **(Figure 7a)** (Stoler, Rogers et al. 2007, Zhou, Feng et al. 2011).

There are many highly debated topics related to CENP-A and although its presence at the centromere is undoubted and its domain architecture is understood, its nucleosome composition or the possible need of the CENP-A nucleosome at the centromere is not (Allshire and Karpen 2008). It has been found in several model systems that treating the centromeric chromatin to partial digestion with micrococcal nuclease yields an atypical smear pattern of digestion. Possible hypothesis state that CENP-A containing nucleosomes might allow for more accessible DNA ends there by leading to less precise termini.

The composition of the CENP-A nucleosome has possibly been reported to be rather different that canonical nucleosomes, with studies on the *S. cerevisiae*'s single CENP-A

nucleosome hinting at the lack of H2A-H2B, being replaced by Scm3, possibly forming a hexamer with $[CENP\text{-}A\text{-}H4]_2$ tetramer. While studies on the nucleosome containing CID, the *D. melanogaster* homologue of CENP-A, seems to indicate the composition to be CID-H4-H2A-H2B tetramer or half nucleosome in the interphase cells, the human CENP-A nucleosome composition was shown to be $[CENP\text{-}A\text{-}H4\text{-}H2A\text{-}H2B]_2$ an octamer. It is quite possible that the composition of the CENP-A nucleosome can vary not only through the progress of cell cycle but also across organisms **(Figure 7B)** (Allshire and Karpen 2008).

Based on these studies it was also observed that the CENP-A nucleosome is structurally distinct, forming a more rigid and compact interaction with H4, in turn a more compact nucleosome that wraps centromere DNA (Miell, Fuller et al. 2013). These findings have been contradicted with recent studies showing that nucleosome height of CENP-A do not differ from that containing canonical histone H3 (Codomo, Furuyama et al. 2014, Walkiewicz, Dimitriadis et al. 2014).

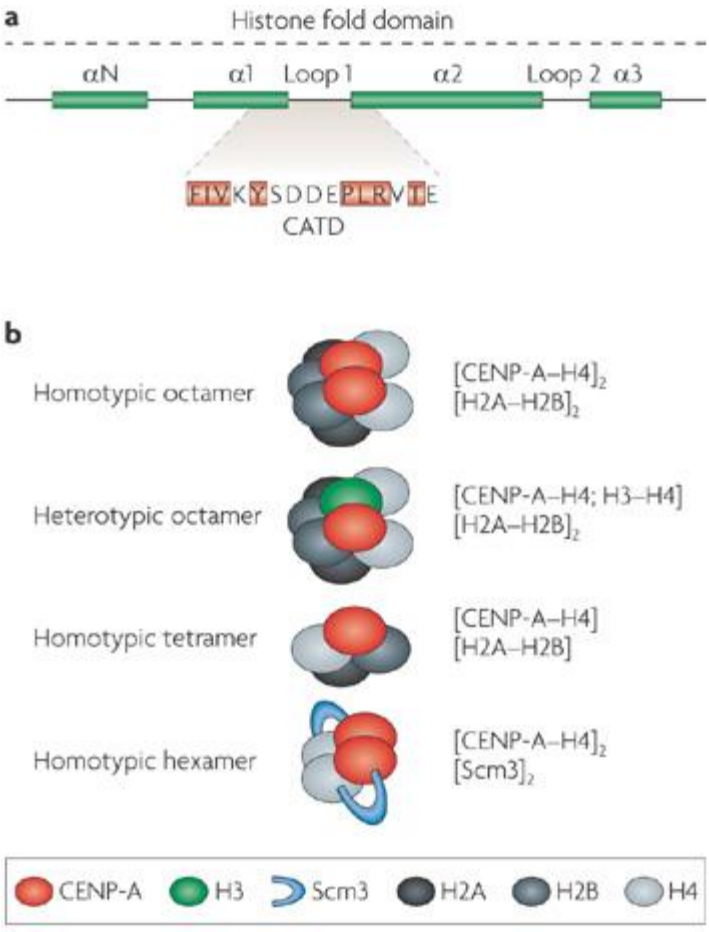

Figure 7. A) CENP-A domain architecture and B) potential nucleosome compositions (Allshire and Karpen 2008).

1.4.2.2e.2 CENP-A nucleosome deposition

Nucleosomes containing canonical histone H3 are loaded as the DNA is replicated in S phase. While the centromere replicates at early S phase in *S. cerevisiae* and mid to late S phase in fruit fly and humans, the CENP-A nucleosomes do not seem to be deposited during the same time, as concluded with canonical H3 organization during S phase (Shelby, Monier et al. 2000, Sullivan and Karpen 2001). CENP-A nucleosomes were found to segregate to sister chromatids after centromere replication, which would mean that CENP-A nucleosomes are diluted two fold (Stellfox, Bailey et al. 2013). Thus these gaps are to be filled or replenished with new CENP-A nucleosomes. It has been shown that the gaps are temporarily filled by histone H3.3, as place holders in S phase (Dunleavy, Almouzni et al. 2011). These findings led to the hypothesis that CENP-A nucleosome loading is replication independent; it was found that in fission yeast the CENP-A level is maximum during early S phase and CENP-A nucleosome is deposited either/both during S phase and G2, keeping in mind that in fission yeast G1 is exceedingly short (Kim, Dubey et al. 2003). In *Drosophila* CID assembly was found to occur during anaphase of mitosis in syncytial embryonic nuclear divisions, where cells cycle between S phase and M phase with nonexistent G1 and G2 (Black and Cleveland 2011).

The findings of CENP-A nucleosome deposition during cell cycle have a profound implication on the mechanics of centromere maintenance and propagation. It raises the question of what the status of centromeric chromatin is during periods of gap in CENP-A loading and also what the status of the molecular bridge that links the centromere with microtubules is, in these states of diluted and varying CENP-A nucleosomes.

1.5 Kinetochore

An important aspect of chromosome segregation involves the movement of chromosomes. Towards this aim of directed movement, chromosomes have to be linked at the centromere to a cellular force that can perform this function. While the microtubules provide the push-

pull force required to move cellular units, including the chromosomes, the linking of centromeric chromatin to the spindle microtubules is performed by a supra-molecular complex called the kinetochore. The kinetochore is a structure that forms on the centromere and is composed of more than 90 proteins in *S. cerevisiae.* The kinetochore, has several functions to carry out in addition to it forming the scaffold between DNA and microtubules, such as regulating sister chromatid cohesion, spindle assembly checkpoint, centromere maintenance and spindle alignment (Biggins 2013). The kinetochore was first identified as a layered structure which contained a dense inner plate that binds centromeric chromatin, an outer plate that contacts the spindle microtubules, a less compact middle linker layer and a " fibrous corona" that extends away from the outer plate (Ris and Witt 1981). Advances in proteomics have enabled the identification of a large number of kinetochore components (Connelly and Hieter 1996, Ortiz, Stemmann et al. 1999, De Wulf, McAinsh et al. 2003, Bock, Pagliuca et al. 2012, Schleiffer, Maier et al. 2012). Yet, while functions of the many kinetochore components have been well worked out, little is known about how they are recruited to the centromere or how they assemble to form the complex kinetochore structure. Other questions include how the timely assembly of the kinetochore is carried out and also how the dis-assembly of the kinetochore takes place.

1.5.1 Ultrastructure

For accurate chromosome segregation it is crucial that the kinetochore assembles on only one site on the chromosome. As previously mentioned, this site of assembly is marked by the presence of CENP-A nucleosomes. The timely deposition of CENP-A nucleosomes at the centromere is important for kinetochore assembly. The chromatin structure subsequently formed upon CENP-A depositions has also been implicated to play a role in kinetochore recruitment and assembly.

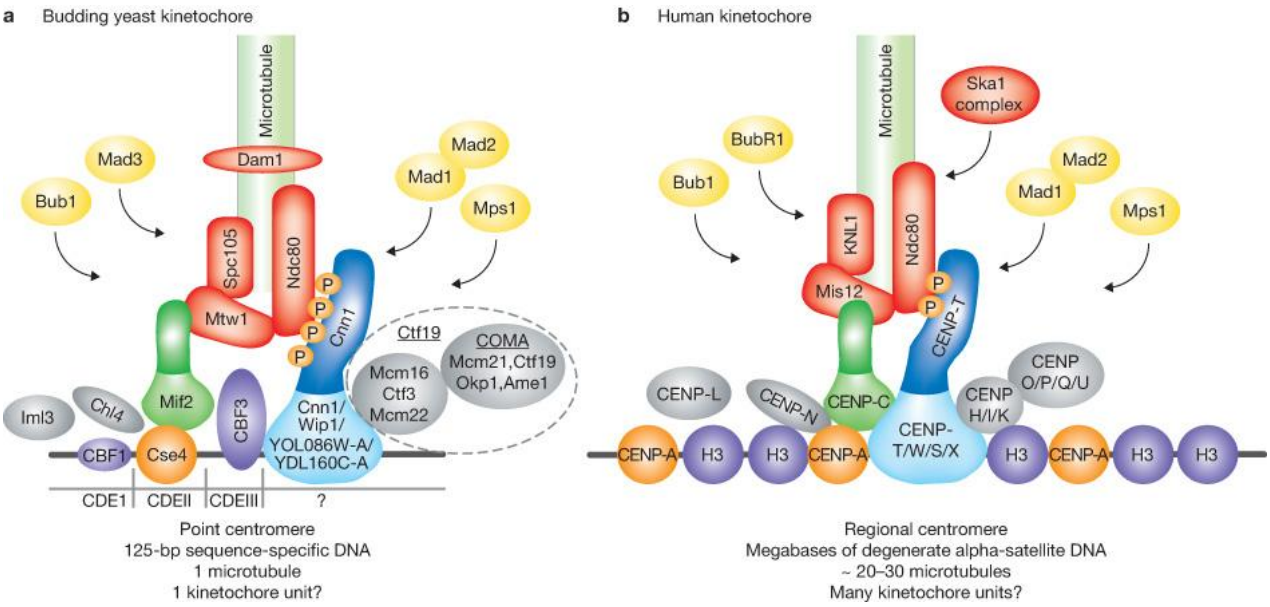

Figure 8. Evolutionary conservation of the gross kinetochore structure from yeast to humans (Gascoigne and Cheeseman 2012)**.**

The kinetochore constitutes three layers, the inner that binds centromeric chromatin, the outer that interacts and modulates microtubule dynamics and the middle layer that connects these two layers. In fungi, the outer kinetochore constitutes the exclusive complex, the Dam1. Recently the Ska complex has been shown to perform similar functions in humans, though it seems not to be homologues to the Dam1 complex **(Figure 8)** (Miranda, De Wulf et al. 2005, Westermann, Avila-Sakar et al. 2005, Hanisch, Sillje et al. 2006). Other protein complexes that bind the microtubule are highly conserved between yeast to humans, including the Ndc80, KNL1 complex and Mis12 complex which are collectively called the KMN network (Cheeseman, Chappie et al. 2006). Although some of the inner kinetochore proteins that show constitutive presence at the kinetochore, are highly conserved such as CENP-C/Mif2 and CENP-A/Cse4, other components of the constitutive centromere association network (CCAN) of proteins have only been implicated to be present at the centromeres of higher eukaryotes and are not sequence conserved. This notion was thrown back with the recent discovery, independently, by Bock and colleagues (Bock, Pagliuca et al. 2012) and Schleiffer and colleagues (Schleiffer, Maier et al. 2012) who through extensive bioinformatics studies identified the homologues of the CCAN network proteins in the budding yeast and several other organisms whose genomes were sequenced.

CENP-T is a key component of the vertebrate kinetochore, whose homologue has now been identified in budding yeast and predicted to exist in other fungi. It is crucial for interacting and forming a scaffold for the Ndc80 complex, which is the major load bearing structure of the kinetochore. Although these recent studies have brought about a revolution in thought amongst similarities that exist in the kinetochore architecture between yeast and humans, some differences do exist **(Figure 8)**. The budding yeast kinetochore contains a unique complex called CBF3 that binds to DNA in a sequence dependent manner (Lechner and Carbon 1991). Hence the requirement of the other CCAN network complexes for chromosome segregation may be less important in yeast. Interestingly, this is what was observed as the identified CENP-T and CENP-W homologues in yeast have been found to be non-essential for viability (Bock, Pagliuca et al. 2012, Schleiffer, Maier et al. 2012). These studies also implicated Ndc80 complex interactions with Mis12 and CENP-T to be mutually exclusive. In compliance, it was observed that artificially tethering CENP-T to a mini-chromosome is sufficient for segregation of a plasmid in *S. cerevisiae*.

1.5.2 Composition of the yeast kinetochore

1.5.2.1 Inner kinetochore

The "inner kinetochore" consists of proteins that are most closely associated with centromeric chromatin. This included the isolation of CENP-A and other associated proteins in vertebrates, identified as the CCAN network (Obuse, Yang et al. 2004, Foltz, Jansen et al. 2006, Izuta, Ikeno et al. 2006, Hori, Amano et al. 2008). This network consists of sub-complexes that include: CENP-C, CENP-H/I/K, CENP-L/M/N, CENP-O/P/Q/U and the histone-fold domain containing proteins CENP-T/W and CENP-S/X (McAinsh and Meraldi 2011, Perpelescu and Fukagawa 2011, Takeuchi and Fukagawa 2012). The inner kinetochore of yeast contains orthologous for most human CCAN proteins.

The CBF3 complex was the first yeast kinetochore sub-complex identified. This unique complex binds to budding yeast point centromeres in a sequence specific manner (Ng and Carbon 1987, Lechner and Carbon 1991). The complex contains Ndc10, Cep3, Ctf13 and Skp1. It has been shown that Cep3 has a zinc-cluster motif that is found in transcription factors, while Ndc10 has a structural similarity with tyrosine DNA recombinases (Dhawale and Lane 1993, Perriches and Singleton 2012). Once the CBF3 complex binds with the centromere, it is stably maintained.

Mif2/CENP-C was one of the initially identified members of the CCAN network and found to co-purify with CENP-A nucleosomes. Due to the extremely low sequence similarity of other CCAN network members, identification was not possible until recently (Schleiffer, Maier et al. 2012). Fluorescent measurements suggest that Mif2 dimerizes and subsequently binds to CDEII (Meluh and Koshland 1995, Meluh and Koshland 1997).

Other components of the yeast inner kinetochore include the COMA complex (Ctf19, Okp1, Mcm21 and Ame1), in addition to other interacting partners. With the exception of Okp1 and Ame1 the other components of this complex have been found to be non-essential; although knock-outs for these non-essential proteins result in increased chromosome segregation defects **(Figure 9)** (Sanyal, Ghosh et al. 1998, Cheeseman, Drubin et al. 2002, De Wulf, McAinsh et al. 2003, Joglekar, Bouck et al. 2006, Schleiffer, Maier et al. 2012).

In addition to CENP-A, CENPT/W and CENP-S/W have a histone fold domain which suggest that they could form novel nucleosome like structures (Bock, Pagliuca et al. 2012, Schleiffer, Maier et al. 2012).

1.5.2.2 Middle and Outer kinetochore components

Unlike the inner kinetochore proteins the distinction between middle and outer kinetochore proteins is less precise. The outer inner and middle kinetochore complexes primary play the role of microtubule binding and in providing the platform for interaction with inner kinetochore proteins. These complexes comprise of Mtw1/Mis12/MIND, Spc105/Knl1/Blinkin, Ndc80, Dam1/Dash as well as a plethora of non-essential components such as motor proteins and checkpoint components **(Figure 9)** (De Wulf, McAinsh et al. 2003, Pinsky, Tatsutani et al. 2003, Westermann, Cheeseman et al. 2003, Hornung, Maier et al. 2011).

The KMN network consists of Mis12 (Mtw1,Dsn1,Nnf1 and Nsl1 at stoichiometric ratios of 1:1:1:1 respectively) (Cheeseman, Drubin et al. 2002, De Wulf, McAinsh et al. 2003), Spc107(composed of Spc105 and Ydc532/Kre28 in ratios of 1:2) (Nekrasov, Smith et al. 2003, Pagliuca, Draviam et al. 2009) and the Ndc80 complex (present in ratios of 1:1:1:1 of Ndc80,Nuf2,Spc24 and Spc25) sub-complexes (McCleland, Gardner et al. 2003, Ciferri, De Luca et al. 2005, Wei, Al-Bassam et al. 2007). This network forms the core microtubule binding activity of the kinetochore. Components of the Ndc80 complex, Nuf2 and Ndc80,

contain a globular head with positively charged Calponin Homology (CH) domains that facilitate binding of this complex to the negatively charged microtubules. The two other components Spc24 and Spc25 form a globular head that interacts with the Mis12 complex (Okada, Cheeseman et al. 2006).

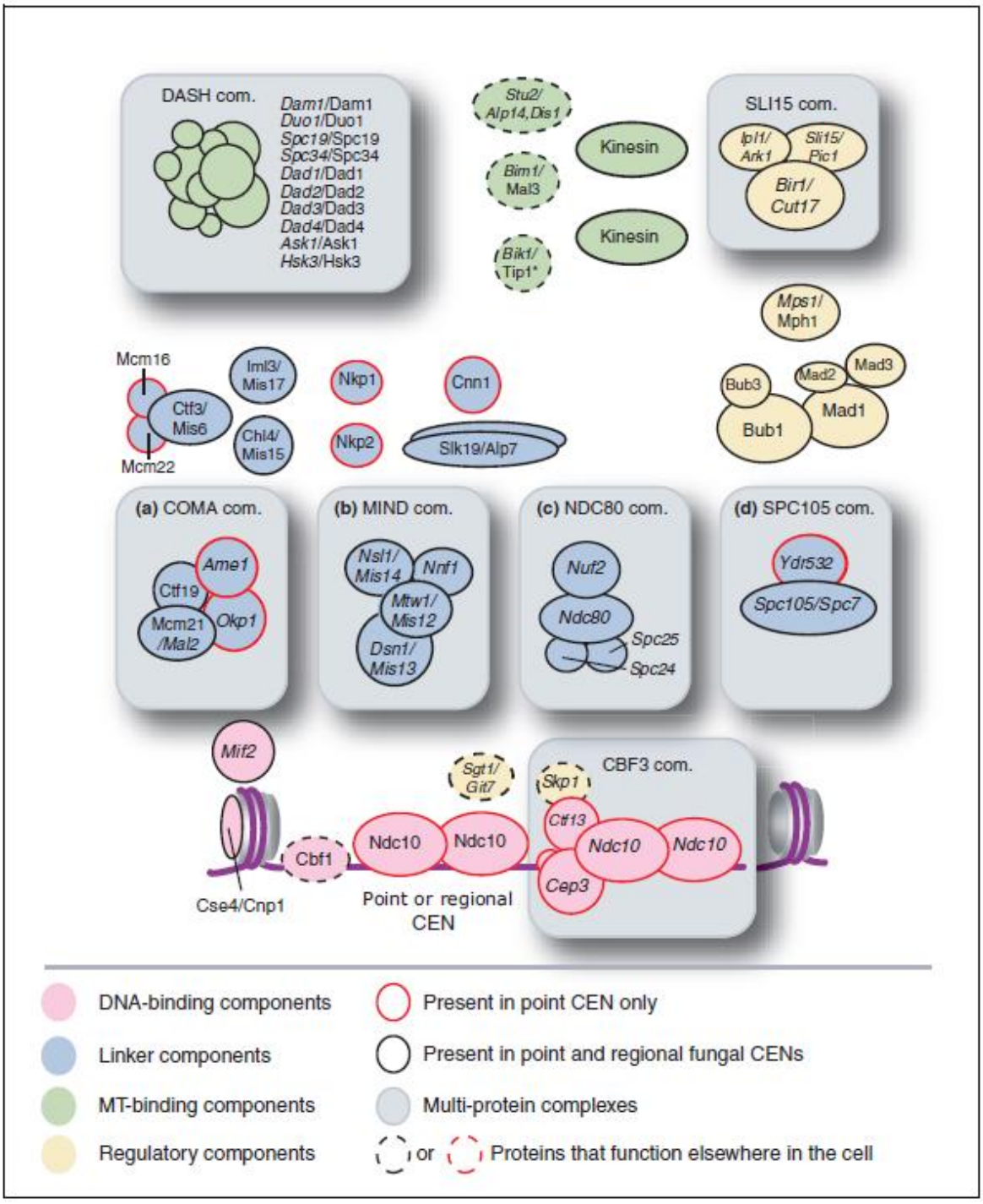

Figure 9. Composition of fungal kinetochores (Meraldi, McAinsh et al. 2006).

While the Spc105 complex has been found to weakly interact with microtubules it has been strongly implicated to be required for the recruitment of spindle assembly checkpoint proteins to the kinetochore (Cheeseman, Chappie et al. 2006).

The major component of the fungal outer kinetochore is the Dam1 complex, which comprises of Ask1, Dad1, Dad2, Dad3, Dad4, Dam1, Duo1, Hsk3, Spc19 and Spc34 **(Figure 9)** (Hofmann, Cheeseman et al. 1998, Cheeseman, Brew et al. 2001, Kang, Cheeseman et al. 2001, Westermann, Avila-Sakar et al. 2005). The entire complex can be reconstituted in vitro through bacterial expressed proteins with each protein present in single copy per complex; with 16 copies of the complex being able to form a ring of ~50nm in diameter (Westermann, Wang et al. 2006, Wang, Ramey et al. 2007). The interactions have been found to be predominantly electrostatic in nature, partially being mediated through N-terminus of Dam1 and possibly the Duo1 protein.

1.5.4 Timing of assembly and turnover of kinetochore proteins during mitosis.

The timing of kinetochore assembly in yeast and metazoans varies. In metazoans only the inner kinetochore, the CCAN network of proteins, is constitutively present at the centromere throughout the cell-cycle (Foltz, Jansen et al. 2006). The subsequent assembly involves the localization of the middle and outer kinetochore layers during mitosis. In contrast to this, the kinetochore proteins of all layers in the budding yeasts *S. cerevisiae and C. albicans* are constitutively present at the centromere (Meluh and Koshland 1997, Sanyal and Carbon 2002, Thakur and Sanyal 2011). While in the fission yeast, *S. pombe,* all except the outer kinetochore protein complex Dam1 are constitutively present; the Dam1 complex localizes to the centromere only during mitosis (Liu, McLeod et al. 2005, Sanchez-Perez, Renwick et al. 2005). This constitutive nature of the yeast kinetochore persists all throughout cell cycle, except for a short duration during S phase when the centromere is duplicated. The completely assembled kinetochores in yeast are also attached to microtubules during these periods.

The kinetochore proteins are highly dynamic, extensive fluorescence recovery after photo-bleaching (FRAP) studies were carried out to address this. It was found that spindle assembly checkpoint proteins that associate with the kinetochore, such as Mad2 have a

half-life of only a few seconds at the kinetochore, while, in contrast the kinetochore components such as the Ndc80 complex and Mif2 have been shown to remain immobile throughout mitosis. Cellular pools of Mis12, CENP-C and CENP-B were shown to have relatively short half-lives at the kinetochore (Shah, Botvinick et al. 2004, Vink, Simonetta et al. 2006, Hemmerich, Weidtkamp-Peters et al. 2008).

Post-translational modifications of kinetochore proteins have also been implicated in playing a major role controlling the dynamics of proteins at the kinetochore. These modifications predominantly being phosphorylation carried out by numerous kinases present during mitosis, such as CDKs and Aurora B (Joglekar, Bouck et al. 2006). The localization and stability of kinetochore proteins at the kinetochore is chiefly dictated by the presence of other kinetochore proteins that assemble on the kinetochore.

1.5.5 Interdependency amongst proteins of the kinetochore

Although the gross architecture of the kinetochore has recently been implicated of being similar and conserved from yeast to humans **(Figure 8)**, it was noticed that the recruitment and localization of various kinetochore proteins to the centromere was dependent on the occupancy of other kinetochore proteins. This interdependency is highly variable across species **(Figure 10)**. A popular mode of analysis of this interdependency is to make use of conditional mutants, where by studying the status of the kinetochore in a non-permissive mutant condition, by microscopy or chromatin immuno-precipitation (ChIP).

Although it is known that CENP-A and its chaperone Scm3 establish centromere identity, in *S. cerevisiae* kinetochore formation is initiated by the CBF3 complex. CENP-A together with the CBF3 complex recruits CENP-C and COMA complexes to the kinetochore (Meluh and Koshland 1997, De Wulf, McAinsh et al. 2003, McAinsh, Tytell et al. 2003). This partially assembled kinetochore is then required by Chl4, Iml3 and the Ctf3 complex for their kinetochore localization. The presence of the COMA complex is also a pre-requisite for the localization of the MIND complex, while the Spc105 and the Ndc80 complexes localize to the centromere independent of the CENP-A but dependent on the CBF3 complex **(Figure 10)**.

Unlike the requirement of CENP-A for CENP-C recruitment in *S. cerevisiae*, CENP-C and CENP-A are mutually required for the other's localization in *S. pombe*. The KMN network proteins in *S. pombe* are interdependent of each other's presence and Mis6 for their kinetochore localization. On the other hand, different subunits of the Dam1 complex have varied requirements for their kinetochore recruitment (Liu, McLeod et al. 2005).

In contrast to these systems where a distinct hierarchal system of kinetochore assembly exists, it was observed in *C. albicans* that the entire kinetochore dis-assembled even when a single essential kinetochore protein was deficient (Thakur and Sanyal 2012). In other words, a kinetochore protein required the presence of all other kinetochore proteins for its centromeric localization.

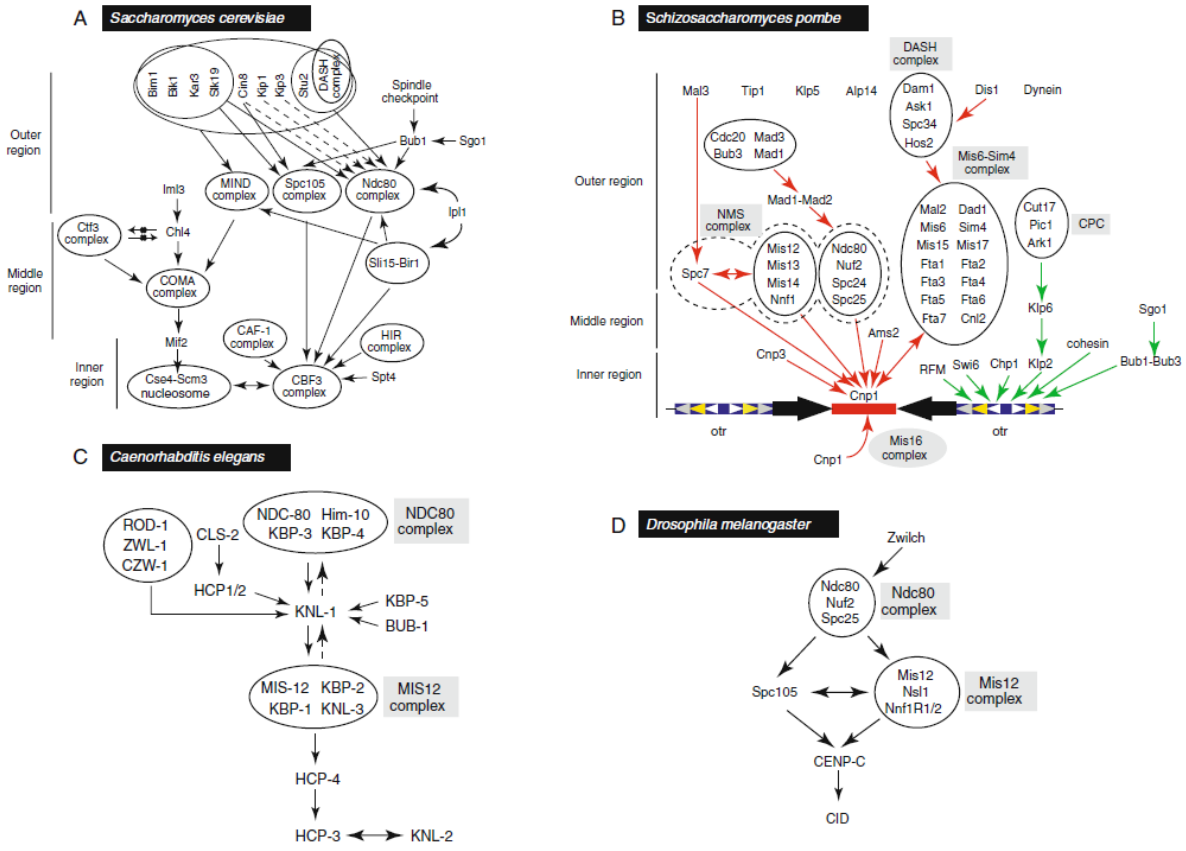

Figure 10. Assembly and interdependency of kinetochore proteins in nonvertibrate organisms (Earnshaw 2009) **.**

Preliminary results in higher eukaryotic systems, point to a hierarchal assembly of kinetochore proteins which predominantly require the CENP-A for their assembly **(Figure 10A and Figure 10B)**.

1.5.6 Clustering of kinetochore is yeast.

In mammalian cells, the centromers of interphase chromatids are spread out uniformly throughout the nucleus with the middle and outer kinetochore layers assembling after the onset of mitosis. Dissimilar to higher eukaryotes, individual budding yeast kinetochores and centromeres are not visible due to its small size. In this system however, kinetochores have been found to cluster throughout cell cycle with a metaphase plate being absent (Straight, Marshall et al. 1997, Jin, Fuchs et al. 2000). The clustered kinetochores are found at the periphery of the nucleus and have been shown to be always associated with microtubules in *S. cerevisiae*. While in *S. pombe* it was observed that the kinetochore only cluster during interphase (Funabiki, Hagan et al. 1993).

1.5.7 Regulation of kinetochore microtubule attachments

The assembly and formation of a complete kinetochore at the centromere is just the beginning of its proposed function: to interact with spindle microtubules and couple the centromere with the push-pull forces of the mitotic spindle. The nature of KT-MT interactions varies during various stages of cell cycle; it starts with the initial capture, followed by progression towards bi-orientation and finally congressing at pro-metaphase; subsequently resulting in separation of sister chromatids during anaphase. During this process, the state of KT-MT attachment is constantly being monitored by the spindle assembly checkpoint (Tanaka, Mukae et al. 2005).

1.5.8 Mitotic spindle

The goal of mitosis is to segregate chromosomes to two daughter cells, a feat that is accomplished by a dynamic macromolecular machine called the mitotic spindle. The mitotic spindle consists of microtubules, in addition to many associated factors which form an anti-parallel, bipolar array. The key components of the mitotic spindle are microtubules (α and β subunits) that arrange in a head-to-tail configuration, with β subunits more at the dynamic plus ends **(Figure 12A)**. The growth and shrinkage of the microtubules provide

the movement force, which is a resultant of addition and loss of tubulin dimers at their ends. This dynamic instability of microtubules result from GTP hydrolysis within the β subunit (Kline-Smith and Walczak 2004).

The organization of the spindle microtubule can be described as an antiparallel array, with the minus ends located at the spindle poles while their plus ends extend outward. There are three types of spindle microtubules depending on the interactions they perform and the direction they extend towards, a) kinetochore microtubules (kMTs) that extend and make contacts with the kinetochores of sister chromatids; b) inter-polar microtubules which form an overlapping array of central spindle and c) astral microtubules that propagate outward toward the cell cortex and are required for the movement of spindle poles into daughter cells **(Figure 12B)**.

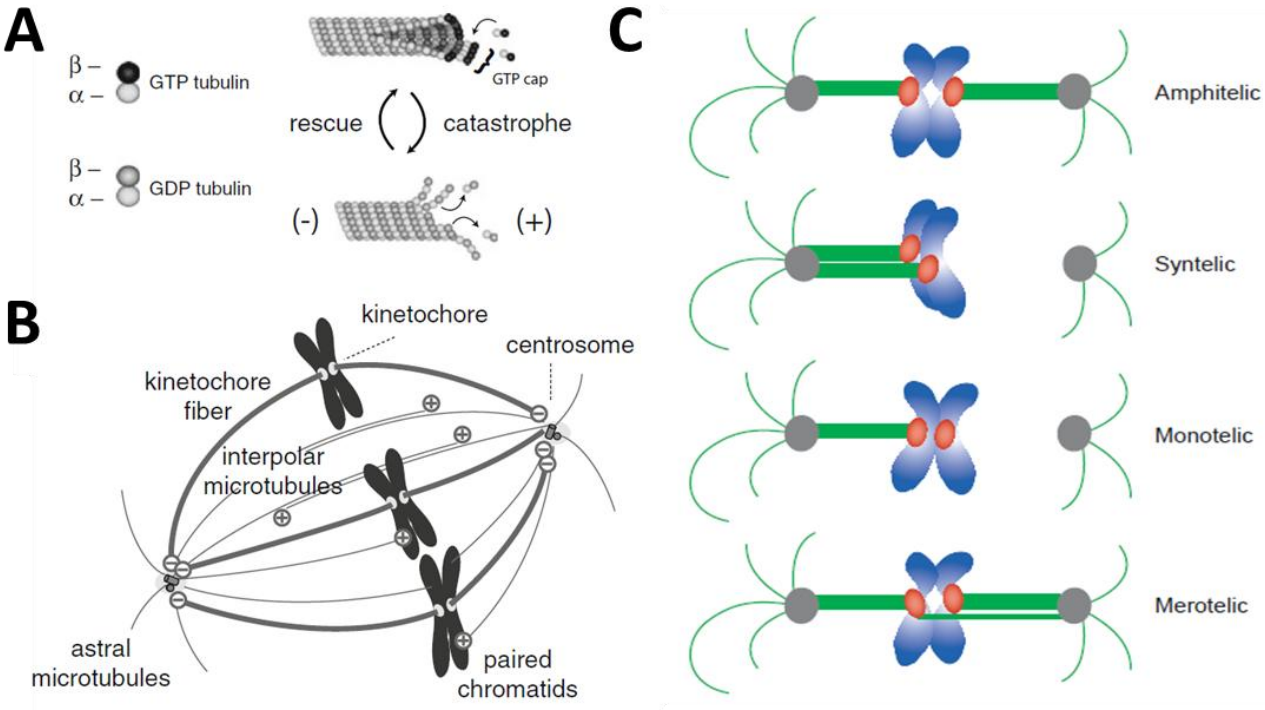

Figure 12. The key components of the mitotic spindle and the attachments made with kinetochores (Pinsky and Biggins 2005, Earnshaw 2009)**.**

The complex movements orchestrated by the mitotic spindle are greatly aided by a vast number of kinesin and dynein motor proteins that modulate spindle dynamics.

1.5.9 KT-MT interphase

In budding yeast the major microtubule binding complexes have been found to be the Ndc80 and the Dam1 complexes. Unlike the Ndc80 complex, the Dam1 complex requires microtubules for its kinetochore localization. Consistent with these observations, it was found that the Ndc80 complex is required for both end-on attachments and lateral attachments; while the Dam1 complex was found to be required only for proper end-on attachments. Elegant in vitro experiments showed that the Dam1 complex forms a ring that enhances the microtubule tip tracking activity of the Ndc80 complex, under load. The Ndc80 complex forms globular head structures, comprising positively charged CH domain, which help it interact with negatively charged microtubules (Maiato, DeLuca et al. 2004, Cheeseman and Desai 2008). In addition to the Ndc80 and Dam1 complexes, many others have been implicated in KT-MT interactions, such as the Spc105 and COMA complexes (Joglekar, Bouck et al. 2006).

1.5.10 Types of attachment

The flawless execution of chromosome segregation requires that the sister chromatids be attached to spindle microtubules, growing outwardly from opposite poles. Although this bipolar attachment is essential, it is achieved rather randomly. This imperfect process can result in several types of incorrect KT-MT attachments that could lead to mis-segregation. Amphitelic attachments or bi-orientation occurs when the kinetochore on opposite facing sister chromatids bind to only the spindle pole it is facing. In contrast monotelic attachments occur when only one of the kinetochores on a sister chromatid bind to one of the spindle poles. While syntelic attachments are defined as KT-MT attachments when both sister kinetochore bind to microtubules emanating from one spindle pole. Monotelic and syntelic attachments are collectively known as mono-oriented. Finally, merotelic attachments take place when either or both sister kinetochores bind microtubules that arise from both poles even though they orient in opposite directions **(Figure 12C)** (Pinsky and Biggins 2005).

It is of utmost importance to the cell that erroneous attachments are corrected and only bi-oriented chromosomes are allowed to go through mitosis. To ensure this, there exists a checkpoint at the transition between metaphase to anaphase, called the spindle assembly checkpoint.

1.6 Spindle assembly checkpoint

The spindle assembly checkpoint (SAC) is a detection and correction system that ensures bi-orientation of chromosomes, resulting in accurate chromosome segregation. The SAC detects both tension and KT-MT attachment defects at the centromere. Although much of the specific events occurring at the SAC are highly debatable, the gross outlines of the signaling and downstream effectors have been well worked out **(Figure 13)**.

Upon detection of an erroneous attachment at the kinetochore, the aim of the SAC is to halt progression of mitosis from metaphase to anaphase. This is carried out by sequestering the pre-requisites for this transition; which are factors (Cdc20) that involve the activation of the anaphase promoting complex (APC) and subsequent degradation of mitotic cyclin **(Figure 13)** by the mitotic checkpoint complex (MCC) (Fang, Yu et al. 1998).

The anaphase promoting complex is a U3 ubiquitin ligase that upon binding Cdc20 is activated and ubiquitinates the anaphase inhibitor, securin , targeting it for degradation by the 26S proteasome machinery (Reddy, Rape et al. 2007). This cascade of events in turn helps in releasing separase to cleave the cohesion present between sister chromatids that allows segregation. APC also ubiquitinates the mitotic cyclin, leading to mitotic exit (Lara-Gonzalez, Westhorpe et al. 2012).

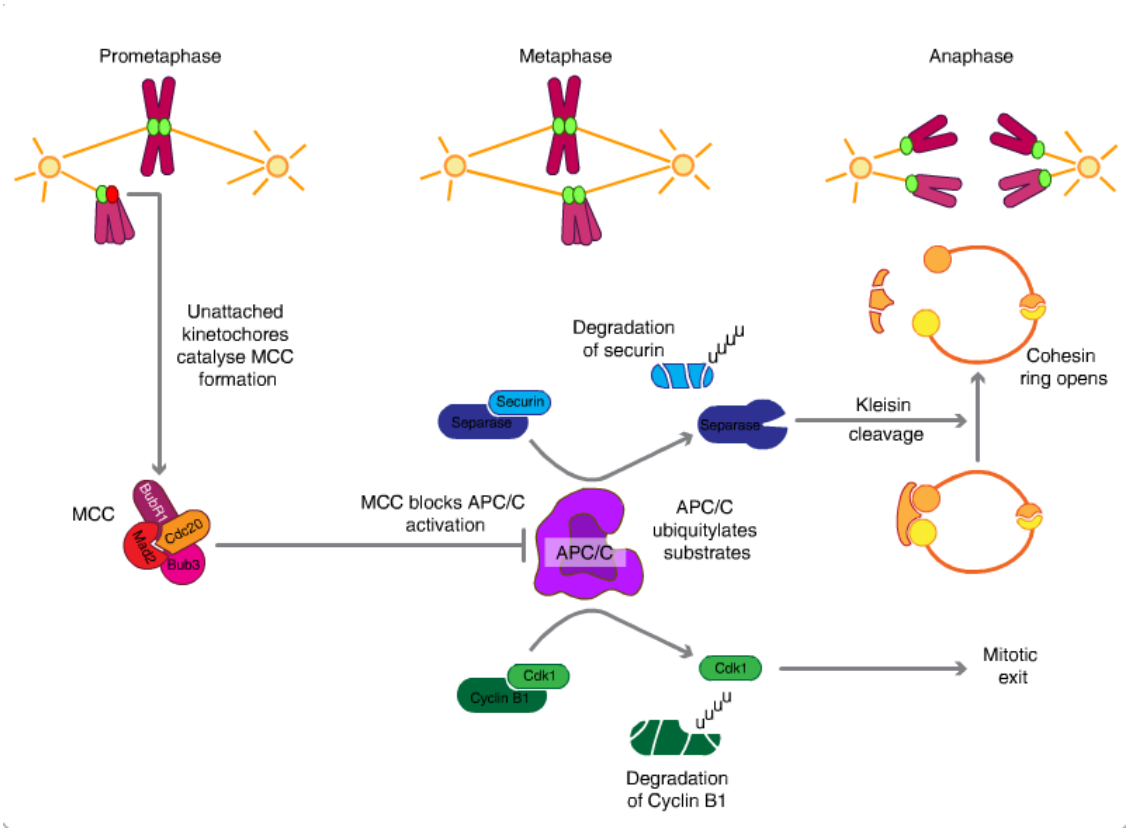

Figure 13. Outline of the conserved spindle assembly checkpoint (Lara-Gonzalez, Westhorpe et al. 2012)**.**

This signal for the sequestration of Cdc20 by the MCC is turned on when the upstream components of the SAC detect the presence of erroneous KT-MT attachments. Until recently this mechanism was not known. It is now hypothesized that the structural change in the kinetochore protein Knl1 recruits Bub1, which is then followed by Mad1 recruiting Mad2 to the kinetochore. The signaling is brought about by the cycling of Mad2 between its open and closed forms. Mad2 is bound to Mad1 in the closed form. This closed form behaves as a prion and converts free open Mad2 into the closed form. The closed, then becomes a part of the MCC leading to the sequestering of Cdc20. The SAC, in addition to sensing the KT-MT attachment by the Knl1 complex, has also been implicated to sense proper tension aided by Aurora B kinase (Rosenberg, Cross et al. 2011, Espeut, Cheerambathur et al. 2012, Shepperd, Meadows et al. 2012). Aurora B activates the SAC by phosphorylating several middle and outer kinetochore proteins which in turn recruit the SAC components on to the kinetochore.

Several mysteries within the SAC are yet to be resolved for understanding the precise mechanism of recruitment of SAC proteins at the kinetochore, and also to understand the mechanism of SAC activation by unattached kinetochore. Also several questions related to the role of the kinetochore scaffold and the role the kinetochore proteins play in the SAC are yet to be thoroughly dissected.

1.7 *Cryptococcus neoformans*, basidiomycete, human fungal pathogen.

Kingdom: Fungi
Phylum: Basidiomycota
Class: Termellomycetes
Order: Termellales
Family: Tremellaceae
Genus: *Cryptococcus*
Species: *Cryptococcus neoformans*

C. neoformans is a basidiomycete fungus, making it evolutionary distinct from other well studied ascomycetous fungus such as *S. cerevisiae, S. pombe and C. albicans. C. neoformans* is closely related to other basidiomycetes such as *Ustilago maydis* than budding yeasts*. C. neoformans* and *S. cerevisiae* have diverged ~500 million years ago; thus studies in this organism have the potential to reveal molecular mechanisms that make them unique as well as establish conserved mechanisms **(Figure 14)**.

C. neoformans is a human fungal pathogen that is known to infect the central nervous system causing meningoencphalitis, which is uniformly fatal if untreated. It is primarily an opportunistic pathogen but can also be a primary pathogen in patients with a compromised immune system. A majority of fatalities due to *C. neoformans* infections are caused in immunocompromised patients predominantly in Africa. This pathogen is currently the fifth largest killer in the world, with more than half a million deaths annually (Srikanta, Santiago-Tirado et al. 2014). Exposure of humans to this pathogen mainly occurs via inhalation of basidiospores formed and released during mating or haploid fruiting (Lin and Heitman 2006).

C. neoformans is found ubiquitously in the environment and is most often associated with pigeon guano or certain tree species. The organism exists as two distinct groups, one that is predominantly found in temperate climates (serotype A and D), which co-incidentally also accounts for the most infections; and serotype B and C that is restricted to the tropical regions.

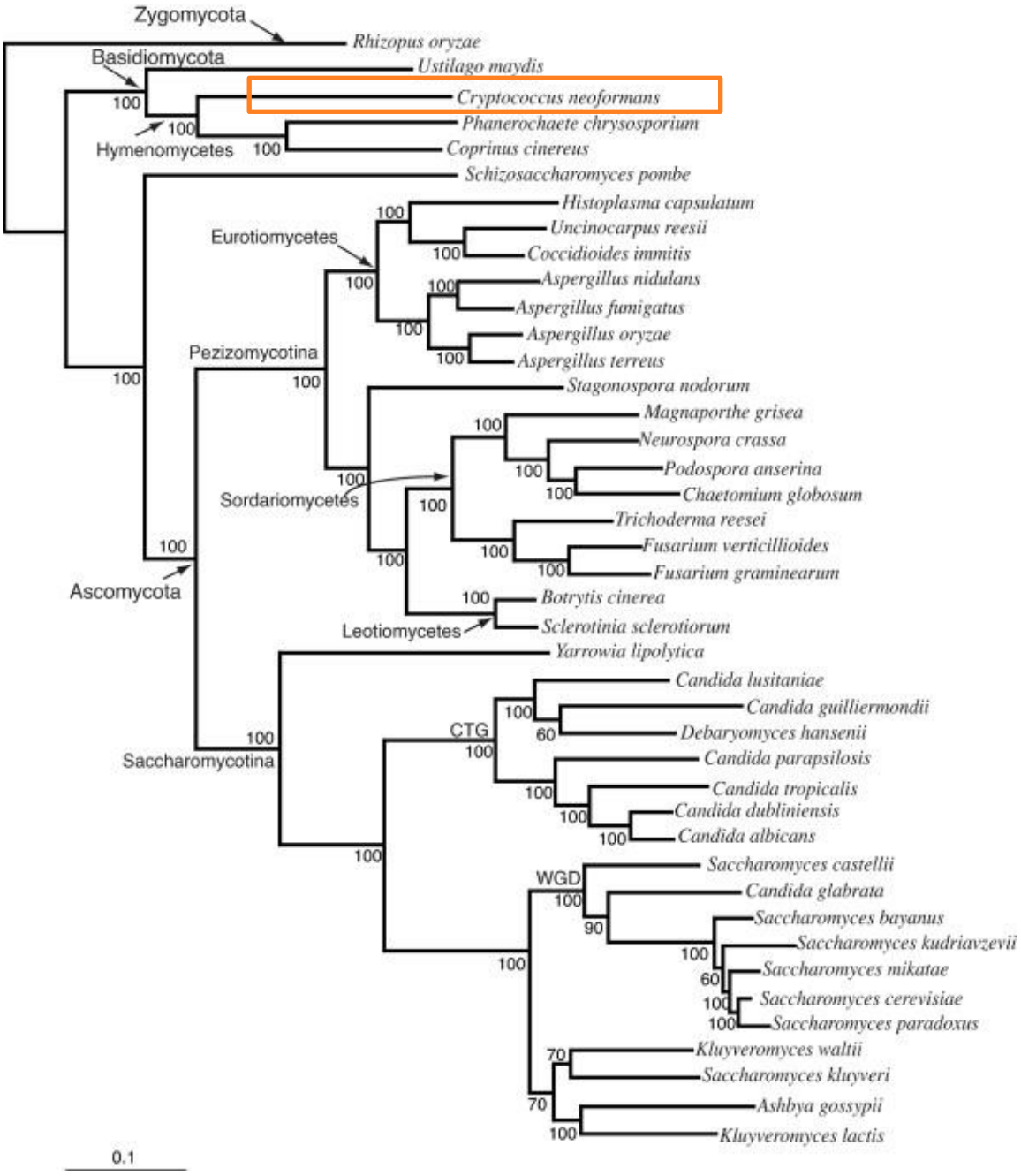

Figure 14. Phylogenetic tree showing the evolutionary distinct fungal phylum basidiomycota, highlighted is *C. neoformans* (Fitzpatrick, Logue et al. 2006)**.**

This fungal pathogen has a defined life cycle, which involves vegetative growth as budding yeast in addition to having the ability to undergo filamentous dimorphic transitions. *C. neoformans* generally exists as haploid budding yeast in the environment with **a** and *α* mating types. Under appropriate conditions of nutrient limitation and stimulation by mating pheromones, the two mating types produce conjugation tubes resulting in cell fusion. Unlike other model yeasts where nuclear fusion occurs immediately, karyogamy is delayed in basidiomycetes and this resulting heterokaryon adopts filamentous growth, into which both parent nuclei migrate (Hull and Heitman 2002). Subsequently a septum forms and one nucleus is transferred to the penultimate hyphal cell via a clamp connection, resulting in fusion between clamp and hyphal cell. Ultimately basidia are formed within which nuclear fusion and meiosis take place. Spores are subsequently formed on the surface of the basidia. In parallel to this process, cells of the α mating type in response to nitrogen starvation and appropriate pheromone signaling can differentiate by a process known as haploid fruiting, leading to filament formation and sporulation **(Figure 15)** (Lin and Heitman 2006).

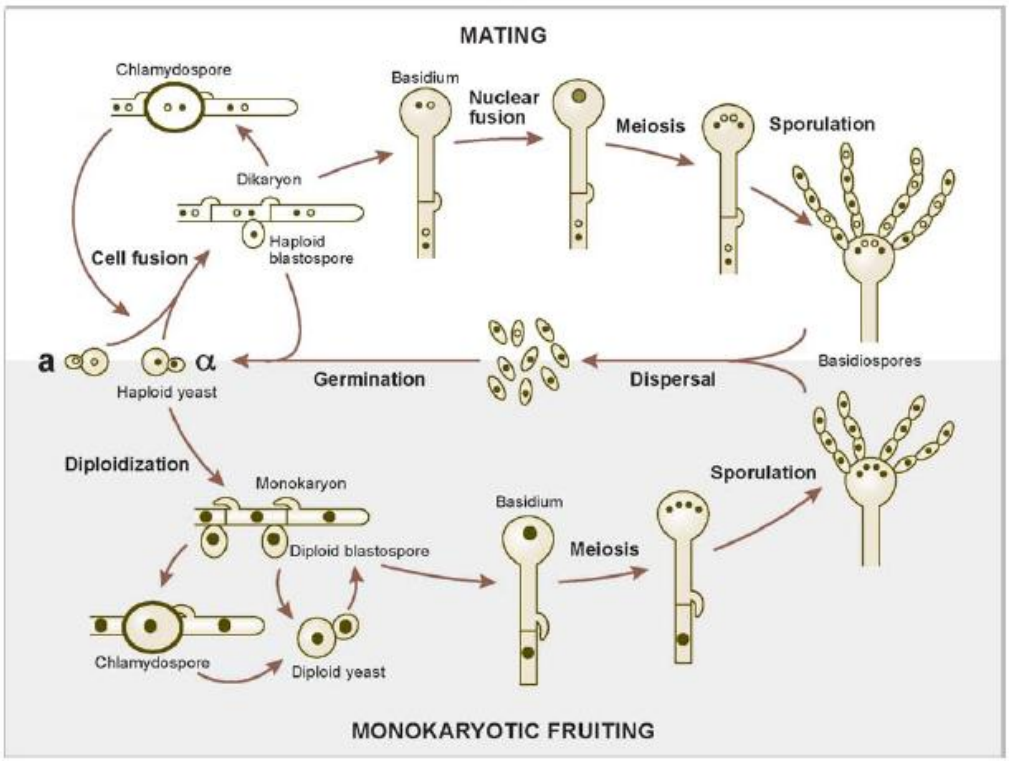

Figure 15. Model of the *C. neoformans* cell cycle (Lin and Heitman 2006)**.**

C. neoformans being evolutionary distinct to other well studied yeast, a troublesome pathogen and evidence of several promising features made us select this basidiomycete as a model system for our studies on chromosome segregation .

To study several essential pathways we utilized the *GAL7* promoter to create conditional mutants. The *GAL7* promoter drives the expression of Galactose-1-phosphate uridlytransferase that converts galactose-1-phosphate to glucose-1-phosphate, a key step in the utilization of galactose. The enzyme is part of the Leloir pathway that catalyzes the conversion of galactose into glucose-6-phosphate. This pathway is activated when glucose is unavailable as a carbon source and galactose is. The concerted action of Gal4, Gal80 and Gal3 with galactose and ATP drive the rapid and high level expression of genes involved in this pathway. The genes encoding the enzymes are tightly regulated at the level of transcription (Ory, Griffith et al. 2004, Baker and Lodge 2012).

Rationale and objectives of the study

The current understanding of the kinetochore architecture is developed, as gleaned from only a few model systems. Although it has been recently found that the gross architecture of a kinetochore is conserved between yeasts to humans, a lot of variation exists with regard to the protein requirement for kinetochore assembly and the process of assembly itself.

In the well-studied ascomyceteous yeasts, *S. cerevisiae* and *C. albicans*, it was observed that they undergo closed mitosis and their kinetochore being constitutively present throughout cell cycle on the centromere. On the other hand, in metazoans, it was seen that the cells undergo open mitosis (the nuclear envelope breaks down) and the kinetochore assembly takes place post-mitotic onset

Much analysis of mitosis has been restricted to ascomyceteous yeast and metazoans hence to better understand mitosis in an evolutionary distinct organism, we choose *C. neoformans*; an evolutionarily unique fungus that belongs to the phylum basidiomycota. During the time of this study we observed that the process of the mitosis in *C. neoformans* involved assembly of the kinetochore concordantly with the partial breaking of the nuclear membrane and clustering of chromosomes, exclusively during mitosis. This process was more similar to the mechanism in known metazoans than other budding yeasts. The observed assembly of the kinetochore in *C. neoformans* also made it possible to score for each step of the assembly process; inner, middle and outer layers.

Another intriguing prospect of the fungal kinetochore was the existence of a fungal specific, microtubule binding, outer kinetochore complex known as the Dam1 complex. This being distinct to fungi could possibly be used as a therapeutic target. The function of the Dam1 complex was found to be associated with maintaining KT-MT interactions, more importantly in fungus that had only one kMT per centromere. Would this property hold true across fungal phylum?

Spurred by these questions and a promising model organism in hand, our objectives were:

1) To understand the spatio-temporal regulation of kinetochore assembly.
2) Understand the interdependency and requirement of various kinetochore proteins for their centromeric localization, which would shed light on the kinetochore architecture.
3) Investigate the essentiality and requirement of the fungal specific Dam1 complex in this pathogenic fungus.
4) To investigate the requirement of the kinetochore for spindle assembly checkpoint function.

Results

A. Defining kinetochore architecture (inner, middle and outer): study of distinct spatial-temporal organization of the microtubule binding protein complexes, the Ndc80 and Dam1 complex, in *Cryptococcus neoformans var. grubii.*

Budding yeasts have been reported to have a distinct mode of kinetochore (KT) assembly (Meluh and Koshland 1997, Hofmann, Cheeseman et al. 1998, Westermann, Cheeseman et al. 2003, Shah, Botvinick et al. 2004, Gascoigne and Cheeseman 2012, Biggins 2013). A kinetochore assembles early in the cell cycle and remain attached to microtubules throughout cell cycle, but for a small duration during S phase when centromeric chromatin is duplicated (Kitamura, Tanaka et al. 2007). Two unique features of the mitotic cycle; closed-mitosis and the presence of spindle pole bodies (SPB) on the nuclear membrane enable the formation of kinetochore-microtubule (KT-MT) interactions within an intact nucleus (Cheeseman and Desai 2008). It is also noteworthy that these observations have been exclusively made upon studying ascomycetes, *S. cerevisiae* and *C. albicans.* Basidiomycetes phylum of fungi constitutes nearly a third of all fungal species known, containing only few known yeasts, one of them being *C. neoformans.* Unfortunately, studies on mitosis and chromosome segregation in basidiomycetes yeasts are nearly absent. To explore the dynamics of kinetochore assembly in *C. neoformans*, previously we uncovered several promising features such as partial opening of the nuclear membrane during mitosis and presence of un-clustered centromeres throughout interphase followed by clustering during mitosis in *C. neoformans* (Kozubowski, Yadav et al. 2013). *C. neoformans* is also highly pathogenic and hence gaining insights into its mode of chromosome segregation would aid towards its combat as a pathogen.

A1. The NDC80 complex: a conserved microtubule interacting and load bearing structure of the kinetochore localizes to the centromere shortly after the onset of mitosis.

The kinetochore is a supra-complex of proteins whose primary function is to link centromeric chromatin with the push-pull force of the mitotic spindle. One of the microtubule binding structures has evolved into a ~570Å long rod with globular ends (Ciferri, De Luca et al. 2005) : the Ndc80 complex. The Ndc80 complex is composed of a tetramer containing equimolar ratios of Ndc80, Nuf2, Spc24 and Spc25. In order to understand the dynamics of localization of the Ndc80 complex at the centromere we identified the *C. neoformans* homologues. *S. cerevisiae* proteins, ScNuf2 and ScNdc80, were used as query sequences for BLAST analysis against *C. neoformans var. grubii* genome database (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html). We subsequently tagged CnNuf2 (SHR 504) and CnNdc80 (SHR506) with a fluorescent reported tag, green fluorescent protein (GFP), using the pCIN19 plasmid construct, at the N-terminal and integrated the cassette at an ectopic loci in the genome. CnGFP-Nuf2 and CnGFP-Ndc80 did not localize similar to previous reports. CnGFP-Nuf2 exhibited a localization pattern similar to that of tubulin and CnGFP-Ndc80 was observed to be omnipresent in the cell with no specific localization. As these constructs did not replace the native copy, expressed by a constitutive H3 promoter and tagged at the microtubule binding globular domain of the protein, these observations were not subsequently pursued although the localization of CnGFP-Nuf2 was intriguing. To overcome these drawbacks of using the pCIN19 plasmid, we made plasmid constructs (GFP-pS2GN and mCherry-pSS02) to tag and replace the native copy of the gene with the fluorescently tagged version. Using these constructs CnNuf2 and CnNdc80 were functionally fluorescent tagged with GFP and mCherry respectively at the C-terminal.

The localization dynamics of Nuf2-GFP and Ndc80-mCherry was then studied by confocal microscopy using strains SHR515 and SHR512 respectively. It was observed that both proteins localized as a single dot only upon onset of mitosis. Gradually through the process of mitosis the single focus divided in the daughter bud and subsequently migrated to the

ends of both daughter buds. Upon completion of anaphase and prior to cytokinesis the localization of Nuf2-GFP and Ndc80-mCherry faded and was no more detectable. Although the individual protein localizations have not been shown, localization in a strain expressing of Nuf2-GFP and Ndc80-mCherry (SHR510) is shown (**Figure A1A)**.

To validate if the Ndc80 complex proteins were localizing at the conventional centromeric location, Nuf2-GFP was co-localized with centromeric marker, Cse4/CENP-A-mCherry (SHR514) **(Figure A1C)**. The dot-like signals of Nuf2-GFP co-localized with those of Cse4-mCherry throughout its appearance during mitosis, indicating that the Ndc80 complex in *C. neoformans* may be localizing to the kinetochore similar to reports in other organisms (McCleland, Gardner et al. 2003, Maiato, DeLuca et al. 2004, Cheeseman, Chappie et al. 2006, Perpelescu and Fukagawa 2011).

These observations strongly hinted at a temporal localization of the Ndc80 complex at the centromere which is distinct with respect to Cse4. To corroborate these results we localized Nuf2-GFP with Mtw1-mCherry, a conserved protein, reported to be part of the middle kinetochore. In the same study, Mtw1 was shown to be transiently localizing at the centromere similar to the Ndc80 complex **(Figure A1B)** (Kozubowski, Yadav et al. 2013). In addition, it was observed that the predicted Ndc80 complex proteins, Nuf2 and Ndc80, co-localized, suggesting that they could be part of the same complex (Figure A1A). Moreover, the stage specific localization of the Ndc80 complex at the centromere may aid in classifying the architecture of the kinetochore based on both function and the timing of localization in *C. neoformans* **(Figure A1D)**.

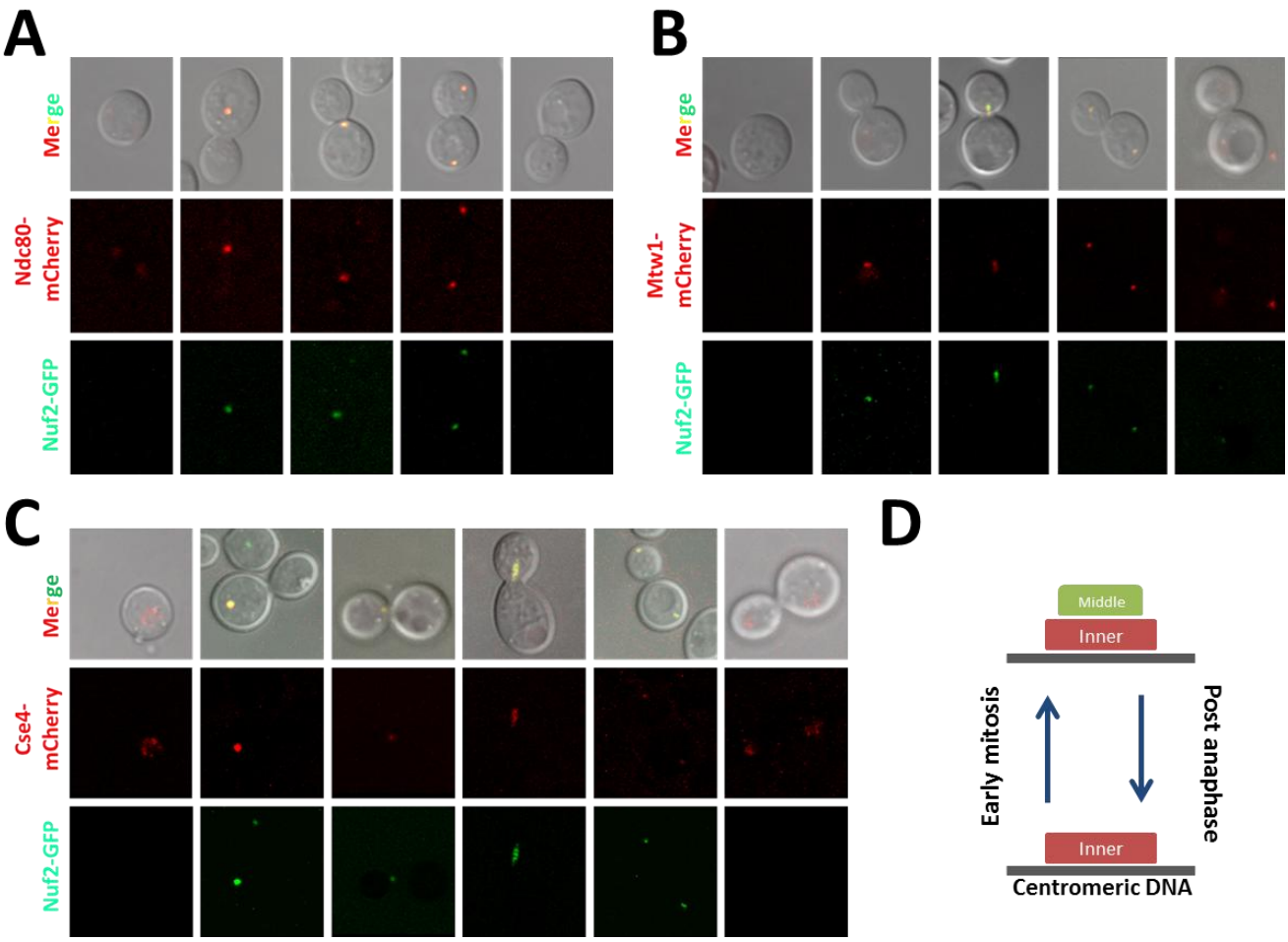

Figure A1. Fluorescent tagged proteins of the Ndc80 complex localize transiently at the centromere during mitosis. A) Ndc80 complex proteins, Nuf2-GFP and Ndc80-mCherry concomitantly appear as single dot during the onset of mitosis and disappear upon completion of anaphase, prior to cytokinesis. B) Nuf2-GFP co-localizes with Mtw1-mCherry, another proposed middle kinetochore protein. C) The Ndc80 complex proteins co-localize with the centromeric marker Cse4. D) Cartoon representing the transient localization of the Ndc80 complex (considered middle with respect to timing of localization).

A2. Dam1 complex subunit, Dad2 localizes to the centromere post-Ndc80 complex localization.

To better understand the localization of microtubule binding kinetochore proteins of *C. neoformans*, we identified CnDad2 (using ScDad2 homologue) a subunit of the fungal specific microtubule binding Dam1 complex, by BLAST analysis. In *S. cerevisiae* it was predicted that Dad2 location in the architecture of the kinetochore was further away from centromeric chromatin than the Ndc80 complex (Joglekar, Bouck et al. 2006).

CnDad2 was GFP-tagged at the N-terminal using the pCIN19 vector construct and integrated ectopically in the genome (SHR507). The localization was monitored using confocal microscopy. The localization dynamics observed were similar to the previously mentioned Ndc80 complex proteins, Nuf2-GFP and Ndc80-mCherry, where Dad2 localized as a single dot only during mitosis and disappeared soon after anaphase, prior to cytokinesis **(Figure A2A)**.

We then proceeded to determine if localization of GFP-Dad2 and Ndc80-mCherry had any temporal order using the strain SHR513 (GFP-Dad2, Ndc80-mCherry). It was observed that GFP-Dad2 was recruited at the centromere post-Ndc80-mCherry localization and disappeared prior to Ndc80-mCherry **(Figure A2C)**. To validate that GFP-Dad2 localized to the centromere it was localized with the centromeric marker Cse4 (SHR 508). GFP-Dad2 co-localized with Cse4-mCherry at all time frames during its transient dot like localization in mitosis **(Figure A2B)**.

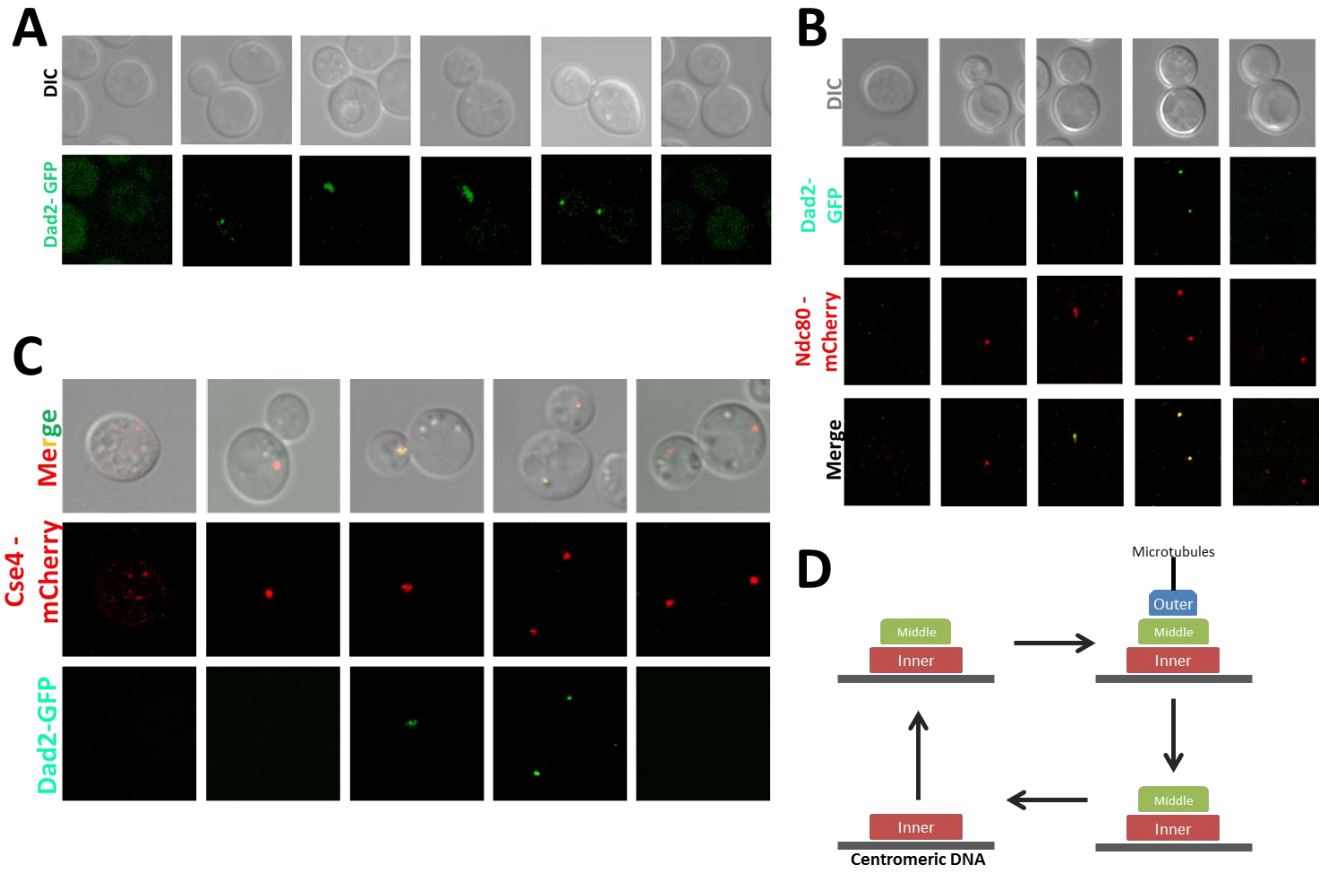

Figure A2. Dad2, a Dam1 complex subunit localizes at the centromere post-Ndc80. A) Mitotic specific localization of GFP-Dad2. B) GFP-Dad2 dot co-localizes with Ndc80-mCherry. It arrives at the centromere after Ndc80-mCherry and leaves before Ndc80-mCherry, post-anaphase but prior to cytokinesis. C) GFP-Dad2 co-localizes with the centromeric marker Cse4. D) Cartoon defining the kinetochore architecture in *C. neoformans* on spatial-temporal parameters. Inner layer (Cse4) is constitutively present followed by the localization of the middle layer (Ndc80 complex) then the outer layer (Dam1 complex) upon mitotic onset. The disassembly dynamics follows the opposite order with outer layer (Dam1 complex) exiting first followed by the middle (Ndc80 complex) leaving the constitutive inner layer (Cse4) being localized at the centromere.

These observations with others made during the same study (Kozubowski, Yadav et al. 2013) clearly point at a kinetochore architecture that was built up gradually during mitosis with the centromere binding protein Cse4 being constitutively localized, followed by recruitment of the Ndc80 and Mis12 complex and finally the Dam1 complex. This temporal localization of kinetochore proteins could be used to classify the architecture into inner, middle and outer layers **(Figure A2D).**

B. Essentiality and requirement of the inner, middle and outer kinetochore proteins for chromosome segregation in *Cryptococcus neoformans.*

The completion of kinetochore assembly in *C. neoformans* occurs soon after entry into mitosis. This observation was in addition to others made during the same study (Kozubowski, Yadav et al. 2013), which pointed towards a distinct mode of kinetochore assembly and mitotic events in this fungus. These events resemble more those of higher eukaryotes than other yeasts. Next, we sought to understand further the assembly, architecture and requirement of various proteins for chromosome segregation, which were found to localize at the kinetochore.

B1. The Dam1 complex, a microtubule binding ring that is unique to fungi is essential in *C. neoformans*, a human pathogen.

C. neoformans (the pathogen) and human (the concerned host), both being eukaryotes, enable biologists to use the former as a viable model system, for they share many conserved features; yet this, in the eyes of a clinician is a burden. For, having shared similar features, it limits the number of unique targets that could lead towards drug discovery against this pathogen. Yet, one such unique system found specific to fungi is a microtubule binding complex called Dam1 complex, which if found essential could be a potential therapeutic target.

The Dam1 complex is composed of ten distinct proteins that come together to form a ring that binds de-polymerizing microtubules at the outer kinetochore (Cheeseman, Brew et al. 2001, Westermann, Wang et al. 2006, Wang, Ramey et al. 2007). The necessity of the Dam1 complex has been explained on the numerical magnitude of kinetochore-microtubule attachments. The Dam1 complex being able to stabilize KT-MT interactions is essential in a condition wherein there exists only one KT-MT attachment per centromere. While its function is partially dispensable when there are several KT-MT attachments exist per centromere, where the stability could be to a small extent compromised on. This argument

was brought forth through observations made in *S. cerevisiae* and *C. albicans* where it is proposed that there exists only one KT-MT attachment and the Dam1 complex was found to be essential (Burrack, Applen et al. 2011, Thakur and Sanyal 2011). While in fission yeast it was found that 2-3 microtubule attachments at each centromere and the subunits of the Dam1 complex were found to be non-essential in the system, though deletion of Dam1 complex proteins did result in reduced chromosome segregation fidelity (Liu, McLeod et al. 2005).

Phylogenetically *C. neoformans* is predicted to be more closely related to fission yeast than budding yeast. To add, we previously have shown that *C. neoformans* exhibits several divergent mitotic processes such as centromere clustering only being observed in mitosis, partial nuclear membrane break and hierarchal kinetochore assembly. Hence we sought to test if the fungal specific Dam1 complex is essential and if other factors govern the requirement of the Dam1 complex in this human pathogen.

To test for this we had to perturb the system, for this we chose to adopt the use of a controllable promoter: the *GAL7* promoter (Baker and Lodge 2012). The expression of the *GAL7* promoter could be controlled by varying the carbon source that was made available to the system. It is induced in the presence of galactose while glucose is absent and shut-off when glucose is present in the system **(Figure B1A)**. Although the controllable expression of the promoter was reported earlier, its use in the context of our requirement was not examined. Hence we created a vector construct, wherein we could simultaneously swap the native promoter with the regulatable *GAL7* promoter and tag a protein at the N-terminal with either GFP or mCherry (**Figure B1B-C),** and sought to examine its functionality. The outline of the assay is represented in **Figure B1D**.

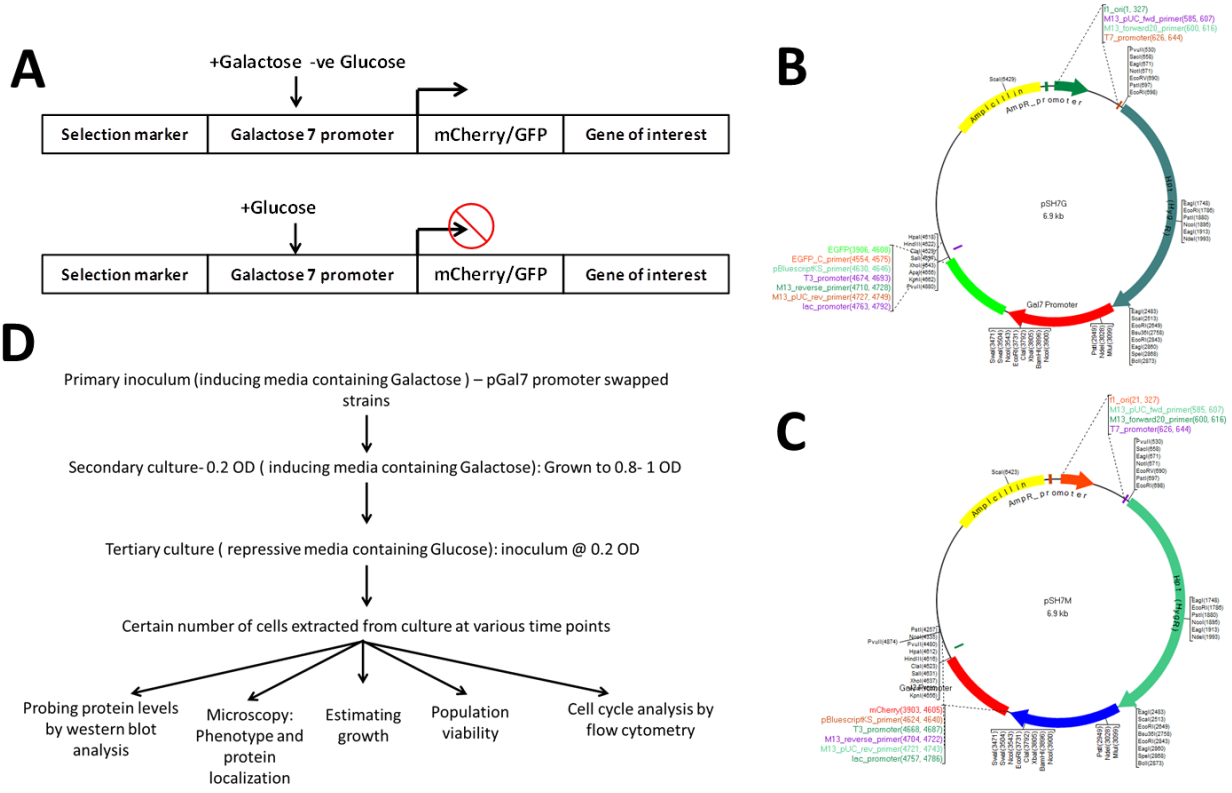

Figure B1. Utilizing the *GAL7* promoter to perturb the system. A) Regulation of the *GAL 7* promoter. B) and C) Vector constructs pSH7G and pSH7M created to promoter swap the native with the *GAL7* promoter and tag the protein with fluorescent reporter, GFP(B) or mCherry(C) respectively. D) A flow diagram of the steps followed for the assay.

Utilizing pSH7G vector construct, the US and DS sequences of Dad1 were cloned. The promoter at the native locus was swapped. This integration via homologous recombination was confirmed by Southern blot analysis (as described in materials and methods) **(Figure B2A and Figure B2B)**. The expression and efficiency of repression was detected by measuring the protein levels of GFP-Dad1 over time using western blot analysis. We proceeded to test the efficacy of the promoter and simultaneously the essentiality of the Dam1 complex protein Dad1 as the promoter showed repression of fusion protein below detectable levels. SHR710 (*GAL7pr-GFP-Dad1*) was streaked and spotted on repressive and permissive plates. To have a gross understanding of the timing of viability loss, cells were repressed for varying time periods in repressive liquid culture and subsequently spotted on permissive plates **(Figure B2D)**. Over extended periods of time it was observed that SHR710 failed to grow in repressive conditions. This result suggests that Dad1 is essential

in *C. neoformans*. The *GAL7pr* being a strong/ high- expression promoter could explain the discrepancy between detected protein level and timing of drop in viability, as the levels of GFP-Dad1 required for cellular function might be small.

To further validate the essentiality of GFP-Dad1, loss of viability under repressed conditions was performed quantitatively and the % viability over time was plotted **(Figure B3C)**. A drastic drop in viability between 9-12 h was observed and it was evident that GFP-Dad1 was essential for viability of *C. neoformans* cells. It also implicated that the *GAL7*pr was efficient to probe essentiality of other kinetochore proteins.

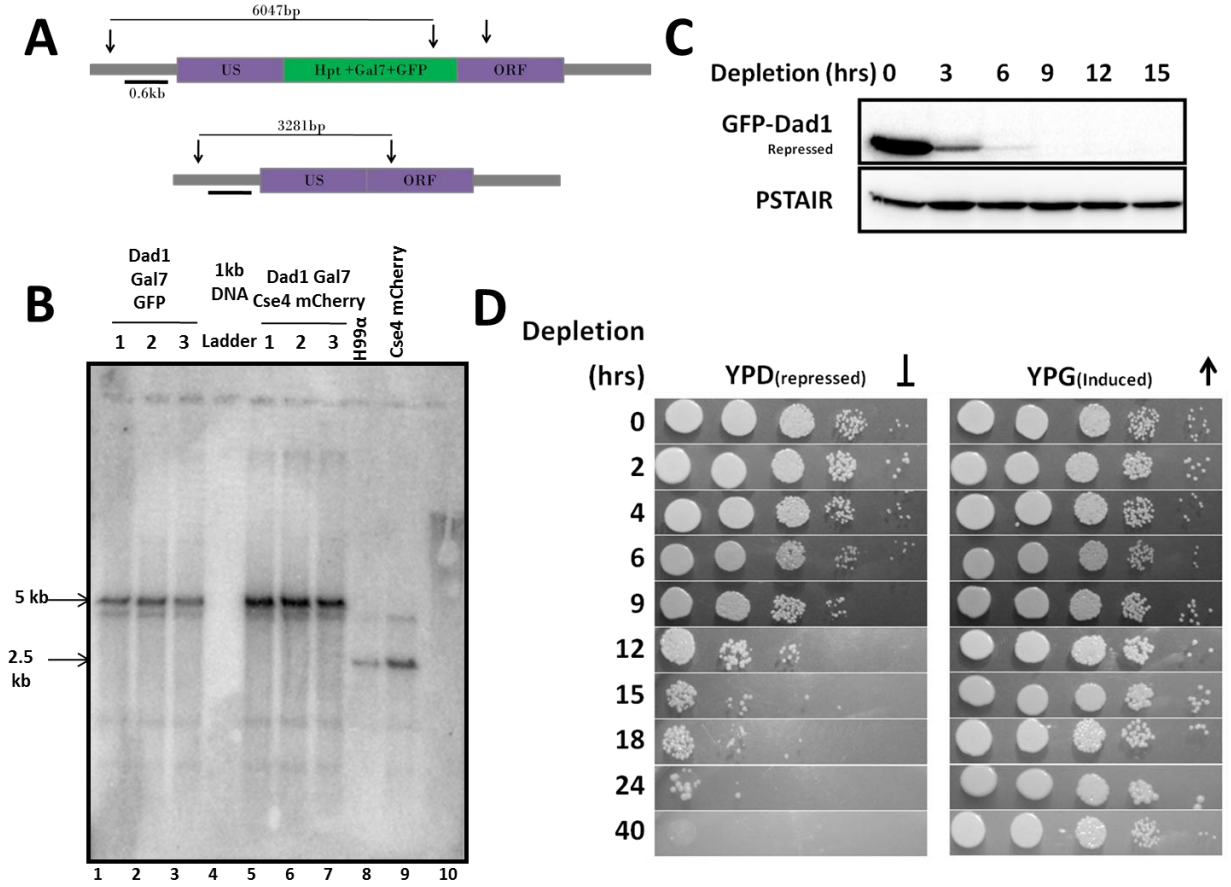

Figure B2. Essentiality of Dam1 complex subunit Dad1. A) Strategy used for southern blot analysis for homologous replacement of native Dad1 gene with the *GAL7* system. B) Southern blot autoradiogram confirming the integration of the transgene at the native Dad1 loci. C) Western blots detecting levels of GFP-Dad1 over time upon shutting off expression of *GAL7pr*. PSTAIR is used as loading control D) Testing the essentiality of Dad1 upon repression of *GAL7pr-GFP-Dad1* via spotting assay.

Subsequently we examined the cellular phenotype of *GAL7pr-GFP-Dad1* (SHR710) upon repression (12h) microscopically. A near absolute population of large budded cells was observed, indicative of a cell cycle arrest **(Figure B3A).** Flow cytometry analysis was performed with Propidium Iodide (PI) staining to assess the effect on cell cycle progression upon *GAL7pr-GFP-Dad1* repression **(Figure B3B)**. The flow cytometry plots suggest an arrest of cells with 2n ploidy, possibly at the G2/M cell cycle checkpoint. In addition to the observed large bud arrest over time, microscopically it was also observed that the bud-neck region widened and cells exhibited fission yeast like cell morphology. To test for these defects in the bud-neck septum, cells were stained with calcofluor white dye **(Figure B3D)**. In comparison to the wild-type, it was observed that the repressed cells had reduced/absence of calcofluor staining the bud-neck septum, indicative of absence/ loss in ability of form the septum.

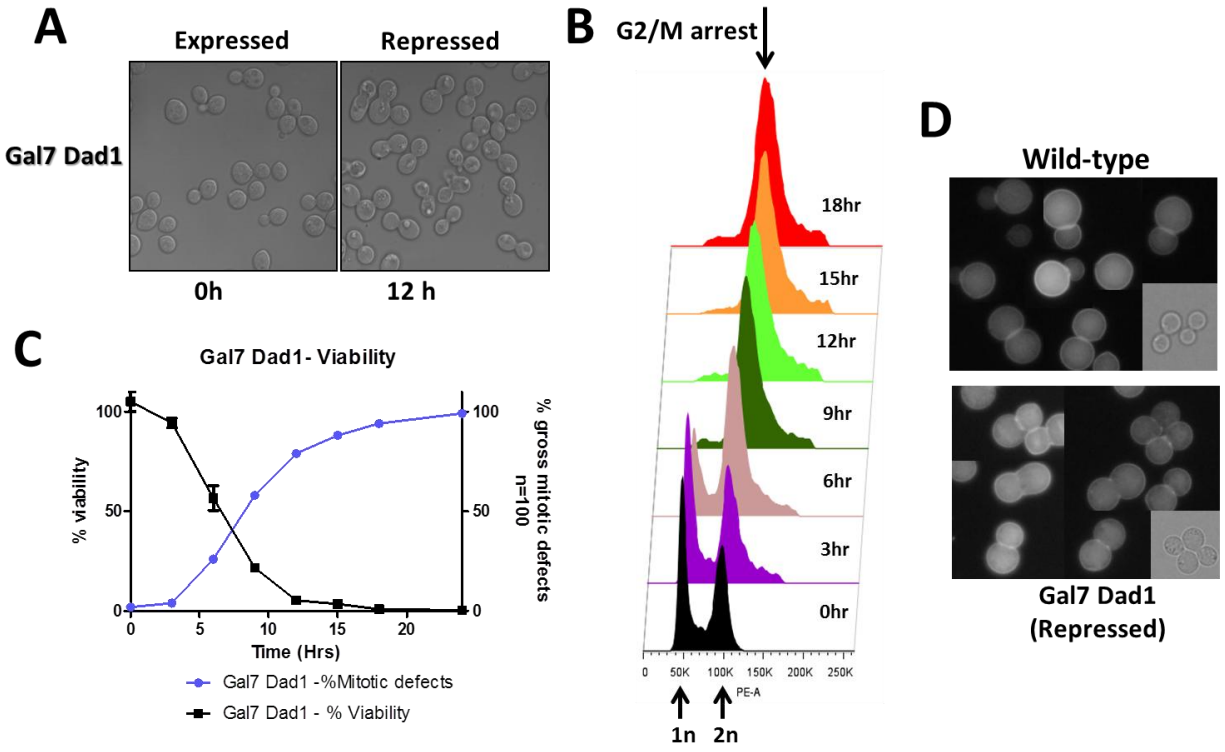

Figure B3. Repression of Dad1 results in G2/M arrest. A) Large bud phenotype observed upon repression of *GAL7pr-GFP-Dad1* for 12h. B) Flow cytometric analysis of cell cycle by PI staining upon repression shows accumulation of cells with 2n ploidy over time. C) Co-relation of drop in viability with the increase in mitotic defects. D) Loss in septum integrity upon repression of Dad1 visualized by calcofluor staining.

A correlation between observed drop in protein levels, cellular viability and cell cycle arrest, upon repression of Dad1 could be drawn; and loss in viability accounted for by the cell cycle arrest which is the result of reduced Dad1 protein levels. Yet, to confirm the observations we performed a similar systematic analysis for a second subunit of the Dam1 complex protein, Dad2 **(Figure B4)**.

To test the requirement of Dad2 for cellular viability, we began by cloning Dad2 in the pSH7M. The transgene of *GAL7pr-mCherry-Dad2* was integrated into the genome by homologous recombination replacing native Dad2 copy. This was confirmed by Southern blot analysis (as described in materials and methods) **(Figure B4A)**. To validate the functional expression of the transgene and effective repression of the promoter western blot analysis against cellular lysates of 0 h and 36 h repressed culture were prepared by TCA method and probed with mCherry antibody **(Figure B4B)**.

Consequently we examined the cells by DIC to observe the effect on cell morphology upon repression **(FigureB4C)**. Similar to Dad1 a uniform large bud arrest was seen, which prompted us to examine its requirement for viability. Cells were spotted on permissive and repressive media upon dilution of 10 for 5 dilutions **(Figure B4D)**. No growth was observed on non-permissive media while single colony growth was seen on permissive plates. It is noteworthy to mention in comparison to Dad1, upon repression Dad2 cells grew to a larger spot size under repressed conditions. Subsequent analysis of cell cycle progress upon repression, over time, via flow cytometry showed a distinct arrest of cells at 2n ploidy similar to GFP-Dad1 depletion **(Figure B4E)**. Yet it was hard to neglect the observation that this complete arrest took at least 15h longer than Dad1 **(Figure B3B)**. It is also worth mentioning that the repressive media was changed at 18h as cells were nearing stationary phase of growth **(Figure B11A)**. Drop in viability was quantified over time by plating a fixed number of cells on permissive media after growth in repressive liquid media **(Figure B4F)**.

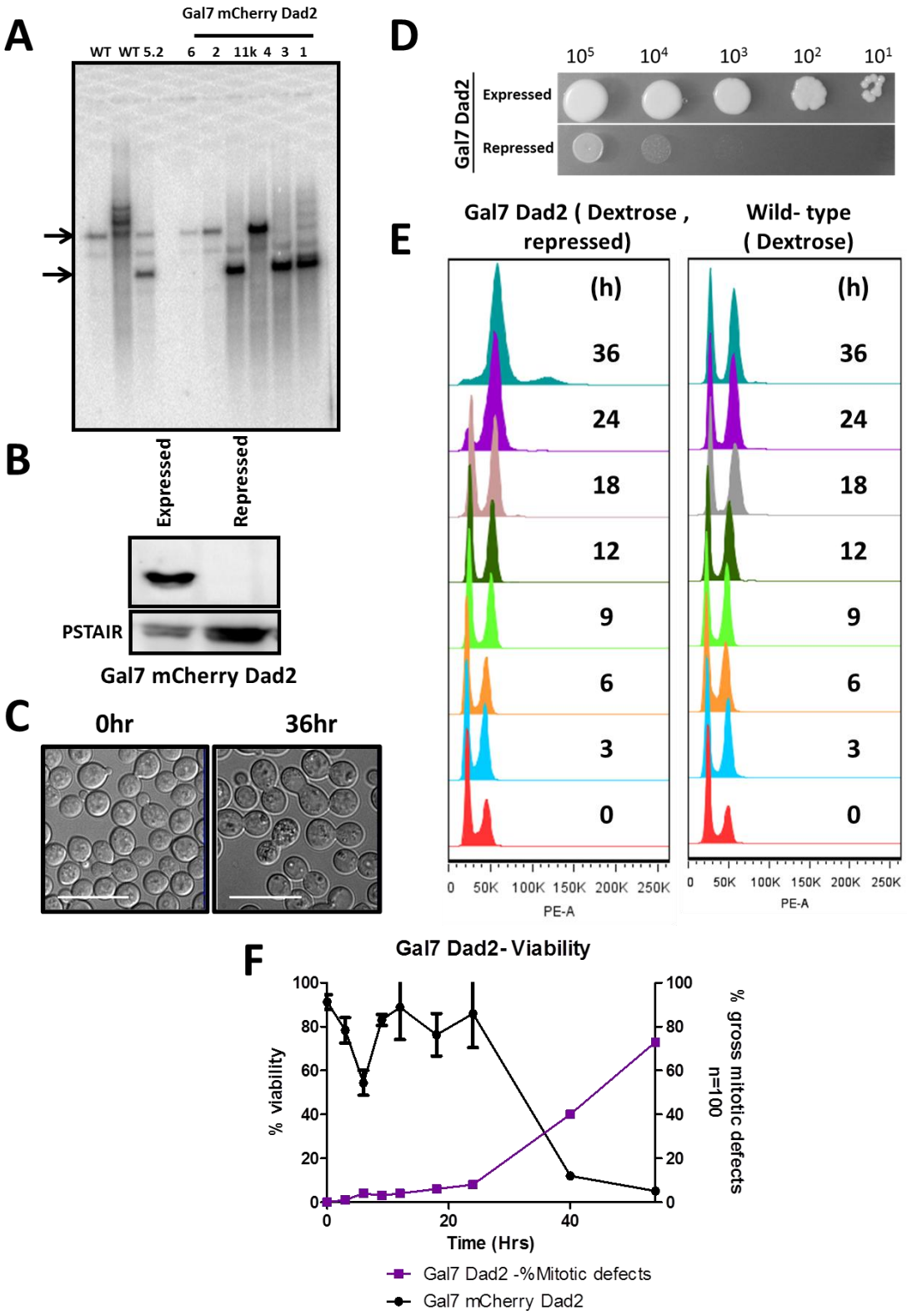

Figure B4. Dad2 is essential for viability and results in large bud arrest upon repression. A) Southern blot confirmation of *GAL7pr-mCherry-Dad2* transgene integration replacing Dad2 native copy. Top arrow points to the expected band for the WT copy while the bottom arrow points towards the band expected upon transgene integration. B) Repression of mCherry-Dad2 by 36h, detection by western blot analysis. PSTAIR is used as loading control. C) Microscopically examined

Dad2 repressed cells exhibited a large-bud phenotype at 36h. D) Spotting of *GAL7pr-mCherry-Dad2* cells at dilutions of 1:10 on permissive and repressive media. E) Arrest of SHR719 upon repression over extended periods of time as analyzed by flow cytometry analysis of PI stained cells (50k- 2n ploidy). F) Quantitative measurement of loss in viability and gross mitotic defects. Scale is 10µm.

With the analysis of the Dam1 complex subunits a strong correlation could be drawn between cell cycle arrest and loss in viability over time upon repression. Yet, the time frame taken to observe the phenotype between the repressions of the two proteins, Dad1 and Dad2, differed significantly.

To rule out the possibility of the fluorescent tag at the N-terminal affecting the observed results, Dad2 was tagged with GFP and also a strain was constructed that contained no tag on Dad2. Tagged (GFP and mCherry) and untagged strain exhibited similar dynamics over equivalent time frames (data not shown). This variable requirement of Dad1 and Dad2 may hint at the redundancy, requirement and stability among the subunits forming the Dam1 complex.

B2. Inner kinetochore proteins are indispensable for accurate chromosome segregation.

The inner kinetochore proteins Cse4 and Mif2, during the current study were shown to be constitutively localized to the centromere throughout cell cycle. It was also observed that they are un-clustered and persist at the nuclear periphery throughout cell cycle except during mitosis, when they transiently come together. To test for the effect of loss among these inner kinetochore proteins on chromosome segregation, Cse4/CENP-A was initially cloned into pSH7M and transformed into WT (KN99a) cells. Replacement of the native copy with the transgene was confirmed by Southern blot analysis (SHR702), as described in materials and methods **(Figure B5A and B5B).**

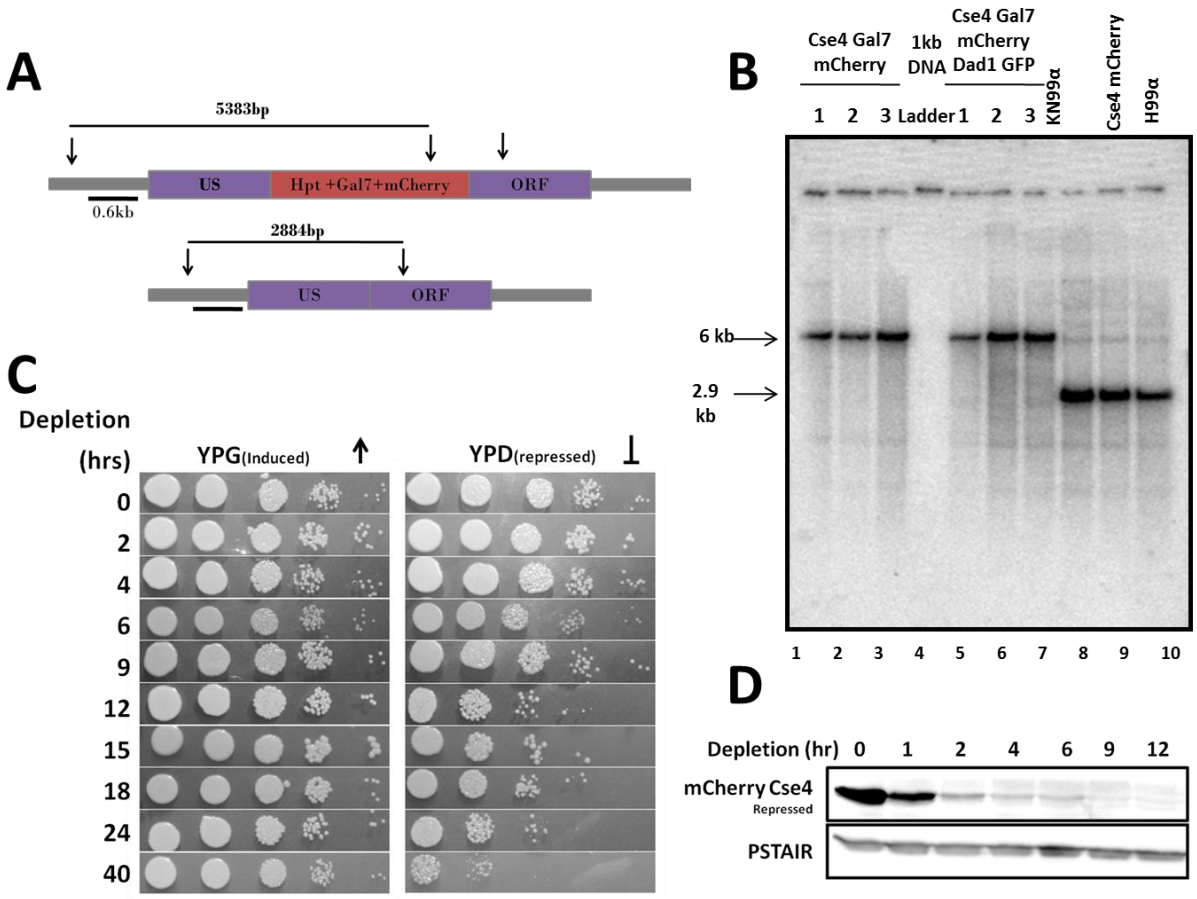

Figure B5. Centromeric histone variant Cse4/CENP-A is essential for viability. A) Schematic of the Southern strategy used to confirm integration of the transgene replacing the native copy by homologous recombination. B) Southern blot confirmation of SHR702. C) SHR702 (*Gal7pr-mCherry-Cse4*) was cultured in liquid media containing either glucose (repressive) or galactose (permissive) carbon sources for various amounts of time and subsequently spotted on permissive plates upon dilutions of 10 for 5 dilutions. Depletion of Cse4 in SHR 702 was found to be essential. D) Western blot analysis of mCherry-Cse4 protein levels upon *GAL7*pr shut down.

Requirement of Cse4 for viability was tested by growing cells in repressive liquid media then spotting them on permissive plates. Drop in viability was observed. To validate the observed effect of Cse4 depletion, viability was performed quantitatively **(Figure B7D)**. These observations were further validated by probing for protein levels in the cell lysates. mCherry-Cse4 protein levels dropped sharply upon repression, 1-6h **(FigureB5D).** A concordant reduction in levels of fluorescent intensity was observed when cells were examined by confocal microscopy, although the fluorescence signal persisted for longer time frames (12h) when compared to protein levels detected by western blot analysis (6h)

(Figure B6). This could be due to limitation in sensitivity of the detection system or could be due to increased Cse4-mCherry stability at the kinetochore.

For subsequent microscopic analysis of mCherry-Cse4 signal upon repression, 15h time point was used as a standard as a complete loss in signal was observed at this time point.

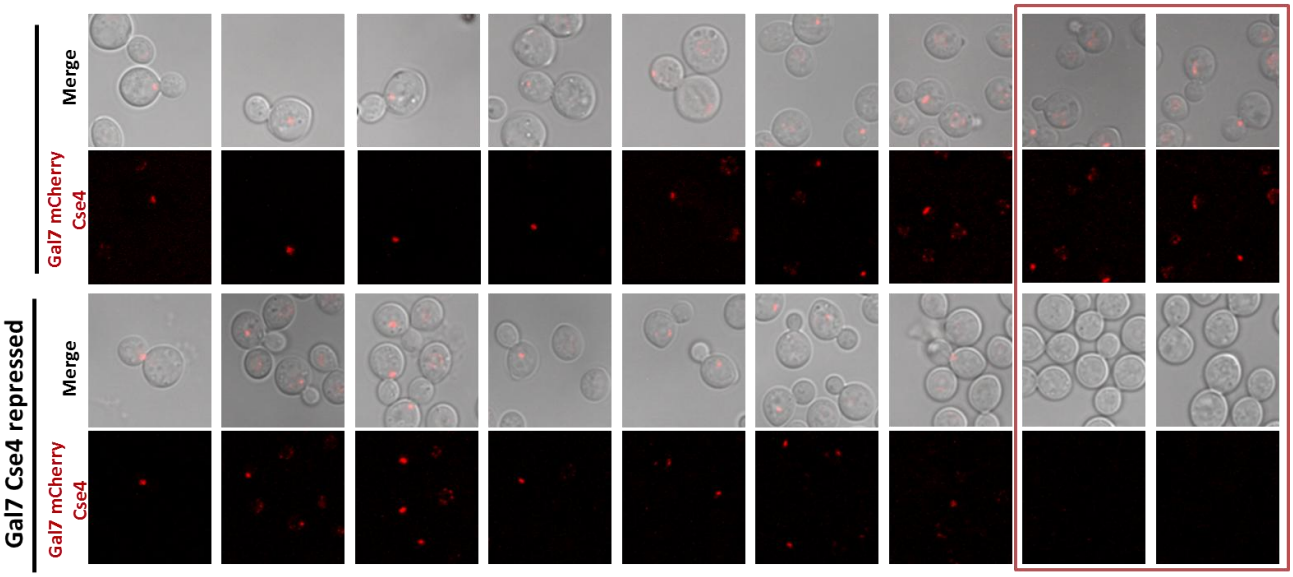

Figure B6. mCherry-Cse4 protein is stabilized when localized at the centromere. 15h time point was used as a standard time point for the subsequent microscopic experiments.

Intriguingly, cellular phenotype upon complete depletion of mCherry-Cse4 was observed to be heterogeneous, cells in all stages of cell cycle persisted **(Figure B7B)**. Thereby, flow cytometry analysis was performed to better understand the state of the cell cycle. In other budding yeasts cell cycle arrest was observed upon depletion of Cse4 (Stoler, Keith et al. 1995, Thakur and Sanyal 2011). Yet, unlike other yeasts, upon depletion of Cse4 in *C. neoformans* did not result in cell cycle arrest. The cells proceeded through cell cycle accumulating defects, indicated by a gradual increase in ploidy beyond 2n and plateauing of the 1n and 2n peaks **(FigureB7A)**. To better visualize this, histone H4 was tagged with GFP in a SHR702 background to stain nuclear chromatin. SHR 705 (*GFP-H4*, *GAL7pr-mCherry-Cse4*) cells were observed upon depletion of Cse4 and defects in nuclear segregation were calculated. When Cse4 was expressed, nuclear chromatin had a uniform distribution encircled by a ring of Cse4 on the nuclear periphery. No cell contained more than one nuclear mass, unless in early anaphase. While in the cells depleted of Cse4 massive nuclear

segregation defects were evident, with some cells were observed having up to 4-5 nuclear mass fluorescent intensities within the same bud, while others exhibited empty cells with no chromatin **(Figure B7C)**. These observable gross defects were scored for; cartoons representing the scored phenotypes are depicted **(Figure B7C)**. This increase in segregation defects could be resulting in the observed loss in cell viability **(FigureB7D)**.

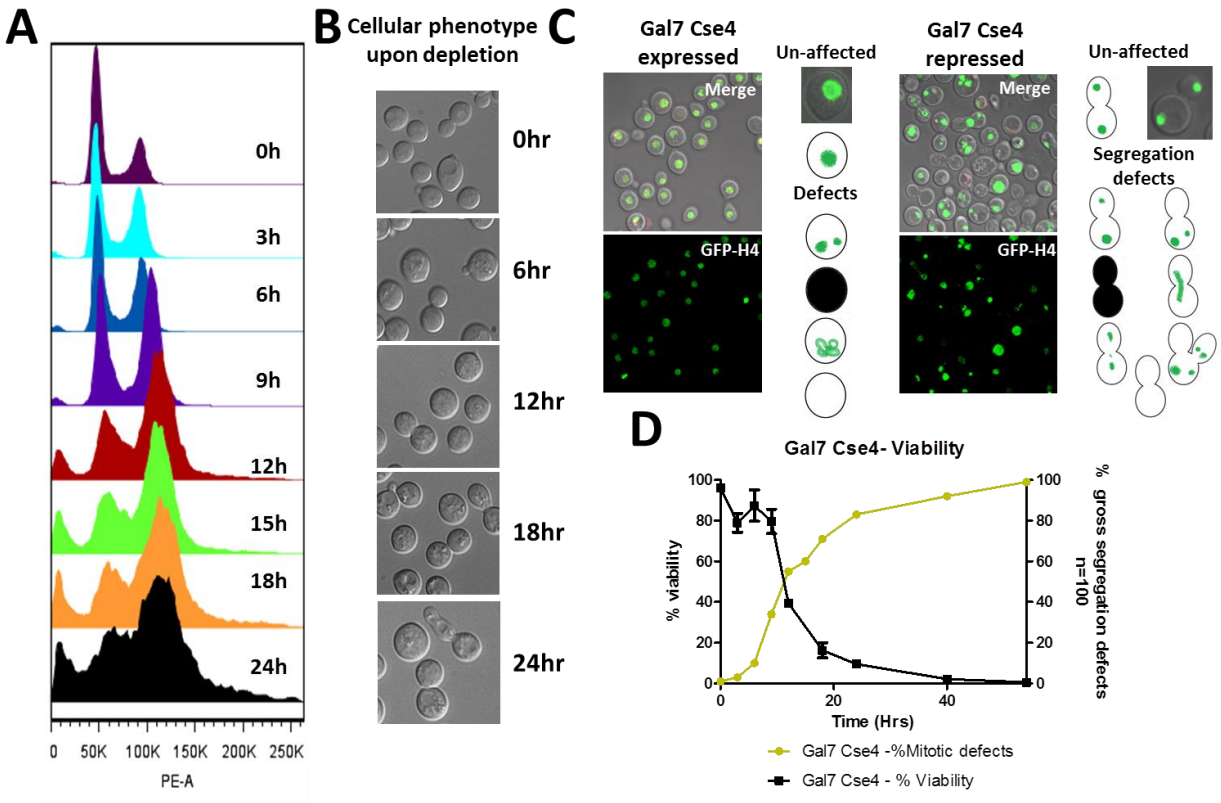

Figure B7. Reduction in Cse4 protein levels result in segregation defects among a large population of cells. A) Flow cytrometric analysis of cells upon Cse4 depletion. B) Microscopic examination revealed cells in all stages of cell cycle were present, no observable cell cycle arrest in Cse4 depleted cells. C) Effect of Cse4 depletion on nuclear chromatin. H4 GFP staining in SHR702 background. WT and mutant phenotype are schematically represented D) Quantitation of viability loss and chromosome segregation defects upon Cse4 depletion show inverse co-relation.

A similar conclusion was drawn when the effect of depletion of another inner kinetochore protein, CnMif2 was examined. The native promoter and gene of Mif2 was swapped with the *GAL7pr* and a GFP tagged transgene respectively, via homologous recombination, resulting in SHR716. Confirmation of the strains by Southern blotting was performed, as mentioned in materials and method **(Figure B8A)**. Positive transformants failed to grow

on repressive plates (+glucose/dextrose, –galactose) **(Figure B8C)**. On performing western blot, viability assay and flow cytometry analysis on *Gal7-GFP-Mif2* repressed population; it was observed that cells were highly sensitive to the drop in levels of Mif2. As although reduction in levels of GFP-Mif2 shared similar dynamics with that of Dad1 and Cse4 **(Figure B8B),** the population of SHR716 cells lost complete viability by 6h of repression. This correlated sharply with the gain in aneuploidy among cells, as observed by flow cytometry **(Figure B8F).** Observed aneuploidy might be the result of large chromosome segregation defects **(Figure B8E)** as no cell cycle arrest was observed upon microscopic or flow cytometric analysis, **(Figure B8D)** similar to Cse4.

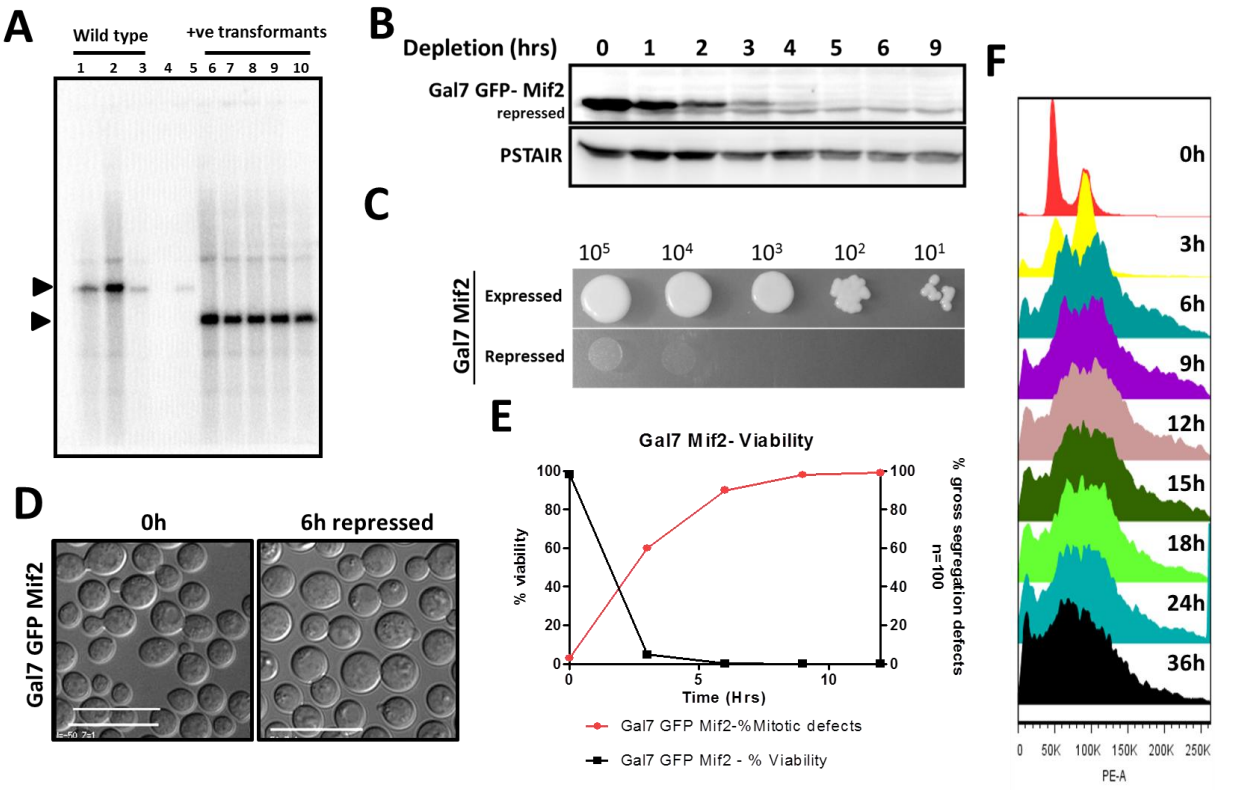

Figure B8. ***C. neoformans*** **is highly sensitive towards reduction in cellular pool of Mif2 protein.** A) Southern blot confirmation of *GAL7-GFP-Mif2* transgene integration. B) Detection of GFP-Mif2 protein pool upon promoter shut down. PSTAIR was used as loading control. C) Mif2 is essential for viability D) Heterogeneity in cell morphology of Mif2 depleted cells, no cell cycle arrest. E) Quantification of cell viability and gross segregation defects over time. Segregation defects were scored for as described for Cse4. F) Extensive gain of aneuploidy in GFP-Mif2 depleted cells as quantified by flow cytometry.

B3. Mtw1 and Ndc80 complex proteins are critical for spindle checkpoint activation.

Depletion of cellular pools of outer and inner kinetochore proteins by utilizing the *GAL7pr* yielded varied dynamics on cell viability, defects and cell cycle. The middle kinetochore consisted of both scaffold proteins as well as protein that interacted with the spindle microtubules. To understand how this linker layer behaves upon depletion, two proteins, Mtw1 and Nuf2 that were previously localized and identified as part of the middle kinetochore layer was studied. Following up, Mtw1 and Nuf2 were cloned into the pSH7G vector construct and transformed into H99α cells, replacing the native copy by homologous recombination **(Figure B10A, Nuf2)**. Expression of the transgene and extent of repression upon promoter shut down was detected in cell lysates prepared from Mtw1 and Nuf2, **Figure B9A and Figure 10C** respectively.

Both tested proteins Mtw1, a structural linker, and Nuf2, an evolutionarily conserved microtubule binding protein, was found to be essential for viability. **(FigureB10D)**. Yet unlike the other microtubule binding complex of Dam1, Nuf2 upon repression did not result in large bud or G2/M arrest phenotype **(Figure B10F)**. This was inferred from microscopic examination of cells repressed for 15h, in addition to the flow cytometric analysis performed on the same population of cells **(Figure B10D)**. Mtw1 upon repression followed similar cell cycle dynamics as Nuf2 when examined by flow cytometry, where cells accumulated aneuploidy over time, increasing ploidy level of cells beyond 2n in addition to the plateauing of the 1n and 2n peaks **(FigureB9D)**. The accumulation of aneuploidy in the cell population occurred though the spindle assembly checkpoint pathway was intact in these cells.

The primary function of the spindle assembly checkpoint is to ensure accurate KT-MT interaction. Hence in the presence of defective attachments the SAC would activate and stall chromosome segregation. While in the absence of an effective spindle assembly checkpoint improper KT-MT attachments would not be corrected and erroneous chromosome segregation would ensue. To test the possibility that depletion of Mtw1 and Nuf2 prevent the spindle assembly checkpoint activation, we scored for segregation defects that result in strains SHR 717 and SHR 718, *GAL7pr-GFP-Mtw1* and *GAL7pr-GFP-Nuf2*

respectively. There was a large increase in segregation defects that could be linked with the loss in viability in this population of cells. Yet this effect observed upon Nuf2 and Mtw1 depletion could not be solely affecting the SAC **(Figure B9E and B10E)**, as it was observed that in wild-type cells deletion of a critical protein in the pathway, Mad2 did not result in such a high rate of segregation defect **(Appendix figure 1)**. Hence depletion of the middle and inner kinetochore proteins was affecting both KT-MT attachments and the functionality of the spindle assembly checkpoint simultaneously. This is yet to be validated.

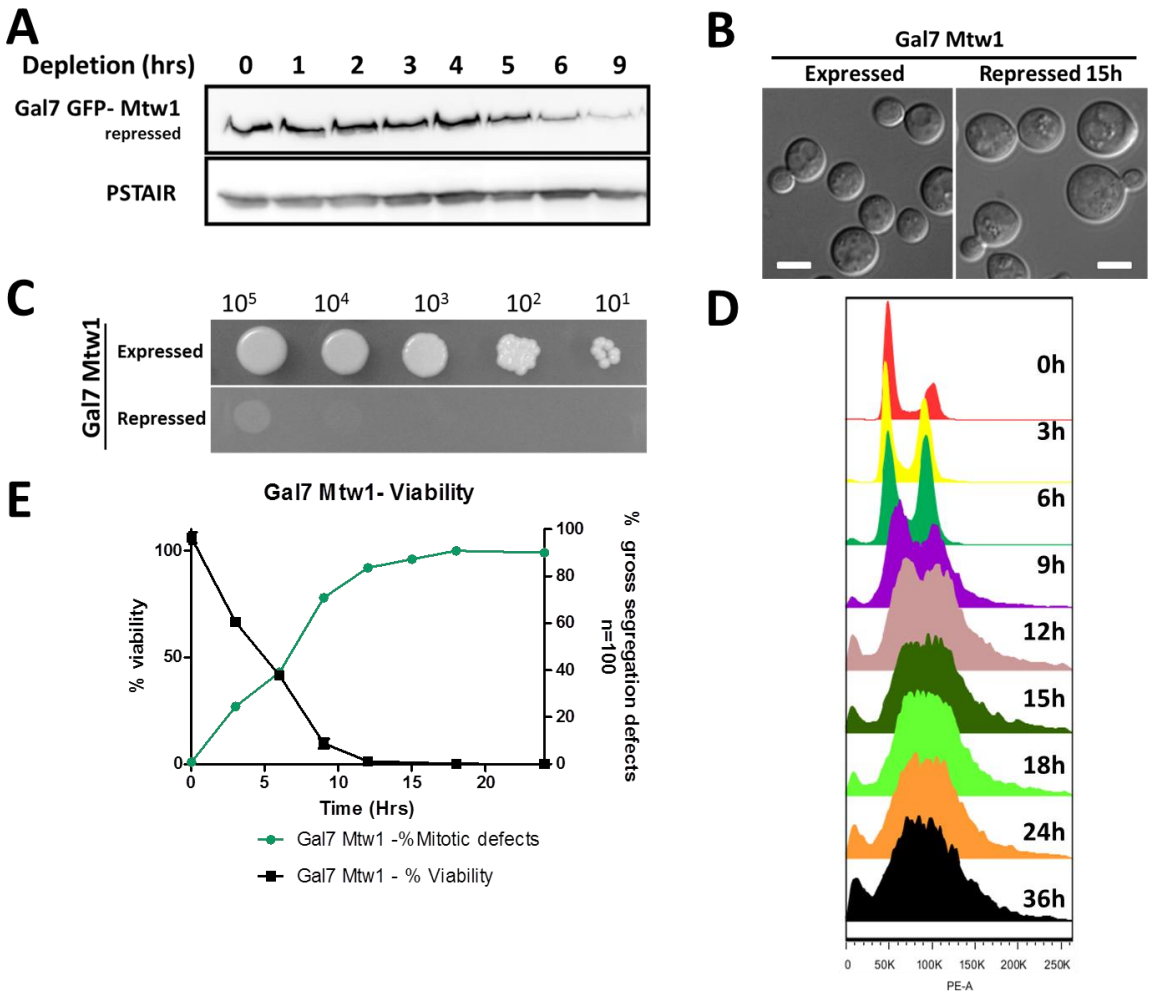

Figure B9. Cells depleted for Mtw1 are impaired in spindle assembly checkpoint. A) Western blot analysis detecting levels of Mtw1 upon repression. PSTAIR was used as a loading control. B) Microscopic examination of SHR715 upon repression. C) Mtw1 is essential for viability. D) Increase in ploidy and accumulation of aneuploidy upon Mtw1 depletion. E) Inverse co-relation between drop in viability and gain in chromosome segregation defects.

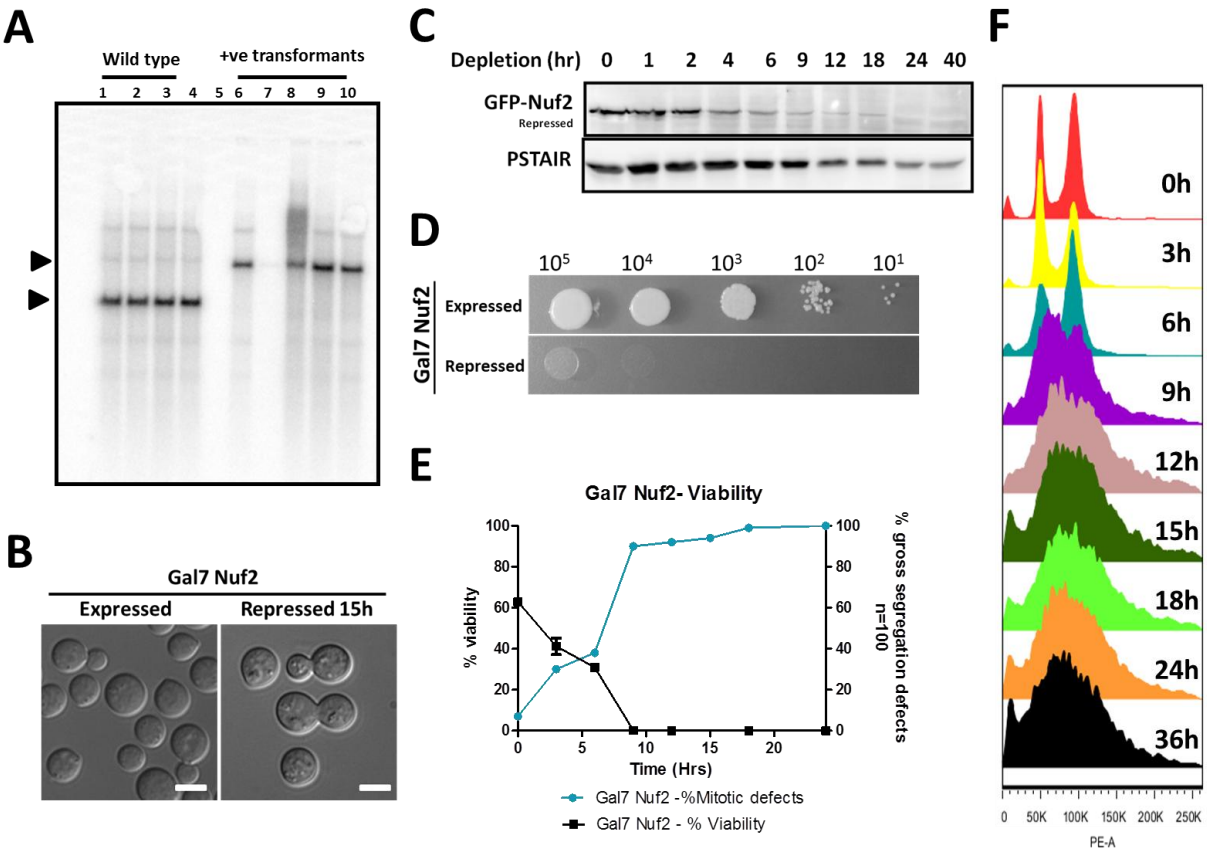

Figure B10. Ndc80, microtubule binding protein complex, subunit Nuf2 is critical for the functional spindle assembly checkpoint. A) Southern confirmation of *GAL7pr-GFP-Nuf2* transgene integration. B) Increase in cell size and failure of cells to arrest at cellular checkpoints. C) Functional expression and repression of transgene over time as detected by western blot analysis. D) Nuf2 protein is essential for viability. E) Nuf2 tagged with GFP at the N-terminal had reduced viability at permissive conditions. Upon repression loss in viability was accounted for by the increase in chromosome segregation defects. F) Nuf2 depleted cells are impaired in spindle assembly checkpoint and become aneuploidy.

B4. Kinetochore proteins have highly divergent effects on chromosome segregation and kinetochore dynamics.

To better understand and compare the effect of various kinetochore proteins upon depletion, on cell growth, the optical density of the repressed cultures was measured over time (@600nm), a minimum of three replicates were performed **(Figure B11A)**. Wild-type (WT) H99α and KN99a strains were used as controls. Optical density of the starting culture was 0.2. In addition, the effect of viability of cells upon depletion of various kinetochore

proteins was quantitated and normalized to wild-type at all-time points and plotted **(Figure B11B)**.

From this analysis it was noticed that the effect on cell growth and viability varied significantly with no specific trend, upon depletion of kinetochore proteins. The effect upon depletion was dependent on the function and properties of the individual protein and not the layer where it was found. These results also allowed us to speculate the half-life and cycling dynamics of these proteins at the kinetochore. Reduction in levels of Mif2 was not accepted well by the cell, wherein cells failed to undergo cell division for more than 2-3 cycles. While on the other end of the scale, Dad2 upon depletion, without the media change, reached stationary phase and exhibited no growth defects **(Figure B11A)**. Yet upon media change, before reaching stationary phase a gradual drop in viability post-24h was observed upon Dad2 depletion. Possible factors that could have induced such as phenotype are: long half-life of the protein, minute molar quantities of the protein being required during each cell cycle or partial complementation of function by other cellular proteins. It was possibly all these factors playing a role upon Dad2 depletion. These factors might have had a varied influence on depletion of other kinetochore proteins, resulting in the diverse data sets observed. No large loss in viability, ~8%, was observed when the spindle checkpoint was rendered defective by deletion of a critical protein, Mad2 **(Figure B11B)**. Yet when Dad1 was depleted in a SAC defective strain the drop in viability was an order of magnitude larger, implying the importance of the spindle assembly checkpoint when cells are stressed. In addition the cell cycle arrest is probably reversible upon subsequent expression of Dad1 at permissive conditions.

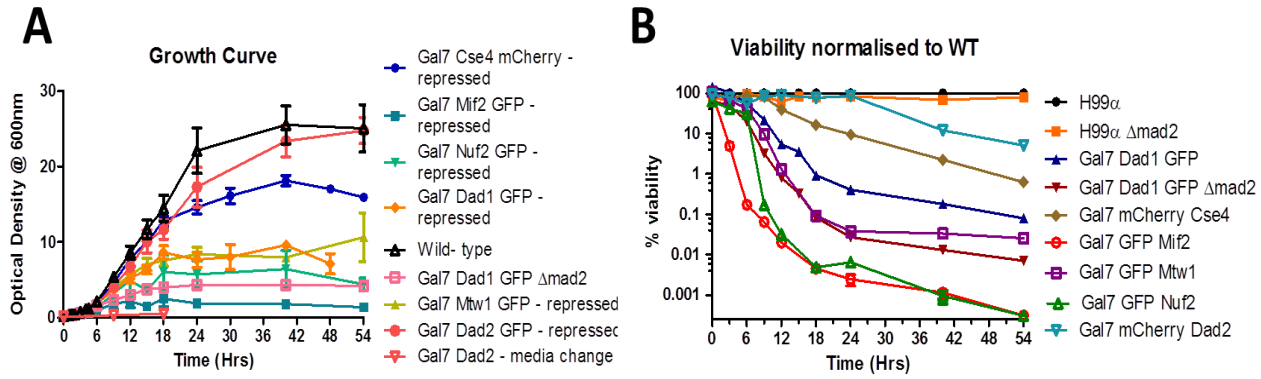

Figure B11. Compilation and comparison of growth (A) and effect on viability (B) in strains depleted for various kinetochore proteins.

It is noteworthy to state here that the effects observed upon depletion of various kinetochore proteins might have been more pronounced if a regulatable promoter that didn't drive expression at such high levels, was used.

B5. Mad2 mediates the cell cycle arrest observed upon depletion of Dam1 complex proteins.

Depletion of Dad1 and Dad2, both subunits of the microtubule binding Dam1 complex were found to arrest in cell cycle with 2n ploidy and large bud phenotype. Inner and middle kinetochore proteins preceded through cell cycle with large nuclear segregation defects that resulted in loss of cell viability. To determine the cellular mechanism behind the observed cell cycle arrest, we deleted a critical spindle assembly checkpoint protein, Mad2. The spindle assembly checkpoint is a cellular fail-safe, which ensures that proper KT-MT attachments have occurred at all chromosomes before anaphase. Mad2 has been implicated as one of the key proteins, where all upstream activator protein signaling pathways converge. This checkpoint activates in mitosis and since this is post S phase; cells arrested have double the nuclear content, resulting in a flow cytometric phenotype similar to that of the Dam1 complex protein depletion, resulting in an absolute 2n peak. Hence *MAD2* was targeted for deletion and was found to be a non-essential component of the cell. This was possibly due to proper KT-MT attachments taking place with high probability in wild-type cells **(Appendix Figure 1)**.

As the ploidy of a large population of cells could be effectively and simultaneously visualized by flow cytometry, *GAL7pr-GFP-Dad1* (SHR710) and *GAL7pr-GFP-Dad1* Δmad2 (SHR 733) cells were grown in repressive media (+glucose) for varying time points, fixed, stained with PI and subjected to fluorescence analysis using FACS Aria III. *GAL7pr Dad1* cells accumulated cells with a sharp peak with 2n ploidy by 9h, while in the Gal7pr Dad1 Δmad2 background; cells proceeded with cell cycle gradually increasing in ploidy by becoming aneuploidy **(Figure B12A)**. Upon microscopic examination of the depleted SHR 733 cells, it was observed that upon deletion of mad2 in SHR 710, cells had lost the large

bud phenotype. The large bud phenotype was a result of nuclear chromatin being stuck/ prevented from segregation and transitioned from metaphase to anaphase of the mitotic cell cycle **(Figure C1C)**. Likewise due to the accumulation of deleterious mutation and improper chromosome segregation upon *MAD2* deletion cell viability was lost **(Figure B12 C and D)**. It was also observed that *MAD2* in a Dad1 over expressed background affected cell viability, hinting at the importance of a correction mechanism in a perturbed system. It also pointed at a situation where in excess of GFP-Dad1 in the cell increased the rate of defects amongst KT-MT interactions.

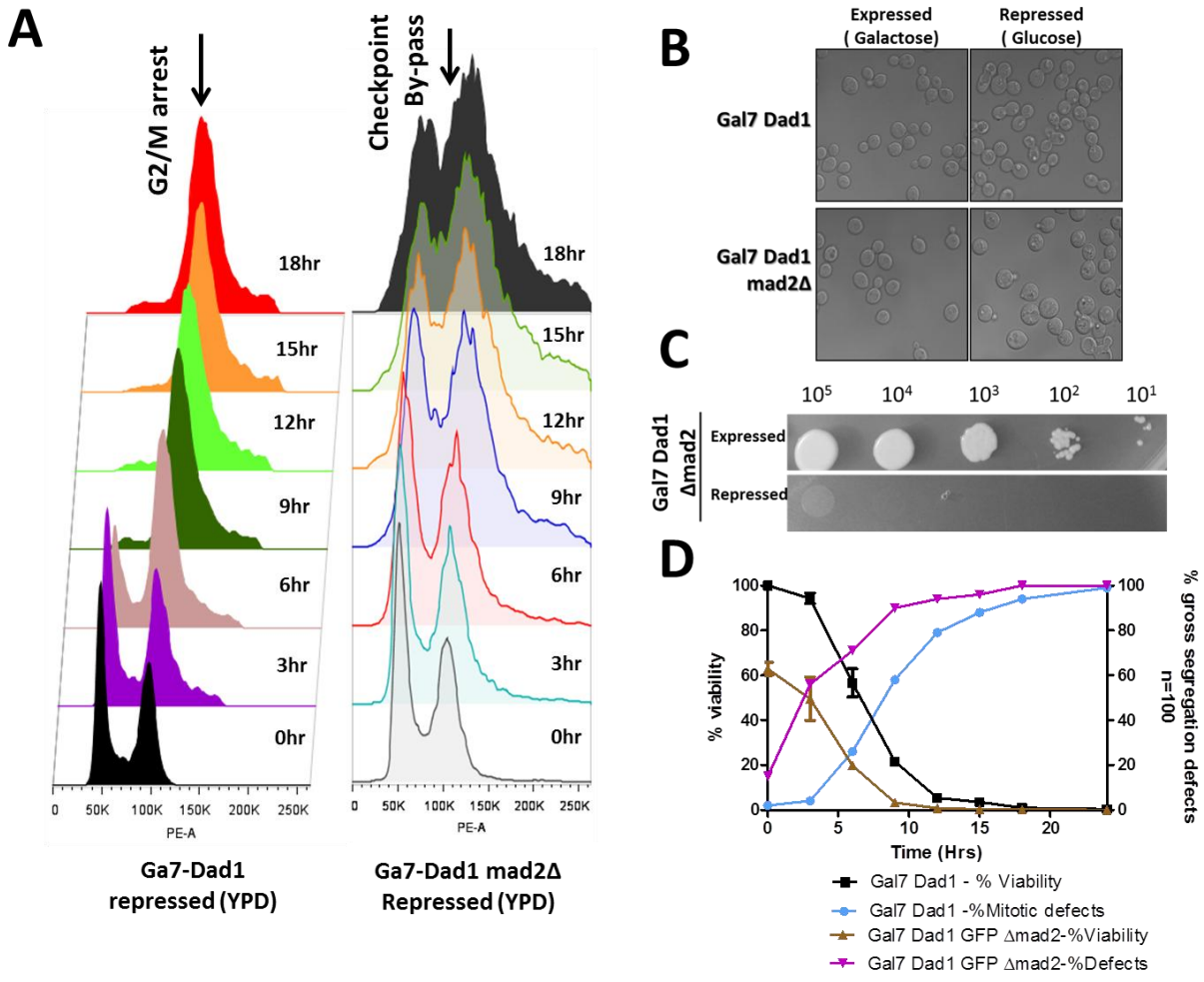

Figure B12. Mad2 mediates the cell cycle arrest resulting from Dam1 complex subunit depletion. A) Flow cytometric analysis of cell cycle: Mad2 deletion abrogated check point arrest upon depletion of Dad1. B) Microscopic comparison between the cell cycle arrest of SHR 710 and SHR 733. C) Maintenance of essentiality upon Mad2 deletion. D) Existence of large errors in segregation is detected upon Mad2 deletion.

C. Intra and inter-dependancy between various layers of the kinetochore : insights into kinetochore architecture.

We have previously shown that the kinetochore architecture and spatial-temporal regulation of kinetochore protein localization on to the centromere is not similar to other yeast. In addition, upon depleting the pools of kinetochore proteins we observed varied effects on the cell cycle of *C. neoformans*. With these encouraging findings we subsequently wanted to address the interdependency between various kinetochore proteins, which would shed light on the precise kinetochore architecture in this organism. 'Precise' I say as previously we described the timely assembly of kinetochore proteins at the centromere, it was crude and did not point to how the constitutive kinetochores (inner kinetochore layer) was assembled, how timing was important and if there was a specific order of protein assembly between proteins of the same layer (as we observed multiple proteins of the same layer localize at the kinetochore simultaneously) or if there exists a master regulator controlling the entire process. These and more could be address only upon fine dissection of the kinetochore architecture.

Towards this aim we utilized the established *GAL7* regulatable promoter to perturb a kinetochore protein and study its effect on other kinetochore proteins. Examination by microscopy was chosen as the most effective means. We began by studying the fungal specific, microtubule binding, essential outer kinetochore complex, the Dam1.

C1. Purturbing the outer kinetochore protein complex, Dam1, does not effect the gross observable kinetochore.

The Dam1 complex proteins Dad1 and Dad2 were previously shown to arrive just prior to KT-MT interaction, which was pre-metaphase, while disassembly from the kinetochore was post-anaphase prior to cytokinesis. It was also observed that cells arrested upon depletion of the Dam1 complex proteins and this arrest with 2n ploidy was mediated by the spindle assembly checkpoint, at the metaphase to anaphase transition.

For microscopic analysis of the effect on kinetochore upon Dad1 repression, various kinetochore proteins from each layer (inner, middle and outer) were tagged with a

complementing fluorescent reporter, mCherry. Consequently we repressed *GAL7pr-GFP-Dad1* by growing them for varying time points in repressive liquid media, isolating cells at each time point and scoring for the fluorescent signal intensity. After initial standardization, 9h was chosen as the optimal time point for scoring the phenotype of other kinetochore proteins, as no GFP-Dad1 fluorescent signal was undetectable; in addition, flow cytometry data showed complete cell cycle arrest by this hour **(Figure C1A)**. On subsequent microscopic analysis for the localization pattern of inner and middle kinetochore proteins, it was observed that kinetochore localization of inner (Cse4-mCherry) and middle (Mtw1-mCheery and Ndc80-mCherry) was similar to wild-type under conditions of GFP-Dad1 expression **(Figure C1C and figure A1).** This centromeric localization of the inner and middle kinetochore layer proteins was not lost upon repression of Dad1. Dam1 complex stabilizes microtubule binding, hence disruption of Dad1 might lead to subsequent loss of the Dam1 complex localization **(Figure C6C)** leading to a loss of tension. This would explain why the middle kinetochore proteins, Mtw1-mCherry and Ndc80-mCherry, appeared as long streaks that bind to the dynamic spindle. Only large bud cells were imaged, during growth in permissive and non-permissive conditions as it is at these specific corresponding cell cycle stages that Dad1 appears to localize as a dot on the clustered centromeres. Staining of nuclear chromatin exemplifies the loss in tension defect and cell cycle arrest where the nuclear mass appeared streaked, unsegregated and present at the bud-neck region of the cell **(Figure C1C)**.

To test if depletion of Dad1 had an epistatic effect on the expression of protein levels of other kinetochore proteins in the cell, western blot analysis was performed against prepared cell lysates from two microscopically examined strains, mCherry-Cse4 and Mtw1-mCherry. No significant reduction in the cellular pools of these kinetochore proteins were seen, within the time frame of the microscopic analysis, 9h **(Figure C1B)**. Although at later time points, decrease in protein levels is observed which could be a result of the drop in cell viability. PSTAIR was used as a loading control.

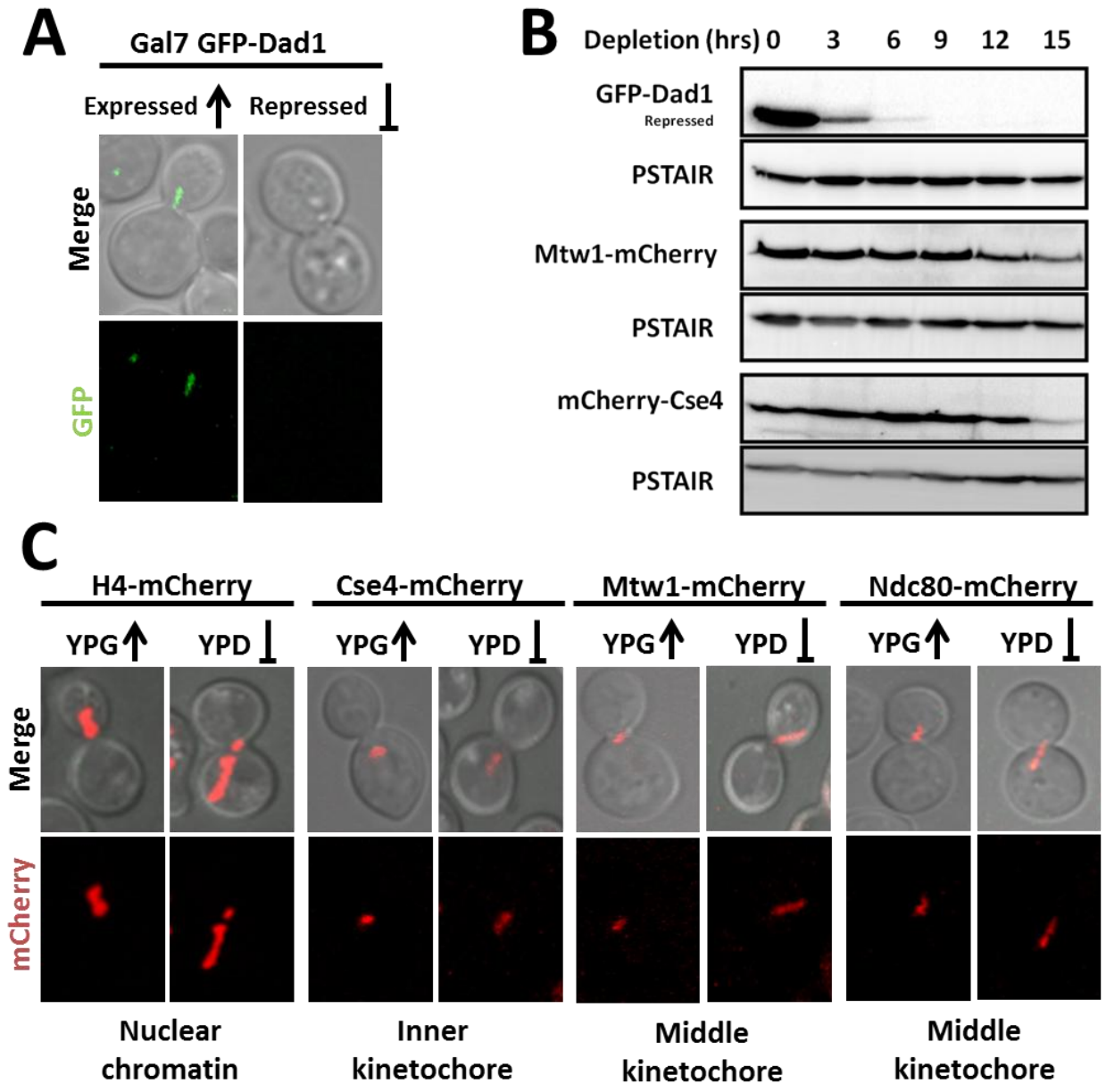

Figure C1. Presence of Dad1 at the centromere is not required for the localization of inner and middle kinetochore layers. A) Detection of fluorescent intensity of GFP-Dad1 in expressed and repressed conditions (9h). PSTAIR is used a loading control. B) Effect of Dad1 depletion on protein levels on Mtw1 and Cse4 detected by western blot analysis. C) Effect on kinetochore localization of nuclear chromatin, inner and middle kinetochore layers upon Dad1 depletion.

C2. Centromeric localization of the NDC80 complex is not affected by perturbing the inner and middle kinetochore proteins.

C2A. Perturbing the inner kinetochore:

Upon testing the localization of kinetochore proteins on Dad1 depletion and not having observed any significant change in kinetochore architecture, we focused on perturbing the centromeric DNA binding proteins Cse4 and Mif2. GFP/mCherry transgene constructs of kinetochore proteins were transformed in SHR702 (*GAL7pr-mCherry-Cse4*) and SHR716 (*GAL7pr-GFP-Mif2*) backgrounds, respectively. 15h of Cse4 repression, as previously described **(Figure B6),** and 6h for Mif2 was observed as optimal time points where the fluorescent signals of the transgenes at the centromere could no longer be detected.

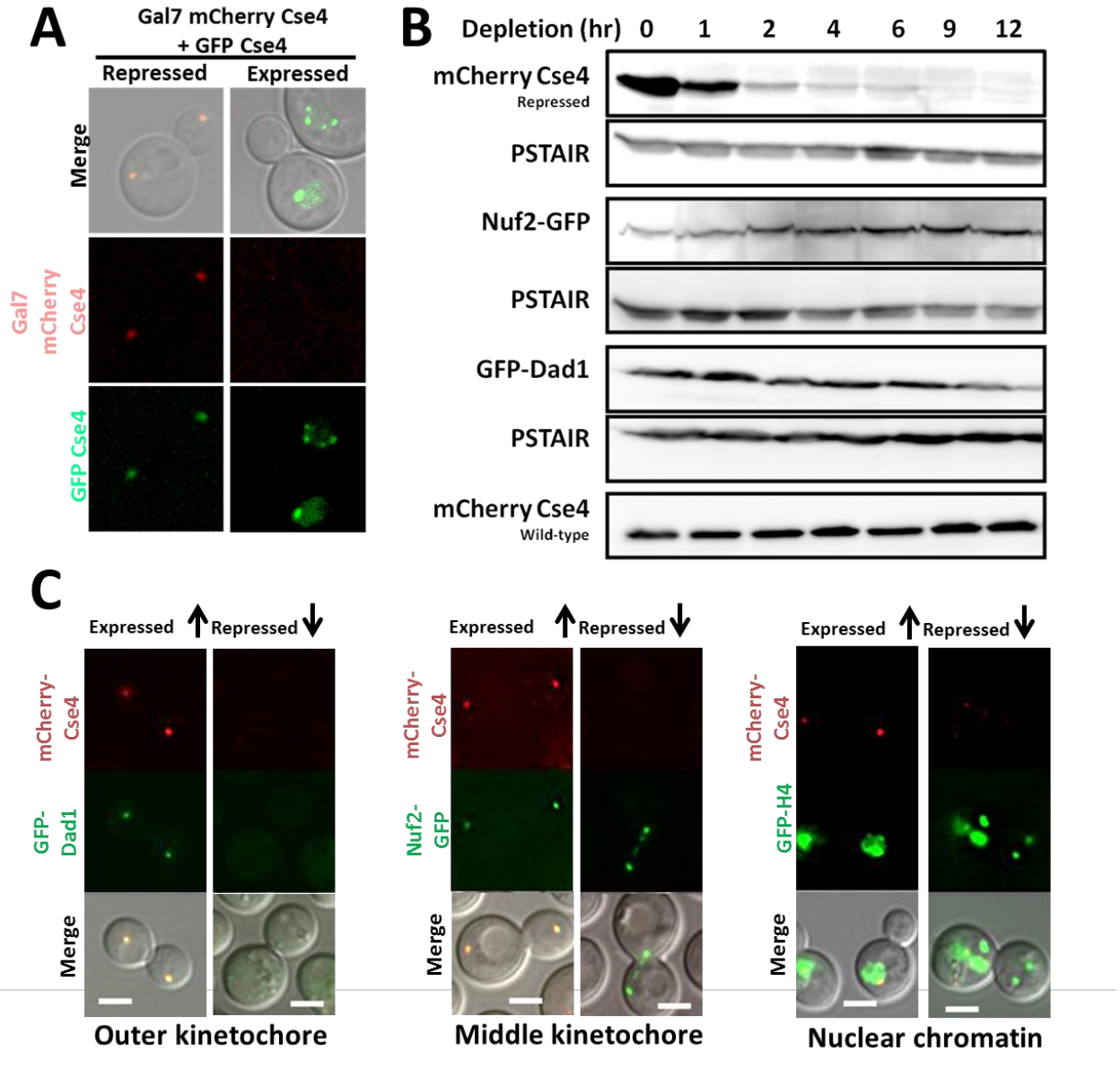

Figure C3. Nuf2 localization at the kinetochore is independent of Cse4. A) Complementation of Cse4, scored for microscopically. B) Western blot analysis for levels of kinetochore proteins upon depletion of Cse4. C) Localization dependency of Dad1, Nuf2 and effect on nuclear chromatin upon Cse4 depletion. Scale 3μm.

To validate the defects that were observed upon Cse4 depletion, SHR702 was complemented with GFP-Cse4 integrated at an ectopic locus and examined microscopically when *GAL7pr-mCherry-Cse4* was expressed and repressed **(Figure C3A)**. The defects upon Cse4 repression were not observed as it was complemented for by the wild type copy.

Subsequently localization of an outer kinetochore, GFP-Dad1, and middle kinetochore protein, Nuf2-GFP, was examined. Cse4 co-localized with both proteins when expressed. Upon repression GFP-Dad1 was found to concordantly disappear from the centromere with Cse4. A comparable phenotype was observed upon Mif2 repression, when scored for Dam1 complex subunit Dad2 **(Figure C4A)**. To our surprise the Ndc80 complex subunit Nuf2 remained localized as a dot, with partial streaking upon complete absence of Cse4 **(Figure C3C)**. A similar observation was made when Mif2 was repressed, wherein mCherry-Ndc80 localization did not vary when scored for **(Figure C4A)**. To further analyze if this phenotype was unique to the dependency of the middle kinetochore with the inner, localization of Mtw1 was analyzed. Unlike the Ndc80 complex, Mtw1 was found to reduce its centromeric presence with that of the inner kinetochore protein Mif2, upon depletion. Western blot analysis was performed on cell lysates upon isolation of cells at various time points to test if the loss in centromeric localization of Dad1 was due to reduction in its protein levels upon Cse4 depletion. No reduction in Nuf2 of Dad1 protein levels was observed, ruling out the possibility that levels of Cse4 regulate the level of cellular protein pools of these kinetochore proteins **(Figure C3B)**.

The loss in centromeric localization of kinetochore proteins upon depletion of inner kinetochore proteins could be explained either by the dependency of other proteins on the inner layer proteins for their centromeric localization or requirement of Cse4 and Mif2 to stabilize and maintain their centromeric localization at the kinetochore.

As reported earlier depletion of Cse4 and Mif2 resulted in large amounts of nuclear segregation defects which were evident when scored for nuclear chromatin labeled by

fluorescent tagging histone H4 with GFP or mCherry, respectively **(Figure C3C and Figure C4A)**.

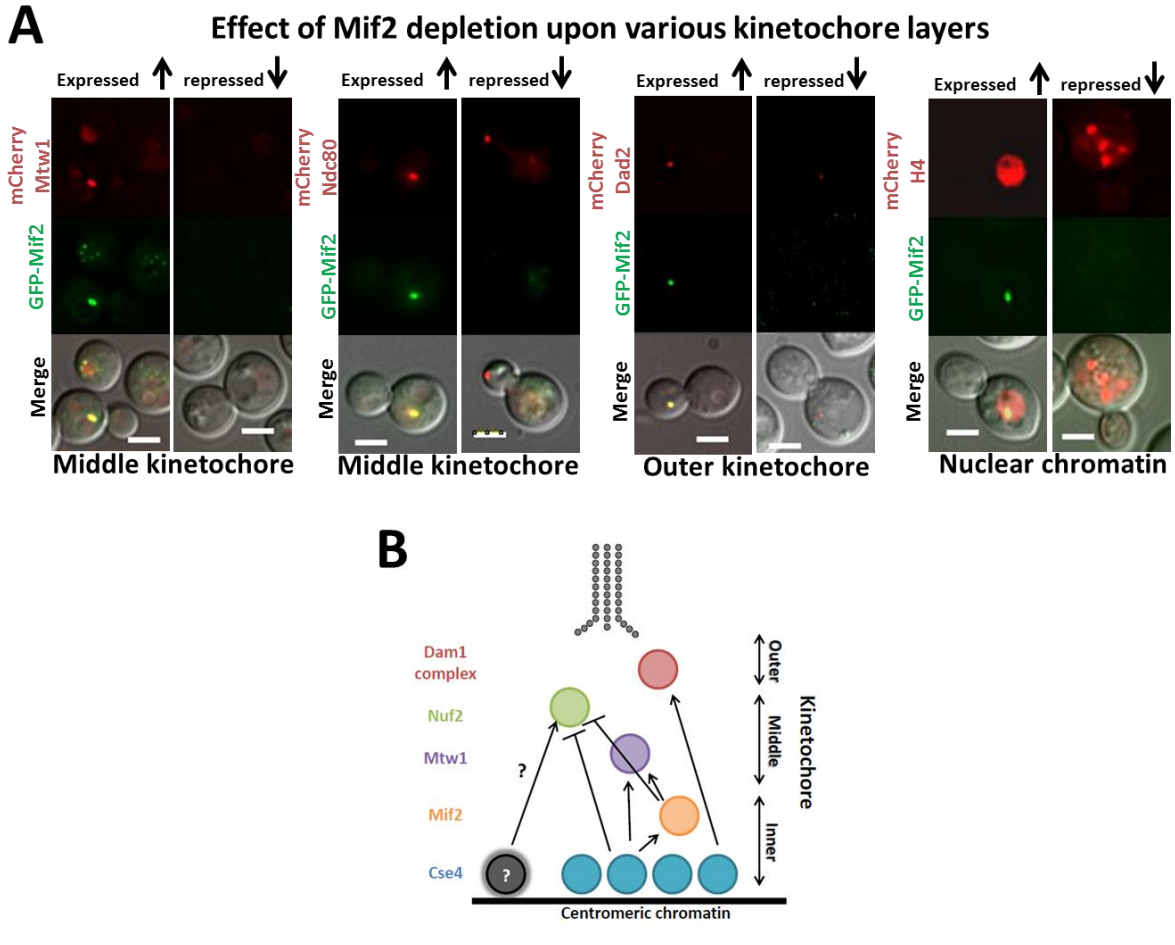

Figure C4. Localization of the Ndc80 complex to the centromere is independent of the inner kinetochore. A) Effect of Mif2 depletion upon various kinetochore layers. B) Schematic representing the interdependencies upon the inner kinetochore. Arrows point towards proteins that are dependent on it for their localization at the centromere. Scale 3µm.

This microscopic study on the localization of middle kinetochore proteins upon depletion of the inner kinetochore, hints at a possible existence of a bi-partite mode of assembly amongst the kinetochore. So, we then tested what the effect of depleting middle kinetochore proteins has on the rest of the kinetochore.

C2B. Centromeric localization and maintenance of the inner kinetochore proteins is independent of the middle kinetochore proteins.

To reciprocally test if the inner kinetochore assembled independently of the middle kinetochore complex and if Dam1 complex exhibited hierarchal assembly with Cse4 and Mtw1; microscopic analysis for localization dependency was performed upon *GAL7pr-GFP-Nuf2* and *GAL7pr-GFP-Mtw1* depletion.

It was found that the inner kinetochore proteins, Mif2 and Cse4, localized to the centromere which is independent of the middle kinetochore proteins, Mtw1 and Nuf2. While the outer kinetochore complex Dam1 subunit, Dad2 required Mtw1 for its localization **(Figure C5)**. The requirement of Nuf2 for Dam1 complex recruitment onto the centromere was not tested. In addition, as described earlier it was observed that upon depletion of middle kinetochore proteins there were large amounts of chromosome segregation defects; wherein these defects were not sensed by the spindle assembly checkpoint **(Figure C5 and figure C6A)**. A possible explanation to this observation is that the middle kinetochore proteins were required for the activation of the spindle assembly checkpoint, which has yet to be tested.

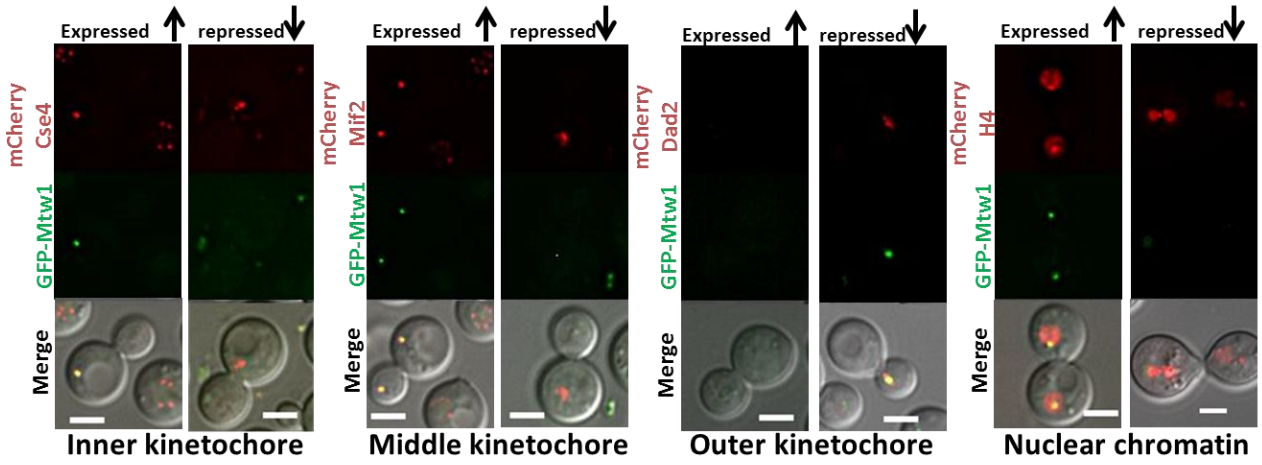

Figure C5. Centromeric localization and maintenance of the inner kinetochore proteins is not effected, while affecting the Dam1 complex upon depletion of Mtw1. Scale 3μm.

Tested kinetochore interdependencies revealed, kinetochore architecture which involves two parallel inter-connected pathways; one which is dependent on the centromeric histone Cse4 and the other, independent of Cse4, and supports the load bearing Ndc80 complex. It has been reported that the Ndc80 complex interacts with a centromeric protein called CENP-T (Bock, Pagliuca et al. 2012, Schleiffer, Maier et al. 2012). Yet this described pathway also involves the requirement of Cse4 for its localization. Hence extensive bioinformatics analysis was performed, yet no CENP-T homologue was found in this organism by us or independently by other groups (Bock, Pagliuca et al. 2012, Schleiffer, Maier et al. 2012) **(Figure C6B)**.

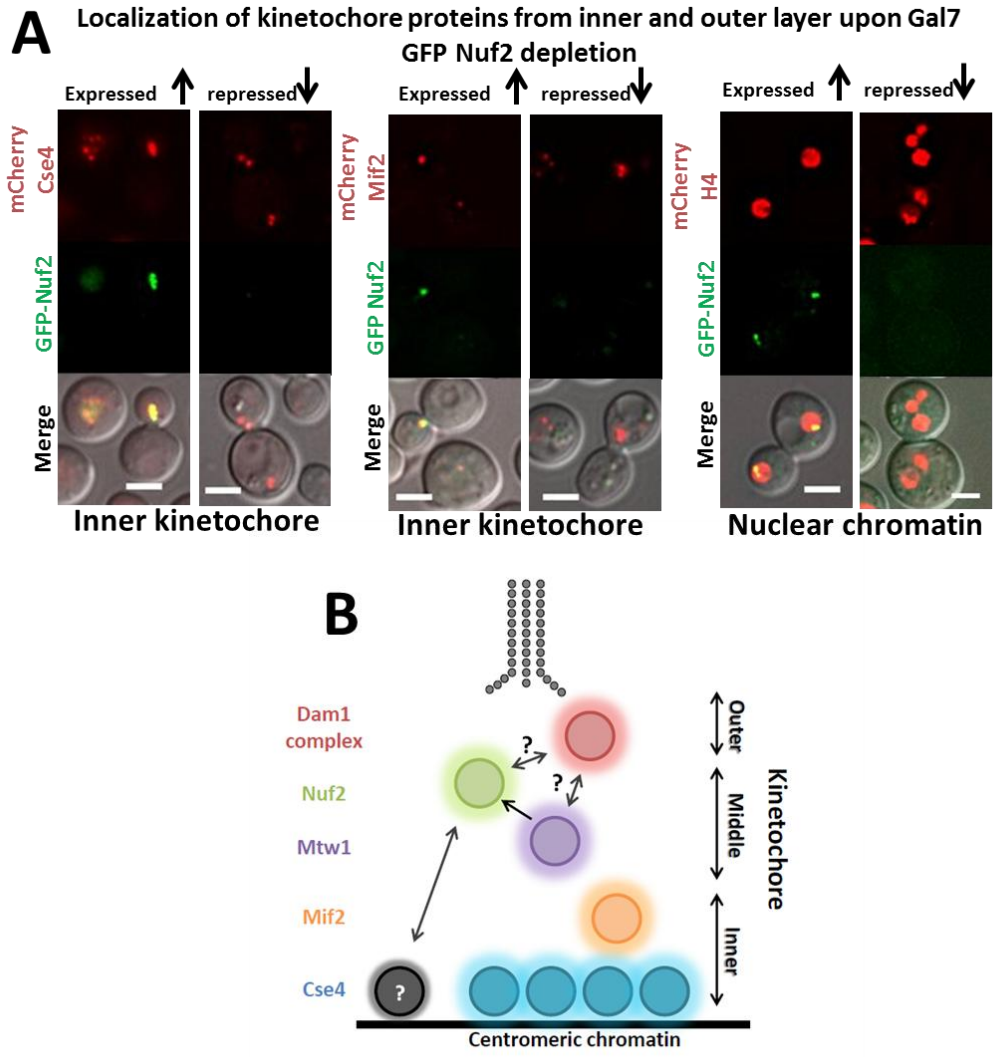

Figure C6. Existence of a Cse4 independent, kinetochore assembly pathway. A) Requirement of the Ndc80 complex protein, Nuf2 for the localization of inner kinetochore proteins. B) Schematic representation of only the interdependencies amongst the middle kinetochore proteins. Scale 3µm.

C3. Hierarchal interplay between kinetochore proteins, within the same layer.

Testing for interdependencies between kinetochore proteins revealed an evident hierarchy of proteins that required Cse4 for its localization and/or stability at the centromere. At the same time it was noticed that the Ndc80 complex did assembly independent of Cse4 and localization was maintained upon complete depletion of any/all of the other kinetochore proteins tested.

We further looked at the interdependencies of proteins that belonged to the same kinetochore layer (inner-inner, middle-middle and outer-outer) to better address the Ndc80 complex localization requirement and timing and recruitment of complexes that arrive at similar stages of mitosis.

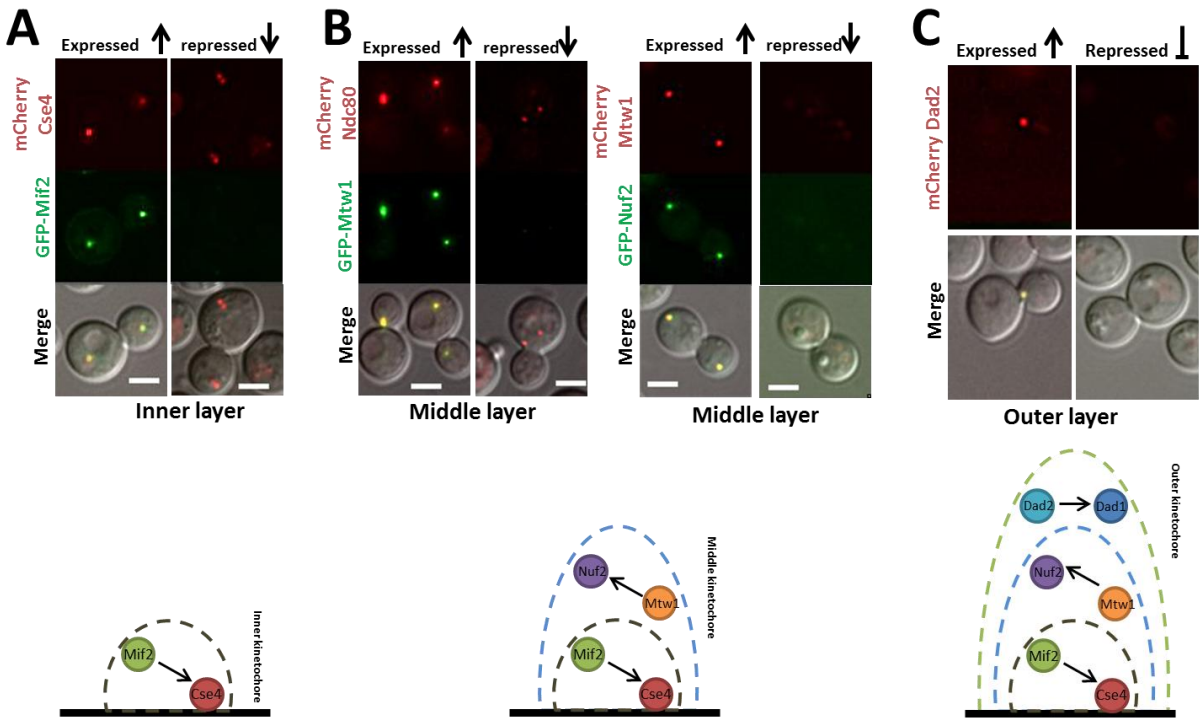

Figure C7. Interdependancy between kinetochore proteins of the same layer. A) Innter kinetochore. B) Middle kinetochore. C) Outer kinetochore proteins of the Dam1 complex. Scale 3µm.

Amongst the inner layer it was observed that Mif2 depended on the centromeric localization of Cse4 for its presence at the centromere **(Figure C7A)**. The reciprocal experiment was not performed, but would be tested subsequently. Amongst the middle

kinetochore proteins it was observed that the Ndc80 complex assembled independent of Mtw1, yet was required for Mtw1's localization at the centromere **(Figure C7B)**. It was also observed that a clear dependency existed between the Dam1 complex subunits Dad1 and Dad2 where Dad2, was dependent on Dad1 for its kinetochore localization **(Figure C7C)**.

This observation in compilation with the earlier study paints a picture of well-regulated interaction network that determines kinetochore architecture. Subsequent studies would be aimed at understanding the requirement of assembly for the Ndc80 complex and the requirement of the possible parallel mode of Cse4 independent assembly observed.

C4. Disruption of mitotic spindle by treatment with nocodazole does not affect kinetochore architecture, although affecting centromere clustering.

To probe further into the requirement of factors for kinetochore assembly, the role played by the mitotic spindle was examined. Towards this we used Nocodazole (100ng/ml), a microtubule de-polymerizing drug, to test the effect on kinetochore assembly and localization at the centromere. Cells were treated with Nocodazole for 3h and subsequently examined microscopically for the fluorescent signal. Examined data revealed a process of kinetochore assembly that is independent, not requiring of the mitotic spindle. Kinetochore proteins from all layers were centromere localized even after extended periods of treatment. It cannot be concluded that the kinetochore assembly is independent of microtubules as, upon scoring for tubulin localization after Nocodazole treatment, it was observed that there was residual microtubules that were attached/ in close proximity to the kinetochore that did not completely de-polymerize **(Figure C8A)**.

Strikingly it was seen that although the kinetochore architecture is not affected by the absence of the mitotic spindle the unique feature of centromere clustering was affected during mitosis **(Figure C7B)**.

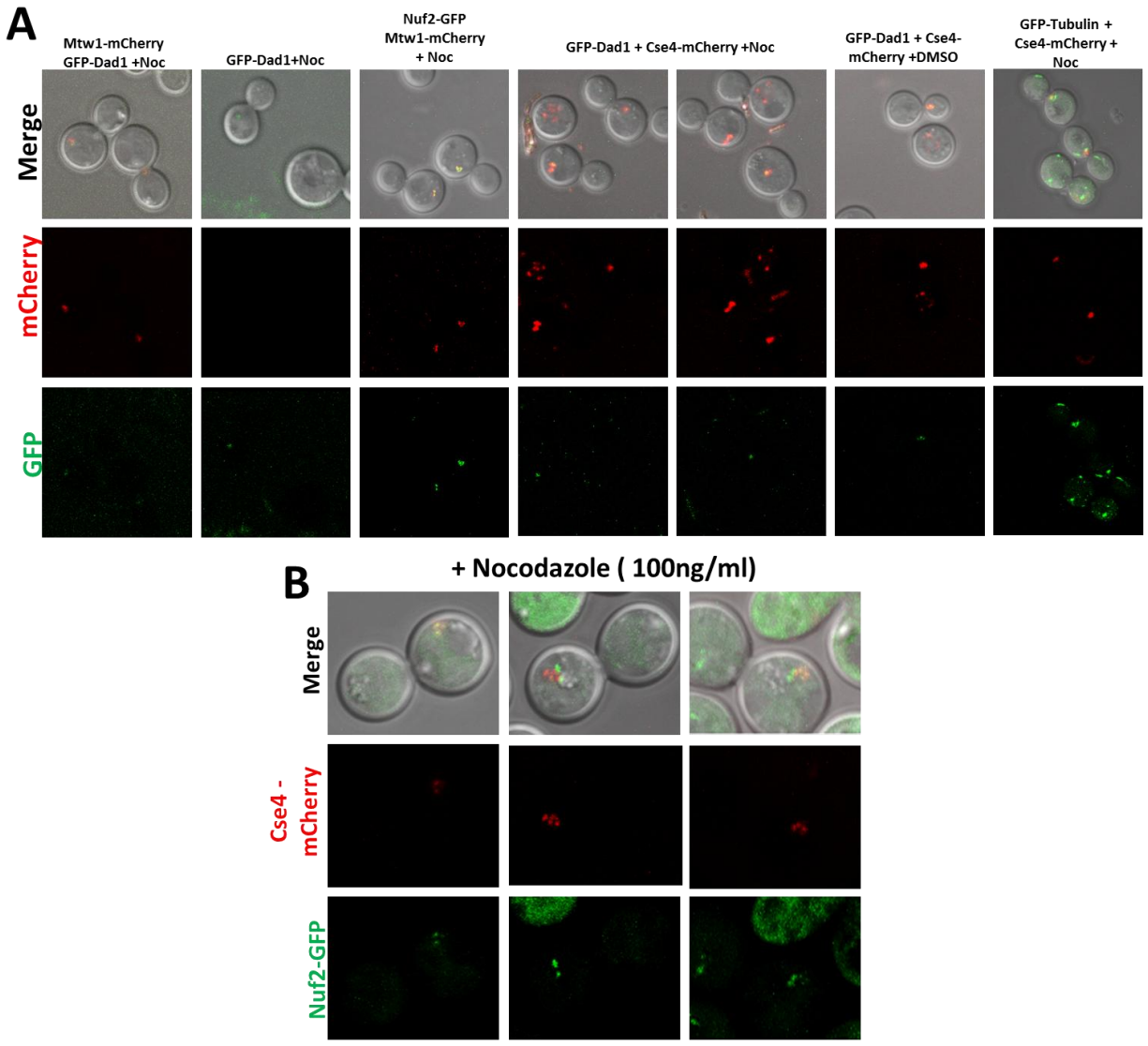

Figure C8. Clustering of centromeres is mitotic spindle dependent while kinetochore assembly in not. A) Localization of various kinetochore proteins upon treatment of cells with Nocodazole. B) Un-clustering of the centromere upon de-polymerization of the spindle microtubules.

Further analysis of kinetochore proteins upon depletion also yielded similar results, where un-clustered centromeres were observed. In conditions similar to microtubule de-polymerization by Nocodazole, kinetochore protein depletions were observed to effect KT-MT tension and attachment **(Figure C7A, Figure C6A and Figure C5B)**. This indicates the requirement of spindle tension/attachment at the kinetochore for bringing the centromeres together into a "clustered" state.

Discussion

A. Defining kinetochore architecture (inner, middle and outer): study of distinct spatial-temporal organization of the microtubule binding protein complexes, the Ndc80 and Dam1 complex, in *Cryptococcus neoformans var. grubii.*

We began the study with the aim of understanding the process of chromosome segregation in *C. neoformans* wherein my study focused on understanding the architecture and assembly of the kinetochore. Towards this we functionally tagged and localized several proteins constituting the primary microtubule binding complexes, Dam1 and Ndc80.

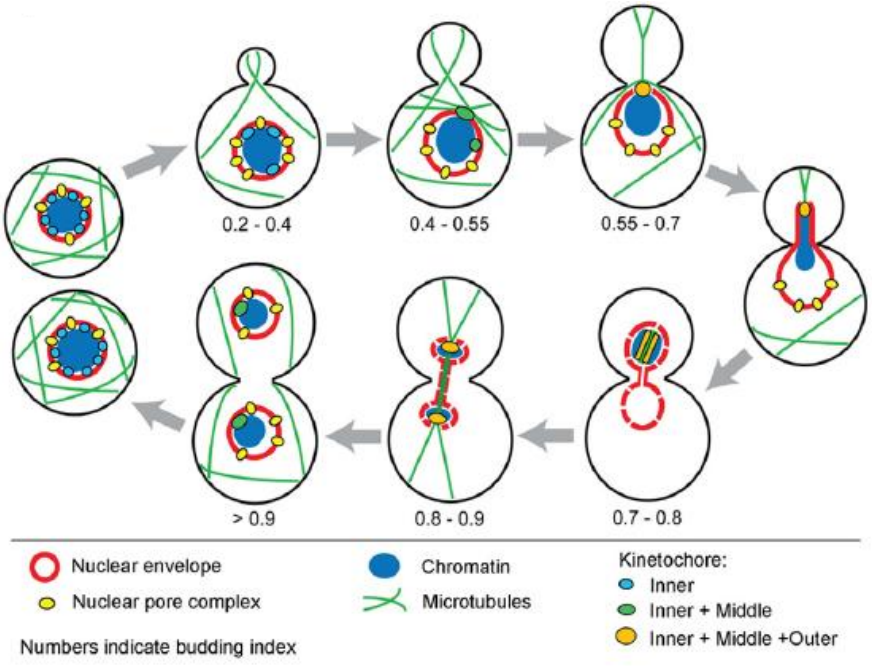

Figure D1. Cell cycle dynamics representing the stages of kinetochore assembly and disassembly in *Cryptococcus neoformans*.(Kozubowski, Yadav et al. 2013)

The spatial and temporal assembly of the kinetochore in this basidiomycetes yeast was found to be dissimilar to the dynamics of kinetochore assembly known in other yeasts and was more similar to that of metazoans. It was observed that the Dam1 and Ndc80 complex proteins localized to the centromere only upon the onset of mitosis, while the inner kinetochore proteins had a constitutive presence at the centromere throughout cell cycle. In addition, the Dam1 and Ndc80 complex proteins that assembled during mitosis exhibited an evident temporal distinction in arrival at the centromere. This temporal assembly of the Dam1 and Ndc80 complex proteins aided us in classifying the kinetochore

into the inner, middle and outer; based on the timing of assembly. The process of disassembly was observed to be hierarchal as well with the Dam1 complex proteins losing their centromeric localization prior to the middle, which occurred post-anaphase and before cytokinesis **(Figure D1)**. The kinetochore assembly in mitosis also took place concordantly with the clustering of centromeres and partial opening of the nuclear membrane in *C. neoformans* (Kozubowski, Yadav et al. 2013). These processes were unlike the constitutive presence of the complete kinetochore throughout cell cycle in budding yeast (Cheeseman, Drubin et al. 2002).

The partial opening of the nuclear membrane may be the restrictive step preventing entry of middle and outer kinetochore proteins into the nucleus thereby preventing premature completion of kinetochore assembly prior to mitosis. This raised several questions: is the clustering of the centromeres dependent on KT-MT interaction occurring at a completed kinetochore? Does the step-wise assembly of the kinetochore hint at the presence of a master regulator, or how are the interdependencies between kinetochore proteins co-ordinated?

B. Essentiality and requirement of kinetochore proteins from the proposed inner, middle and outer layers for chromosome segregation in *Cryptococcus neoformans.*

The reason behind the functional requirement of the fungal specific Dam1 complex is highly debated with recent reports offering an explanation: the microtubule binding Dam1 complex is essential in yeast where there exists only 1 kMT per centromere and aids in maintaining this attachment (Burrack, Applen et al. 2011, Thakur and Sanyal 2011). It was also noted that in these yeasts the kinetochore is assembled and bound to microtubules throughout cell cycle. In parallel, the Dam1 complex is non-essential in fission yeast where 2-3 microtubules attach to a centromere and the KT-MT interaction are brought about only on the onset of mitosis.

To test the validity of the argument we chose *C. neoformans* a basidiomycetes yeast, distinct from the previously studies exclusively performed on ascomycetes fungi, which clusters centromeres concordantly with the assembly of the kinetochore and results in establishing

KT-MT interactions subsequently. Preliminary studies were performed by creating a conditional mutant of the Dam1 complex subunits Dad1 and Dad2 by placing them under a *GAL7* regulatable promoter.

The Dam1 complex was found to be essential in this yeast as cells lost viability and failed to grow on repressive media. It could indicate at a state where there exists only 1 kMT per centromere in *C. neoformans,* yet this possibility has not been confirmed. Understanding this is a current priority as it would aid in explaining the requirement of the Dam1 complex exclusively in fungi in addition to why it might have been lost during evolution in other eukaryotes.

In a similar fashion, inner and middle kinetochore proteins were tested to understand if they were essential for viability in a system that has been shown to exhibit unique kinetochore dynamics. Utilizing the previously established *GAL7* controllable promoter, the kinetochore proteins studied, Cse4, Mif2, Mtw1 and Nuf2 were found to be essential. While performing these studies it was noticed that although these proteins form the same supra-molecular complex, upon depletion the observed phenotype varied significantly.

The depletion of Dam1 complex subunits, Dad1 and Dad2, resulted in large bud arrest with cells containing 2n ploidy and loss in viability was an outcome of cell cycle arrest **(Figure D3B)**. Intriguingly, upon depletion of inner, Cse4 and Mif2, and middle, Mtw1 and Nuf2, kinetochore proteins it was observed that no cell cycle arrest was observed and cells undertook erroneous chromosome segregation. This resulted in massive amounts of aneuploidy proving detrimental to cell viability over time. The absence of cell cycle arrest was also noticed when cells were examined microscopically, wherin a heterogenous population of cells present representing all stages of cell cycle was observed **(Figure D2)**.

The SAC had been known to delay cells in response to faulty KT-MT attachments, which arrested cells at metaphase to anaphase transition. At this checkpoint arrest which occurs post S-phase, analysis of DNA content of cells exibits a 2n ploidy. To validate the cell cycle arrest observed in response to the Dam1 complex protein depletion, *MAD2* an essential component of the cell cycle was deleted in this SHR710 (*GAL7pr-GFP-Dad1* background). Upon subsequent depletion of Dad1, cells failed to arrest with 2n ploidy and large bud phenotype was lost **(Figure D3D)**. The cell cycle profile observed upon mad2 delection

was similar to defects observed when inner and middle kinetochore proteins were depleted.

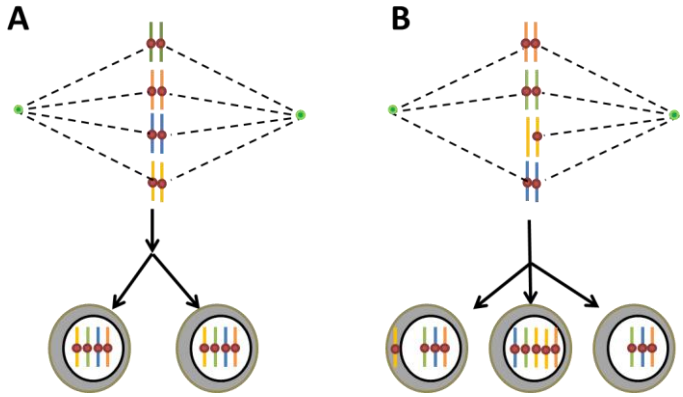

Figure D2. Segregation defects observed upon depletion of inner and middle kinetochore proteins. A) In a wild-type state the presence of a complete kinetochore and active spindle assembly checkpoint encure effective chromosome segregation. B) Depletion of inner and middle kinetochore proteins resulted in large segregation defects that accumulated over time, with the SAC unable to correct for them. While upon depletion of Dam1 complex proteins it was observed that cells arrested similar to A/B top panel , at the metaphase to anaphase transition, with segregation not being allowed.

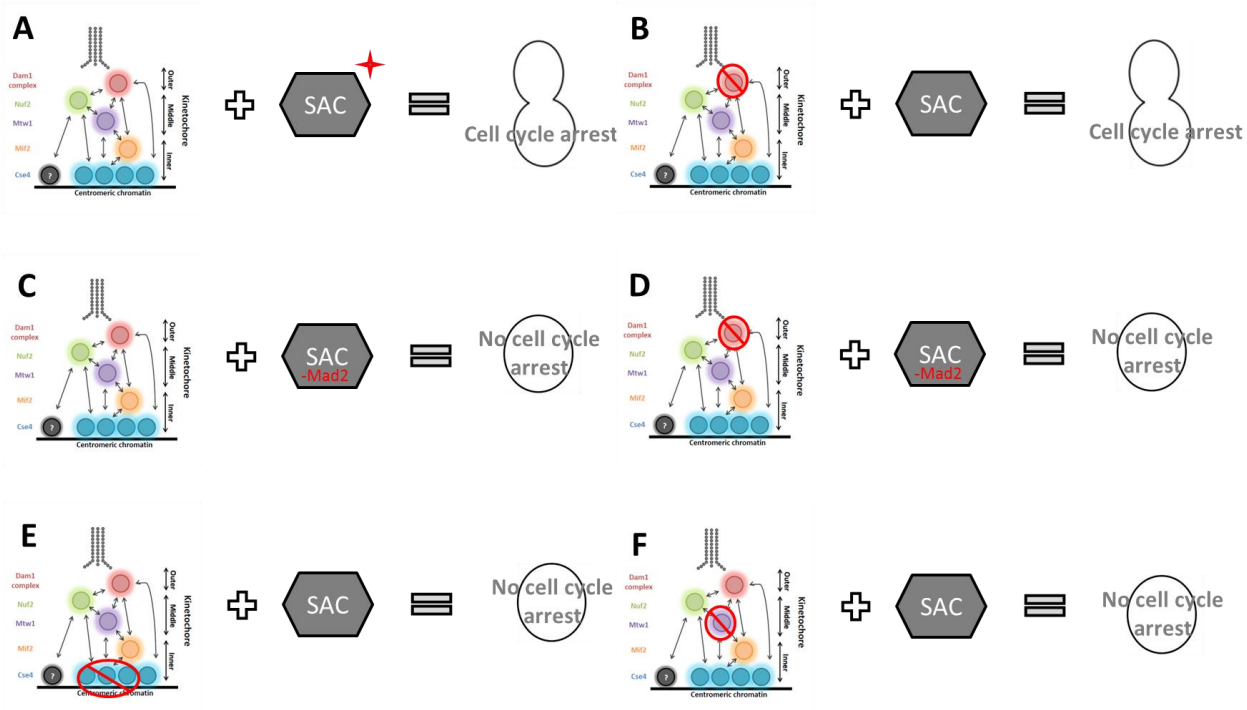

Figure D3. Interplay between the kinetochore and spindle assembly checkpoint (SAC). A) Wild-type cells delay the onset of mitosis when defects in KT-MT interactions exist, requiring both,

a functional the kinetochore and SAC components. B) Upon perturbing the KT-MT interaction by depleting Dam1 complex proteins and with a functional SAC, cells arrest at large-bud stage. C) and D) This arrest is abrogated when Mad2, a critical component of SAC signaling is deleted. E) And F) perturbing the inner and middle kinetochore complexes which effects KT-MT interactions do not activate SAC.

Hence we postulate that the inner and middle kinetochore proteins have a critical role to play in the spindle assembly checkpoint. Which might be at the level of activation or its subsequent down-stream signalling which we are yet to dissect **(Figure D3 E and F)**.

C. Intra and inter-dependancy between various layers of the kinetochore: insights into kinetochore architecture.

Much had been gleaned about the spatio-temporal assembly of kinetochore proteins from the earlier studies, by localizing various kinetochore proteins and following them throughout cell cycle. Although we observed a step wise assembly of the kinetochore we were unable to dissect out the timing of localization of various kinetochore proteins from similar layers, that assembly simultaneously onto the centromere. This approach had several drawbacks which limited the information we were able to extract regarding kinetochore architecture and assembly in *C. neoformans*. In addition, the scaffold required for the stability various kinetochore proteins and/or localization to the centromere could not be gathered.

In previously constructed conditional mutants using the *GAL7* regulatable promoter, we tagged various kinetochore proteins with fluorescent reporters so as to be able to follow them over time and determine their centromere occupancy using fluorescence microscopy, upon perturbing various kinetochore proteins.

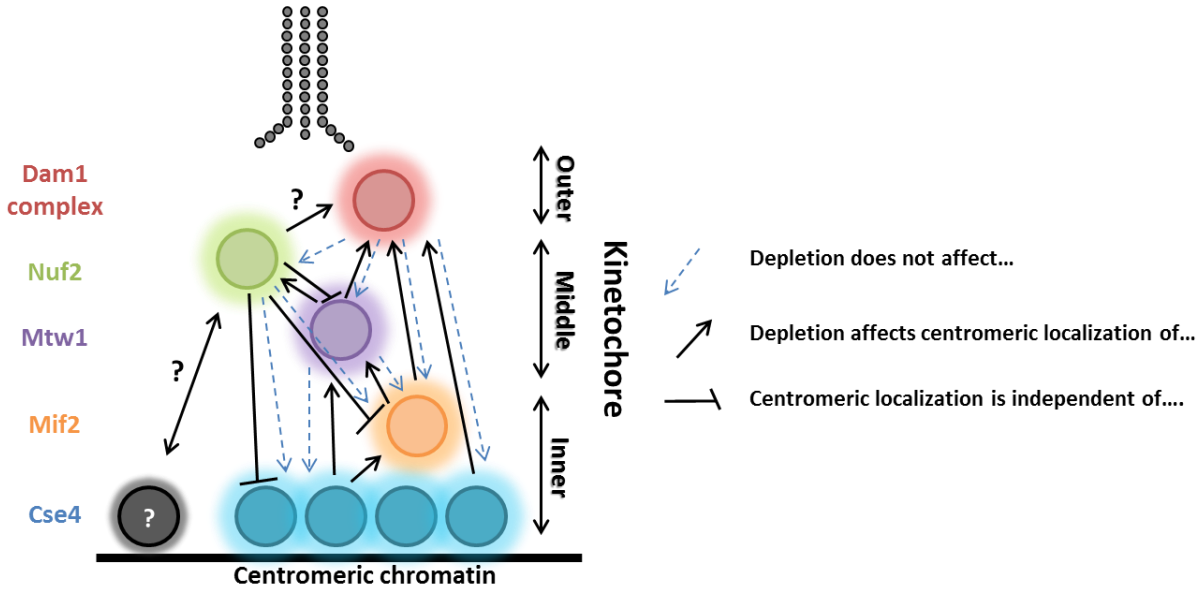

Figure D4. Network of interdependencies between kinetochore proteins in *Cryptococcus neoformans.*

Through this method we were able to determine at a complex interplay between various kinetochore layers and complexes **(Figure D4.)**. We observed that affecting the localization of a kinetochore protein further away from centromeric chromatin did not affect the localization of the underlying kinetochore proteins, while affecting the inner layer proteins affected the entire structure of the kinetochore. Yet, an exception to this structural independency was the localization of the Ndc80 complex that was not dependent on any of the complexes we tested. But this complex affected the localization of the Mtw1 and Dam1 complex proteins. These observations hint at the possibility of a novel/ parallel mode of kinetochore assembly being present in *C. neoformans.*

Reports in higher eukaryotes have described of a centromeric protein, CENP-T that interacts directly with centromeric chromatin and plays the role of a platform for the Ndc80 complex. This CENP-T complex was also recently identified in yeast (Bock, Pagliuca et al. 2012, Schleiffer, Maier et al. 2012). However ours and the efforts of other groups, via bioinformatics have not yielded any positive hits for the presence of such homologue in *C. neoformans*. Interestingly analysis in its close basidiomycete relative, *Ustilago maydis* have yielded putative homologues. Hence the question, could there exist a novel protein holding

up the Ndc80 complex? Or is the complex directly binding to centromeric chromatin in a Cse4 independent protein? These questions are to be answered in the near future.

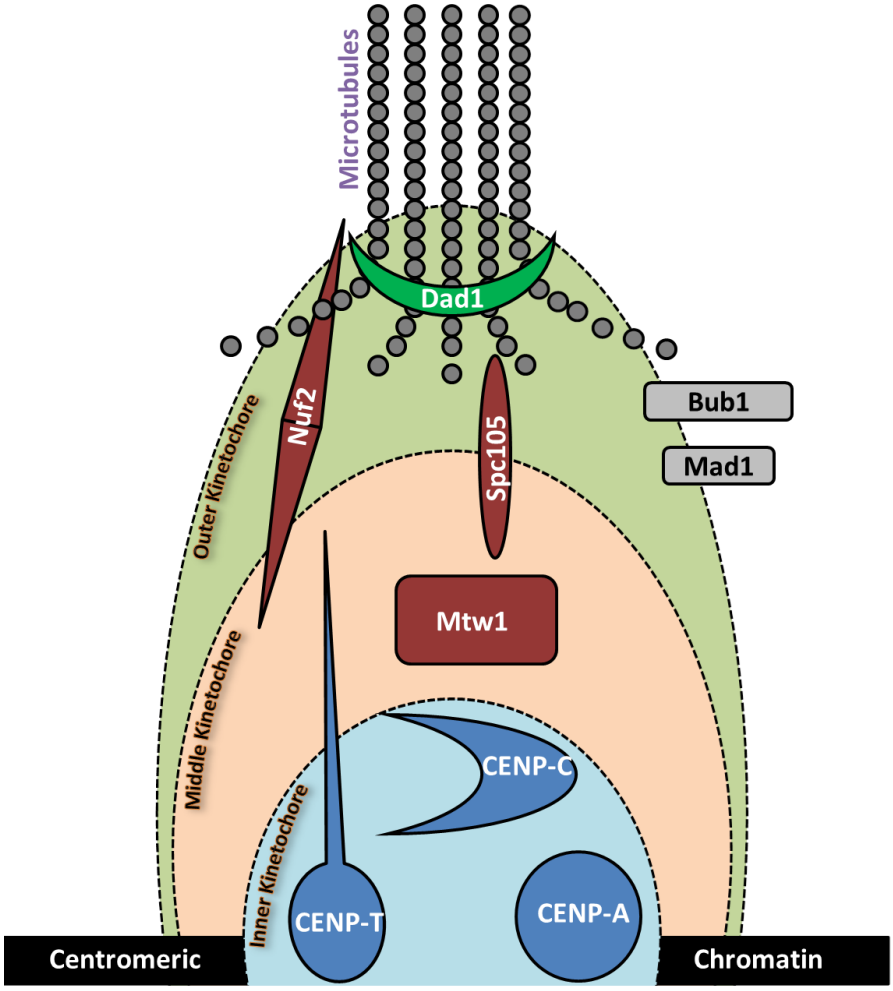

Figure D5. Proposed model of the *C. neoformans* kinetochore.

In addition, it was also observed that there existed a unique pattern of dependence between proteins proposed to be part of the same layer. If was found that Mif2 required Cse4 for its centromeric localization and Mtw1 required Nuf2. Amongst the Dam1 complex also it was found that Dad2 required the presence of Dad1 for its centromeric localization.

Further studies on these and other kinetochore complexes could shed much light about an evolutionary distinct mode of kinetochore assembly in this basidiomycete. This study could also introduce *C. neoformans* as a worthy new model system to study mitotic process, more so the kinetochore architecture for it presents several distinct phenotypes at each stage of kinetochore assembly that could be scored for easily.

Material and methods

M1. Identification of kinetochore protein ORF's

BLAST analysis was performed using the sequence alignment tool available at http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/Blast.html to identify *C. neoformans* homologues of *S. cereveisiae NUF2, NDC80 and DAD2,* which was used as query sequence for the blast searches. The putative *CnNUF2* Orf CNAG_00680 exhibited significant homology with an Expect score of 2.272E-17. This identified Orf was 1916 nt in length coding for 450aa. Similar search analysis yielded *CnNDC80*, CNAG_07635, a putative homologue that had 27% identity with the *ScNDC80* and was highly conserved at the globular head region with an Expect value of 1E-34. The putative *CnDAD2* Orf identified was as CNAG_00327. This putative homology of *ScDAD2* was poorly conserved with 18% identity and an E value of 1.4. (Appendix table 1)

M2. Strain construction: Tagging of kinetochore proteins with a fluorescent reporter.

The entire Orf of *NUF2, NDC80* and *DAD2* was cloned into the vector construct pCIN19 by single site cloning using BamHI, tagging the proteins with GFP at the N-terminal and this transgene copy was integrated at an ectopic loci. Subsequently to replace the native copies of these kinetochore proteins, *NUF2 US* and *DS* (~1 kb) was cloned into pS2GN using the restriction sites XbaI/PstI and SalI/ApaI respectively. This cassette was transformed into Wild-type (H99α) cells replacing the native copy with a C- terminal GFP tag yielding the strain SHR515. To replace the native NDC80 Orf with a mCherry tagged construct, overlap PCR was performed. The C-terminal mCherry tagged transgene was transformed to replace the native copy of the gene by homologous recombination yielding SHR512.

M3. Construction of conditional kinetochore mutants.

A vector construct was created by cloning 1 kb of the *Gal 7* promoter into pJAF15 plasmid using the restriction sites BamHI/SpeI. An insert containing *HPT-Galactose 7* promoter was subsequently excised and cloned into pBSII KS (-) at sites NotI/BamHI. This was selected by α-complimentation of β-Galactosidase, using X-GAL and IPTG in the media. Subsequently

GFP/mCherry was clones downstream of the Galactose7 promoter, while excluding the stop codon at sites BamHI/HindIII yielding the plasmids pSH7G and pSH7M respectively.

For the creation of conditional kinetochore mutants ~1 kb of the US and DS sequences for CSE4, MIF2, MTW1, NUF2, NDC80, DAD1 and DAD2 was cloned into both pSH7G and pSH7M, independently of GFP was replaced with mCherry upon completion of cloning or vice versa, using the restriction sites SacI/NotI to clone the US sequences of CSE4, MIF2, MTW1, NUF2, NDC80, DAD1 and DAD2. To clone the DS sequences HindIII/XhoI sites was used for CSE4 and MIF2, HpaI/XhoI sites used for MTW1/DAD2, HindIII/KpnI to clone the DS of NUF2 and HpaI/KpnI sites were used to clone DAD1 DS. The cloned and confirmed cassettes were released and used for transformation.

Southern blot analysis was used to confirm the replacement of the native copy with the transgene. The genomic DNA from PCR confirmed transformants were prepared by glass bead lysis method, digested, blotted transferred onto Zeta-Probe membranes (BIO-RAD) and probed with Phoshorus-32 labeled DNA fragment sequence present outside the cassette region used for transformation.

Sl.No	Strain name	Cassette used to replace native copy	Enzyme used	Fragment lengths expected(bp)		Figure
				Wild-type	Transgene	
1.	SHR740/SHR702	*Gal7pr-GFP/mCherry-CSE4:HYG*	ClaI	2884	5383	B5
2.	SHR716/SHR735	*Gal7pr-GFP/mCherry-MIF2:HYG*	StuI	2575	3718	B8
3.	SHR717/SHR736	*Gal7pr-GFP/mCherry-MTW1:HYG*	EcoRV	4050	2976	Not shown
4.	SHR718/SHR737	*Gal7pr-GFP/mCherry-NUF2:HYG*	ClaI	2890	4803	B10
5.	SHR710/SHR739	*Gal7pr-GFP/mCherry-DAD1:HYG*	ClaI	3281	6047	B2
6.	SHR738/SHR719	*Gal7pr-GFP/mCherry-DAD2:HYG*	EcoRV	4113	6822	B4
7.	SHR734	*mad2:NEO*	NcoI	3208	4538	Not shown

Table M1. Tabulation of enzymes used and fragment lengths expected upon southern blot confirmation of kinetochore conditional mutant strains

M4. Construction of *mad2* null strain.

To construct the deletion cassette for *MAD2* ~1 kb of US and DS was cloned into pLK25 using SacI/SpeI and NotI/BglII respectively. The cassette was release and transformed to delete the native copy with selection for Neomycin resistance.

M5. Media and growth conditions

Strains containing fluorescent tagged kinetochore proteins were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose/glucose) with shaking (180rpm) at 30^{o}C. While strains containing kinetochore proteins under the control of the *GAL7* promoter were grown in permissive conditions containing YPG (1%yeast extract, 2% peptone and 2% galactose) under shaking at 180 rpm maintained at 30^{o}C. For non-permissive media the aforementioned YPD media was used.

M6. *C. neoformans* transformation by biolistic method

Cells were grown in YPD/YPG to late log phase, pelleted; supernatant discarded, re-suspended in water and plated onto YPD/YPG containing 1M Sorbitol plates for transformation. The cells were then bombarded with 0.6µm gold beads (BIO-RAD) that were coated with DNA of the required construct, using 1M CaCl2 and 1M spermidine free base and washed with 100% ethanol. Bombardment of the cells with the gold beads was performed using the Biolistic® PDS-1000/He Particle delivery system at 1350Psi. Following transformation cells were incubated for 4-6 h at 30^{o}C on non-selective media for recovery. Upon rescue cells were recovered from the transformation plate by using water and plated on selective plates containing recommended concentration of antibiotics. Transformants were observed in 3-5 days post-transformation.

M7. Viability assays and measuring chromosome segregation defects.

Conditional mutants of *CSE4, MIF2, MTW1, NUF2, DAD1* and *DAD2* were grown overnight in permissive media (+galactose –glucose). Innoculated at 0.2 OD into permissive media the following day and grown until 0.8-1OD. Pelleted, washed and resuspended in repressive media (+glucose) at 0.2OD. Cells at specific time points were harvested, counted, serially diluted and plated on permissive plates and grown for 2days at 30^{o}C. Colonies were counted and viability curve was plotted.

M8. Calcofluor staining

Cells were harvested as required, a drop of cell suspension placed on a clean glass slide, one drop of Calcofluor staining solution (Cat. No. 18909, Sigma-aldrich) and 10% Potassium Hydroxide was added. A coverslip was places and incubated for 1min at RT before examination under UV light at 1000X magnification.

M9. Flow cytometry analysis

Conditional mutants for kinetochore proteins and wild type cells were grown and harvested at various time points as performed for viability assays and fixed with 80% ethanol added drop-wise while mixing the cell suspension in 100µl of water. Cells were fixed overnight at 4^{0}C. Pelleted and washed twice with RNAse buffer and subsequently resuspended in RNAse buffer containing 1mg/ml RNAse (Cat.No.R4875, Sigma-aldrich) and incubated for 4-6h at 37^{0}C with shaking. Cell suspension was then pelleted and washed with 1X PBS, repeated twice and stored at 4^{0}C until required. Propidium iodide (Cat.No.81845, Sigma-aldrich) staining was performed at 10µg/ml final concentration. Fluorescent intensity was acquired using 561nm laser, BD®FACS Aria III system.

M10. Nocodazole treatment

An overnight culture of cells was grown, subsequently inncoulated into fresh media @0.2OD containing low or high concentration of Nocodazole (Cat.No. M1404, Sigma-

aldrich) 100ng/ml or 10µg/ml respectively. Cells were observed under a bright field and fluorescent microscope at 1000X magnification at varying time points to observed cell phenotype and kinetochore protein localization.

M11. Fluorescence microscopy, image capture and processing

To observe the localization of various kinetochore proteins tagged in a wild-type background, cells were grown in YPD to early log phase, pelleted, washed and resuspended in distilled water. A drop of the cell suspension was placed on a clean glass slide and observed under the microscope. To observe the localization of kinetochore proteins upon depletion cells were grown in permissive media and subsequently transferred to non-permissive media, mentioned in M6. Harvested cells at various time points was pelleted, washed and resuspended in distilled water. Images were captured either using Carl Zeiss confocal laser scanning microscope (LSM 510 META) or DeltaVision system (Olympus X1-71 base) equipped with a CoolSnap HQ^2 high-resolution charge-coupled-devise (CCD) camera and a 100X objective (100X/1.40 oil , differential interference contrast [DIC] /0.17/FN26.5, UIS2 series). The filters used (Live cell) were GFP/fluorescein isothiocyanate (FITC) 475/28, mCherry/AF594 575/25 for excitation and GFP/FITC 525/50, mCherry/AF594 632/60 for emission and a Dual em pass GFP/mCherry filter for high-speed imaging. For confocal microscopy, Ar 488 and HeNe 543 lasers were used for excitation of GFP and mCherry, respectively. The image processing was done using the Zeiss image processing software LSM5 Image Examiner, ImageJ, Image-Pro Plus, or Photoshop (Adobe Systems, San Jose, CA).

M12. Western blot analysis

Western blot analysis was carried out to confirm the expression of tagged kinetochore proteins and reduction in cellular protein pools, upon growth in repressive media.

Cells were harvested at various time points and lysates were prepared by TCA precipitation protocol. Harvested cells were suspended TCA solution of final concentration of 15%, stored at -70^0C / -20^0C overnight. Subsequent cells were thawed on ice, pelleted, re-

suspended in lysis buffer and bead beated until 90% lysis was achieved. Cells were then resuspended in SDS-loading buffer. Lysates were subjected to electrophoresis using 10% /12% SDS PAGE. Proteins were then transferred onto nitrocellulose membranes for 30min at 2A by semi-dry transfer method. Transfer of proteins onto the membrane was confirmed by 0.1 %(w/v) Ponceau staining. Membranes were blocked with 5% skim milk for 1h followed by incubation with primary antibody in 2.5% skim milk, overnight at 4^0C. Washes were given with 1XPBS+0.1% tween 20 and incubated with secondary antibody in 2.5% skim milk for 3h at RT. Blots were subsequently developed using Genesys® (Syngene systems, Inc.).

M13. Antibodies

Primary antibodies used for western blot analysis: mouse anti-GFP antibody 1:5000 (Cat.No.11814460001, Roche), rat anti-mCherry antibody 1:2000(Cat.no.632496, Clontech) and mouse anti-PSTAIR antibody 1:10000(Cat.No.ab10345, Abcam®).

Secondary antibodies used for western blot analysis were anti-mouse HRP conjugated antibody 1:2000 (Bangalore Genei Cat # HP06) and anti-rat HRP conjugated antibody 1:2000(Bangalore Genei).

M14. Strains, plasmids and primers used for the study

The constructed strains and plasmids and also the primers used for this study are tabulated in the Appendix tables A2, A3 and A4 respectively.

Appendix

A1. Proteins under study:

Protein name	ORF Number^
CnNdc 80	CNAG_07635
CnNuf2	CNAG_00680
CnDad2	CNAG_00327
CnHistone H4	CNAG_01648
CnNdc1	CNAG_01045
CnCENP-A	CNAG_00063
CnCENP-C	CNAG_05391
CnDad1	CNAG_02082
CnMis12	CNAG_04157
CnMad2	CNAG_01638

^ As annotated in *Cryptococcus neoformans var. grubii genome* database.

A2. *Cryptococcus neoformans* strains constructed:

Strain	Genotype	Source
***C. neoformans* var. *grubii* (serotype A, congenic to H99α and KN99a)**		
SHR501	*MAT*a *DAD2-mCherry:NEO*	This study
SHR502	*MATa DAD2-mCherry::NEO GFP-CSE4:NAT*	This study
SHR503	*MAT*a *GFP-NUF2::NAT CSE4-mCherry::NEO*	This study
SHR504	*MATa GFP-NUF2::NAT*	This study
SHR505	*MAT*a *GFP-NDC80:NAT CSE4-mCherry::NEO*	This study
SHR506	*MATa GFP-NDC80:NAT*	This study
SHR507	*MATa GFP-DAD2:NAT*	This study
SHR508	*MAT*a *GFP-DAD2:NAT CSE4-mCherry::NEO*	This study
SHR509	*MATa NDC80-mCherry::NEO GFP-CSE4:NAT*	This study
SHR510	*MAT*a *NDC80-mCherry::NEO NUF2-GFP:NAT*	This study
SHR511	*MATa NDC80-mCherr:: NEO GFP-DAD1:NAT*	This study
SHR512	*MAT*a *NDC80-mCherry::NEO*	This study
SHR513	*MATa NDC80-mCherry::NEO GFP-DAD2:NAT*	This study
SHR514	*MATa NUF2-GFP::NAT CSE4-mCherry::NEO*	This study
SHR515	*MATa NUF2-GFP::NAT*	This study

SHR516	*MAT*a *NUF2-GFP::NAT MTW1-mCherry::NEO*	This study
SHR519	*MAT*a *GFP-NDC80:NAT MTW1-mCherry::NEO*	This study
SHR701	*MAT*a/*a GFP-CSE4:NAT GAL7pr mCherry-CSE4::HYG*	This study
SHR702	*MAT*a *GAL7pr mCherry-CSE4::HYG*	This study
SHR703	*MATa GFP-NDC1 NAT:GAL7pr mCherry-CSE4::HYG*	This study
SHR705	*MATa GFP- H4 NAT:GAL7pr mCherry-CSE4::HYG*	This study
SHR706	*MATa GFP- DAD1:NAT GAL7pr mCherry-CSE4::HYG*	This study
SHR707	*MATa GFP- aTUB:NAT GAL7pr mCherry-CSE4::HYG*	This study
SHR710	*MATa GAL7pr GFP-DAD1::HYG*	This study
SHR711	*MATα CSE4-mCherry:NEO GAL7pr GFP-DAD1::HYG*	This study
SHR712	*MATα MTW1-mCherry:NEO GAL7pr GFP-DAD1::HYG*	This study
SHR713	*MATα NDC80-mCherry :NEO GAL7pr GFP-DAD1::HYG*	This study
SHR714	*MATα H4-mCherry:NEO GAL7pr GFP-DAD1::HYG*	This study
SHR801	*MAT*a *rdp1::NEO GAL7pr mCherry-CSE4::HYG*	This study
SHR802	*MATardp1::NEO GFP-H4:NAT*	This study
SHR803	*MATardp1::NEO NUF2-GFP:NAT*	This study
SHR804	*MAT*a *rdp1::NEO GAL7pr GFP-DAD1::HYG*	This study
SHR805	*MATα ago1::NAT MIF2-mCherry:NEO*	This study
SHR806	*MATα ago1::NAT MTW1-mCherry:NEO*	This study
SHR807	*MATα ago1::NAT NDC80-mCherry:NEO*	This study
SHR715	*MATα DAD2-mCherry:NEO GAL7pr GFP-DAD1::HYG*	This study
SHR716	*MATa GAL7pr GFP-MIF2::HYG*	This study
SHR717	*MATa GAL7pr GFP-MTW1::HYG*	This study
SHR718	*MATa GAL7pr GFP-NUF2::HYG*	This study
SHR719	*MAT*a *GAL7pr mCherry-DAD2::HYG*	This study
SHR720	*MATα NDC80-mCherry:NEO GAL7pr GFP-MIF2::HYG*	This study
SHR720	*MATα MTW1-mCherry:NEO GAL7pr GFP-MIF2::HYG*	This study
SHR721	*MATα DAD2-mCherry:NEO GAL7pr GFP-MIF2::HYG*	This study
SHR722	*MATα H4-mCherry:NEO GAL7pr GFP-MIF2::HYG*	This study
SHR723	*MATα CSE4-mCherry:NEO GAL7pr GFP-MIF2::HYG*	This study
SHR724	*MATα NDC80-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR725	*MATα CSE4-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR726	*MATα MIF2-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR727	*MATα DAD2-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR728	*MATα H4-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study

SHR729	*MATα CSE4-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR730	*MATα H4-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR731	*MATα MIF2-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR732	*MATα MTW1-mCherry:NEO GAL7pr GFP-MTW1::HYG*	This study
SHR733	*MATα mad2::NEO GAL7pr GFP-DAD1::HYG*	This study
SHR734	*MATα mad2::NEO*	This study
SHR735	*MATa GAL7pr mCherry-MIF2::HYG*	This study
SHR736	*MATa GAL7pr mCherry -MTW1::HYG*	This study
SHR737	*MATa GAL7pr mCherry -NUF2::HYG*	This study
SHR738	*MAT*a *GAL7pr GFP-DAD2::HYG*	This study
SHR739	*MATa GAL7pr mCherry-DAD1::HYG*	This study
SHR740	*MAT*a *GAL7pr GFP-CSE4::HYG*	This study
SHR741	*MATα GFP-H4:NAT mad2::NEO*	This study
SHR742	*MATα GFP-NDC1:NAT mad2::NEO*	This study
SHR743	*MAT*a *GFP-H4:NAT GAL7pr mCherry-DAD2::HYG*	This study
SHR744	*MATα mad2::NEO GAL7pr GFP-MIF2::HYG*	This study
SHR745	*MATα mad2::NEO GAL7pr GFP-MTW1::HYG*	This study
SHR746	*MATα mad2::NEO GAL7pr GFP-NUF2::HYG*	This study
SHR747	*MATα mad2::NEO GAL7pr mCherry-CSE4::HYG*	This study
SHR748	*MATα mad2::NEO GAL7pr mCherry-DAD2::HYG*	This study
SHR749	*MAT*a *GFP-NDC1:NAT GAL7pr mCherry-DAD2::HYG*	This study
SHR750	*MATα GFP-NDC1:NAT GAL7pr mCherry-MTW1::HYG*	This study
SHR751	*MATa GFP-TUB:NAT GAL7pr mCherry-MTW1::HYG*	This study
SHR752	*MAT*a *GFP-TUB:NAT GAL7pr mCherry-DAD2::HYG*	This study
CNV108	*MATα GFP-H4:NAT*	Kozubowski, et al., mBio, 2013

A3. Plasmids constructed:

Strain	Genotype	Source
pSS01	*Dad2 mCherry NEO Amp^R*	This study
pSS02	*mCherry NEO Amp^R*	This study
pSS04	*Gal7pr GFP Dad1 HYG Amp^R*	This study
pSS05	*Gal7pr mCherry Cse4 HYG Amp^R*	This study
pSS06	*H3pr GFP H3.4828 NAT Amp^R*	This study

pSS07	*Gal7pr GFP Nuf2 HYG Amp^R*	This study
pSS08	*H3pr GFP Ndc80 NAT Amp^R*	This study
pSS09	*H3pr GFP Dad2 NAT Amp^R*	This study
pSS10	*Nuf2 GFP NAT Amp^R*	This study
pSS11	*H3pr GFP 08cdN NAT Amp^R(opposite orientation)*	This study
pSS12	*H3pr GFP Dad2 NAT Amp^R(opposite orientation)*	This study
pSH2	*Gal7pr HYG Amp^R*	This study
pSH7G	*Gal7pr GFP HYG Amp^R*	This study
pSH7M	*Gal7pr mCherry HYG Amp^R*	This study
pS2GN	*GFP NAT Amp^R*	This study
pFUN2	*H3pr GFP Nuf2 NAT Amp^R*	This study
pSS13	*Gal7pr GFP Mif2 HYG Amp^R*	This study
pSS14	*Gal7pr GFP Mtw1 HYG Amp^R*	This study
pSS15	*Gal7pr GFP Nuf2 HYG Amp^R*	This study
pSS16	*Gal7pr GFP Cse4 HYG Amp^R*	This study
pSS17	*Gal7pr GFP Dad2 HYG Amp^R*	This study
pSS18	*Gal7pr mCherry Mif2 HYG Amp^R*	This study
pSS19	*Gal7pr mCherry Mtw1 HYG Amp^R*	This study
pSS20	*Gal7pr mCherry Nuf2 HYG Amp^R*	This study
pSS21	*Gal7pr mCherry Dad2 HYG Amp^R*	This study
pSS22	*Gal7pr mCherry Dad1 HYG Amp^R*	This study
pSS23	*Δmad2 NEO Amp^R*	This study

A4. Primers used:

Primer name	Sequence(5'- - - - - 3')
pCIN19-GFP FP	GTGCGGATTCATGGTGAGCAAGGGCGAGG
pCIN19-GFP RP	GTGCAAGCTTGTTAACGTACAGCTCGTCCATGCCGTG
mCherry-FP	GTGCGGATCCATGGTGAGCAAGGGCGAG
mCherry-RP	GTGCAAGCTTGTTAACCTTGTACAGCTCGTCCATGC
pCIN19-GFP-NAT FP	CAAACTGCAGATGGTGAGCAAGGGCGAG
pCIN19-GFP-NAT RP	ACCCAAGCTTAGGATGTGACCTGGAGAGC
Cm-Nuf2 FP	GTCCGGATCCATGTCGCAGCAGAATCGCAGAGTGC
Cn-Nuf2 RP	GTCCGGATCCTCACCTGCAAACAGCATGGTAATGC

Cn-DAD1 US -FP Gal7	GTGCGAGCTCGCTAGCTTCTCCAAGATGGGTGTCACG
Cn-DAD1 US- RP Gal7	GTGAGAATGCGGCCGCCTTGGAGTGCTAGTTTTCCTGC
Cn-DAD1 FP Gal7	ATGTCTTTATCAAGACCATCGAATGCCTACGATGC
Cn-DAD1 RP Gal7	GTGCGGTACCGAGCTCATGCCTATGAAGTCCAGC
Gal7-Cse4-US-FP	CAACGAGCTCGGTAAAAGGTCACCAGTAGCAG
Gal7-Cse4-US-RP	ATAGTATTGCGGCCGCATTCCTTCCGATTGTTTCG
Gal7-Cse4-G-FP	CAGCAAGCTTATGGCAAGAACAGTAACGAGC
Gal7-Cse4-G-RP	GTAGCTCGAGCATGATTGTCACCCTCTTTGC
Cn-Nuf2-GU- FP	CTCCTCTAGAGTATAATCTGACACTAGCTCGCAGAG
Cn-Nuf2-GU- RP	CTATCTGCAGGTCAAACTTGGTCTGCAAGTACAACTG
Cn-Nuf2-DS-FP	ACGCGTCGACCATGCTTCCTATACAAATAGTTTCC
Cn-Nuf2-DS-RP	CATAGGGCCCGGATCTAGAATGCAGAAAGTAATAAGGTAG
Cn-Nuf2-UP- RP2	CTATCTGCAGCAGTTGTACTTGCAGACCAAGTTTGAC
Cn-Nuf2-DS-RP2	CATAGGGCCCCTACCTTATTACTTTCTGCATTCTAGATCC
Dad1-GS-FP2	GTGCAAGCTTATGTCTTTATCAAGACCATCGAATGC
NDC 80-U-Mc-FP	GAAATTGGAGGATGAATATGTCCAGCTGATGAG
NDC 80-U-Mc-RP	CTCGCCCTTGCTCACCAT TTGAGTCTCTGCCAACGCTCTAATAC
mC-neo-N-FP	GTATTAGAGCGTTGGCAGAG ACTCAAATGGTGAGCAAGGGCGAG
mC-neo-N-RP	CGAGGAAGAACAGAAGCCAGAAG CCAAGCTTGGTACCGAGCTC
NDC 80-D-Neo-FP	GAGCTCGGTACCAAGCTTGG CTTCTGGCTTCTGTTCTTCCTCG
NDC 80-D-Neo-RP	TGCTGCTGCTGTTGCTGGGATTCC
Cn-H3-4828-Pc-FP	GACGGATCCGCT ATGGGTAAGTACCTTTTTTACATGC
Cn-H3-4828-Pc-RP	GTACACTAGTCTCTCTGGACCCATTGAAGATGGAG
Dad1-GS-FP2	GTGCAAGCTTATGTCTTTATCAAGACCATCGAATGC
NDC 80-U-Mc-FP	GAAATTGGAGGATGAATATGTCCAGCTGATGAG
NDC 80-U-Mc-RP	CTCGCCCTTGCTCACCAT TTGAGTCTCTGCCAACGCTCTAATAC
mC-neo-N-FP	GTATTAGAGCGTTGGCAGAG ACTCAAATGGTGAGCAAGGGCGAG
mC-neo-N-RP	CGAGGAAGAACAGAAGCCAGAAG CCAAGCTTGGTACCGAGCTC
NDC 80-D-Neo-FP	GAGCTCGGTACCAAGCTTGG CTTCTGGCTTCTGTTCTTCCTCG
NDC 80-D-Neo-RP	TGCTGCTGCTGTTGCTGGGATTCC
Cn-H3-4828-Pc-FP	GACGGATCCGCT ATGGGTAAGTACCTTTTTTACATGC
Cn-H3-4828-Pc-RP	GTACACTAGTCTCTCTGGACCCATTGAAGATGGAG
Cn-SH-CG7-OC-FP	CACCGATACGTACGGCTTGAACG
Cn-SH-CG7-OC-RP	GTTAGGCAGGAGACTTTGCAATGG
Cn-SH-D1G7-OC-FP	CACTCACGTTGCAGTGTCATGG

Cn-SH-D1G7-OC-RP	GTATTGCTCCCATCTTCATCTCG
Cn-S72M-Nuf2-US-FP	GACTGAGCTCCTTGCACTCTTACAGAAGCCTCC
Cn-S72M-Nuf2-US-RP	TCACATGCGGCCGCGATTGCTGAATGCAAATGCAG
Cn-S72M-Nuf2-DS-FP	GACTAAGCTTATGTCGCAGCAGAATCGCAG
Cn-S72M-Nuf2-DS-RP	GACTGGTACCGATTTCAAGCTGTGTGACGATACG
Cn-S72M-dad2-US-FP	CTTGCGTACTCTGCAGCTGC
Cn-S72M-dad2-US-RP	CTGCAGATATCCATCACACTGGAGTTACAGTGGAAATTAAGG
Cn-S72M-dad2-MD-FP	CCTTAATTTCCACTGTAACTCCAGTGTGATGGATATCTGCAG
Cn-S72M-dad2-MD-RP	CATTTCTATTGATGGACGGGACATCTTGTACAGCTCGTCCATGC
Cn-S72M-dad2-US-FP	GCATGGACGAGCTGTACAAGATGTCCCGTCCATCAATAGAAATG
Cn-S72M-dad2-DS-RP	CATCTTTATCCTGTTGTTGGAAGC
Dad2-G7M-US-FP	AGCTTGAGCTCCTTCGAGATATACAGCTCC
Dad2-G7M-US-RP	TTTAAGCGGCCGCCACTCGAGAGTTACAGTG
Dad2-G7M-DS-FP	CATCAAGCTTGGTGGTATGTCCCGTCCATCAATAGAAATG
Dad2-G7M-DS-RP	AACTCTCGAGGTGAGATAGGGTTGAAGGAGC
Mad2 –US-FP	TCGTGAGCTCGTCTCAACAATTTGGTTACTGATCAAGG
Mad2-US-RP	AGGACACTAGTTTCGTGGGGTAGAAACTGGAAG
Mad2-DS-RP	TTATGGGCCCGTAATATTATCTAGTTCAACGTTCACG
Mad2-DS-RP	ACCCTTAGATCTGTGAATTCCTTTTATCCATTTTCC
Dad2-G7M-US-FP	AGCTTGAGCTCCTTCGAGATATACAGCTCC
Dad2-G7M-US-RP	TTTAAGCGGCCGCCACTCGAGAGTTACAGTG
Dad2-G7M-DS-FP	CATCAAGCTTGGTGGTATGTCCCGTCCATCAATAGAAATG
Dad2-G7M-DS-RP	AACTCTCGAGGTGAGATAGGGTTGAAGGAGC
Mad2 –pCIN-US-FP	TCGTGAGCTCGTCTCAACAATTTGGTTACTGATCAAGG
Mad2-pCIN-US-RP	AGGACACTAGTTTCGTGGGGTAGAAACTGGAAG
Mad2-pCIN-DS-RP	TTATGGGCCCGTAATATTATCTAGTTCAACGTTCACG
Mad2-pCIN-DS-RP	ACCCTTCCAGATCTGTGAATTCCTTTTATCCATTTTCC
Mad2-DS-pLK-RP	TTTAAAGCGGCCGCGTAATATTATCTAGTTCAACGTTCACG
Mtw1-G7G-US-FP	AGCTGAGCTCCAAATCCACAACATCTGAAATACG
Mtw1-G7G-US-RP	AAATTTGCGGCCGCGAACGTAGAGACGATTATGAATGC
Mtw1-G7G-DS-FP	GCTGTTAACGGTGGTATGGTCCCGAGGAAGCCAG
Mtw1-G7G-DS-RP	AGGTCCTCGAGCATTGGCAAGCTAACTAAATTAATGGAACG
Mif2-G7-US-FP	AGCTGAGCTCCAAGTCTCTTGTCGACATCTCTCC
Mif2-G7-US-RP	TTATTAGCGGCCGCGTTGAAGATGTTCTGGAGAAGTGC
Mif2-G7-DS-FP	AACCCAAGCTTATGTCCCACATAACACCCTCAAGA

Mif2-G7-DS-RP	TCGTCTCGAGCTTTCCATCTGCTTGCTTCTTTGG
Dad2-G7-SH-FP	GTATTCTCGGAGGTCTTGATCC
Dad2-G7-SH-RP	CTTATCGCTTACCTGCAGC
Mif2-G7-SH-FP	GATGATAGAAATTGCATGTCC
Mif2-G7-SH-RP	CATACCTTCTTTCTCCTGC
Mtw1-G7-SH-FP	GTGAAGTAGCTGATGTACCTG
Mtw1-G7-SH-RP	CATACCTTCTTTCTCCTGC
Nuf2-G7-SH-FP	GAAAGCTAAAGCAGTGACG
Nuf2-G7-SH-RP	CATATTCTTGCACGAACG
pGAL7-CFM732-FP	GTATCCATTGCATATCTTATCG
pGal7-CFM192-RP	GAAACTGCAAGAGATTGTCAG

A5. Appendix figures:

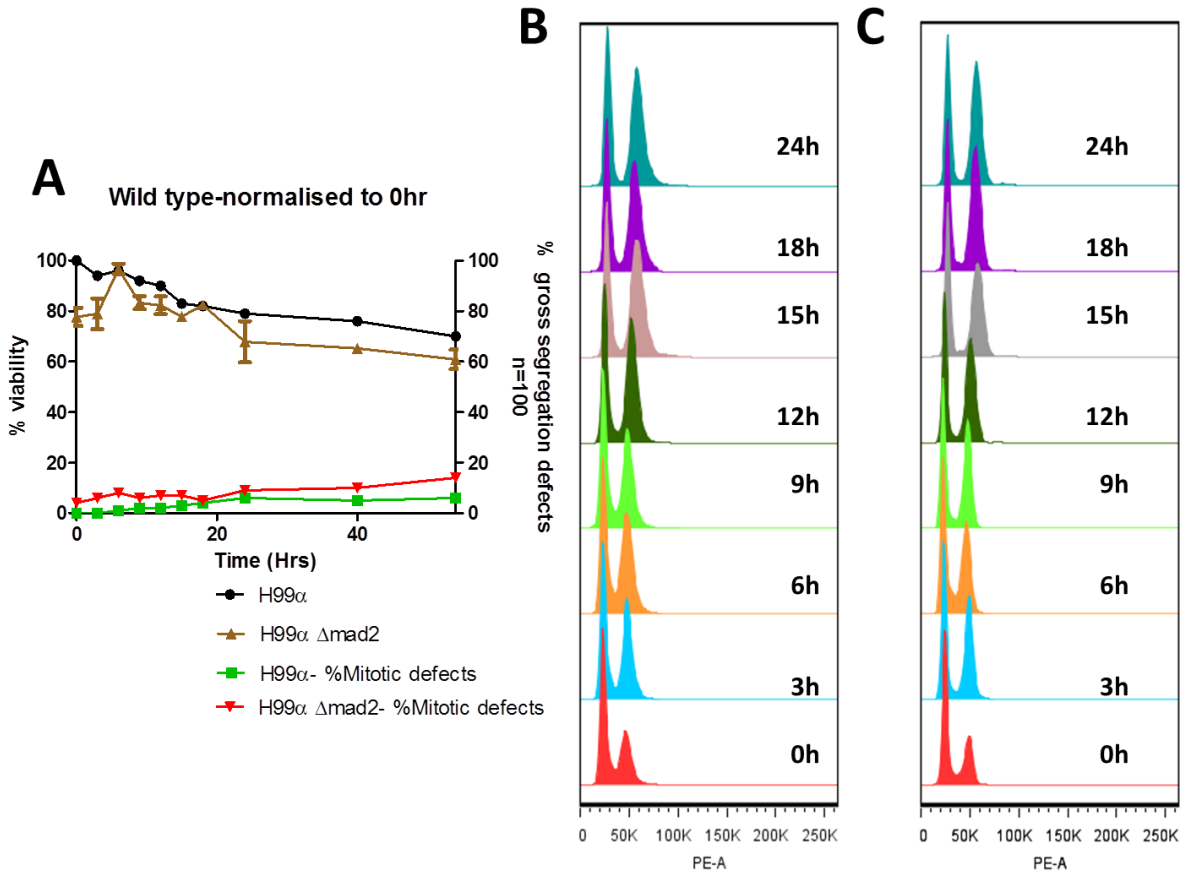

Figure 1. Quantitation of Wild-type phenotype. A) Normalized viability plots comparing viability between wild-type and Δmad2 background. B) Cell cycle analysis of wild-type and Δmad2 cell population over time in media containing glucose.

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